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*EDITORIAL*

# Animal models of portal hypertension

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## Abstract

Animal models have allowed detailed study of hemodynamic alterations typical of portal hypertension and the molecular mechanisms involved in abnormalities in splanchnic and systemic circulation associated with this syndrome. Models of prehepatic portal hypertension can be used to study alterations in the splanchnic circulation and the pathophysiology of the hyperdynamic circulation. Models of cirrhosis allow study of the alterations in intrahepatic microcirculation that lead to increased resistance to portal flow. This review summarizes the currently available literature on animal models of portal hypertension and analyzes their relative utility. The criteria for choosing a particular model, depending on the specific objectives of the study, are also discussed.



**Key words:** Cirrhosis; Nitric oxide; Portal vein

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## INTRODUCTION

As for all pathologic conditions, the use of animal models is of enormous importance for the study of pathophysiological disturbances of portal hypertension, since they allow comprehensive study of questions that cannot be addressed in human studies. The historical evolution of the knowledge of portal hypertension clearly illustrates this. The concept that portal hypertension is not only the consequence of an increased resistance to portal blood flow, but also of an increase in portal inflow could not be definitely demonstrated until the development of an adequate methodology to conduct detailed hemodynamic studies in experimental animals[1,2]. Later, the isolation and *ex vivo* study of the two vascular beds implicated in the syndrome, the mesenteric bed and the liver vasculature, allowed the physiological characterization of the vasoactive mediators involved in mesenteric vasodilation and in the increased vascular tone of the cirrhotic liver. The next step was the introduction of introduction of molecular biology tools to molecular biology to identify the alterations in the signaling pathways responsible for the dysregulation of these mediators[3]. This allowed the development and validation of therapeutic targets that have been ultimately tested in patients with cirrhosis and portal hypertension.

This review will cover the most commonly used animal models of portal hypertension and their relative utility for the study of different aspects of this syndrome. In the selection of an animal model for the study of portal hypertension some general concepts, which apply to every animal model, must be considered[4] (Table 1). The final choice will largely depend on the specific alteration of the pathophysiology of portal hypertension to be studied, because not all models express all disturbances characteristic of the portal hypertension syndrome. The first step is species choice. Rat and rabbit are the species used most often. More recently, the methodology for hemodynamic studies in rat and rabbit have been implemented to mice[5-7]. This advance has enormously widened the research possibilities due to the availability of *knock out* and transgenic mice. The use of animals like dogs or pigs[8] offers an advantage for the instrumentation and dissection of vascular structures due to their size, but this practice has been abandoned due to high cost. This review will focus on commonly used models at present.

## MODELS OF PORTAL HYPERTENSION

The portal pressure gradient is the result of the interaction between portal blood flow and the vascular resistance that opposes that flow. This relationship is defined by Ohm's law in the equation:

$$\Delta P = Q \times R$$

where $\Delta P$ is the portal pressure gradient (the difference between portal pressure and inferior vena cava pressure), Q is blood flow within the entire portal venous system (which in portal hypertension includes also the portal-systemic collaterals), and R is the vascular resistance of the entire portal venous system. It follows that portal pressure may increase because of an increase in portal blood flow,

**Table 1 General considerations in choosing animal models (modified from Mullen & McCullough[4])**

| |
|---|
| **Reproducibility:** % of animals reaching the desired state. Consistent time frame to attain desired state. |
| **Specificity:** The model should have the desired abnormality without other complicating problems. |
| **Costs:** Consider not only the direct costs, but also indirect costs such as animal housing (and, therefore, the time to achieve the desired state). An expensive but reliable model could be cheaper than a cheap but inconsistent model. |
| **Safety:** Animal and induction method should not be a risk for the personal. |
| **Size:** Blood volume sample requirements or need for vascular access may determine the size of the animal. The size also determines drug spending. |
| **Ethics:** Different ethics committees can have different opinions about the acceptability of one model. |
| Feasibility: Whether the laboratory has the expertise, manpower facilities, *etc*, to generate or handle the model. |

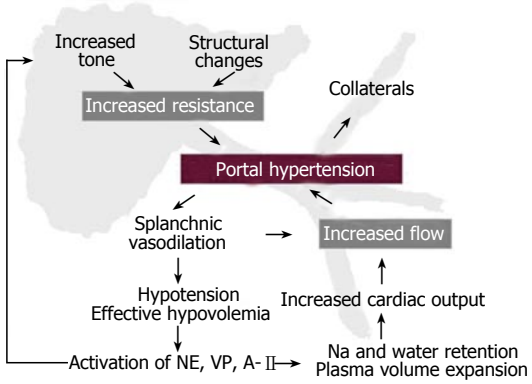


**Figure 1** Summary of the pathophysiology of portal hypertension. The increase in hepatic resistance leads to an increase in portal pressure. This leads to a cascade of disturbances in the splanchnic and systemic circulation characterized by vasodilation, sodium and water retention and plasma volume expansion, that are major players in the pathogenesis of ascites and hepato-renal syndrome. Additionally, these alterations lead to an increase in portal blood inflow that contributes to maintain and aggravates portal hypertension. Another characteristic feature is the development of porto-systemic collaterals that are resposible for complications such as variceal bleeding and hepatic encephalopathy.

an increase in vascular resistance, or by a combination of both[3]. It is well established that the primary factor leading to portal hypertension is an increased resistance to portal blood flow[9-11]. This increased resistance can be prehepatic (portal vein thrombosis), intrahepatic (liver cirrhosis) or posthepatic (Budd-Chiari syndrome). Independently of the cause, portal hypertension is associated with severe disturbances in the systemic and splanchnic circulation, characterized by vasodilation, hypotension, activation of vasoactive systems, plasma volume expansion and increased cardiac output[12]. This is known as the hyperdynamic circulatory syndrome, and it leads to an increase in portal blood inflow that contributes to maintain or worsen portal hypertension despite the development of portal-systemic collaterals (Figure 1). This means that for the study of hyperdynamic circulation both models of pre-hepatic and intra-hepatic portal hypertension are useful. For the study of the intrahepatic circulation specific models of disease are needed.

### *Models of pre-hepatic portal hypertension*

**Partial portal vein ligation:** Partial portal vein ligation model (PVL) has been widely used in the study of the pathophysiology of portal hypertension. This model has been developed in rats[1,13,14], mice[6,7] and rabbits[15]. The portal vein is freed from surrounding tissue after a midline abdominal incision. A ligature (silk 3-0) is placed around a blunt-tipped needle lying along the portal vein. Subsequent removal of the needle yields a calibrated stenosis of the portal vein that has the diameter of the needle. In the conventional rat PVL model a 20G needle is used (0.889 mm diameter)[1,13,14]. By using needles of greater caliber, less severe stenosis and thus less severe degrees of portal hypertension are induced[16,17]. The diameters of the needles and resulting levels of stenosis are as follows: 16G: 1.651mm, 18G: 1.270 mm, 20G: 0.889 mm. The conventional needles for mice and rabbits are 27G[6,7] and 18G[15], respectively. Surgery must be conducted in aseptic conditions. Numerous websites provide information on surgical techniques and pre- and post-operative care of the animals, including information on anesthesia, analgesia and antibiotic prophylaxis (http://info.med.yale.edu/yarc/vcs/). For portal vein ligation no antibiotic prophylaxis is needed.

PVL model has been extensively used because the procedures are easy to perform, inexpensive, reproducible and portal hypertension develops very fast. One week after portal vein ligation rats develop the complete portal hypertensive syndrome, with hyperdynamic circulation and portal-systemic shunting. Portal-systemic shunting is already detectable at two days. The percentage of portal-systemic shunting; i.e. the amount of portal blood inflow diverted to collaterals, approaches 100% after the 7$^{th}$ day[10]. Mesenteric vasodilation and increased cardiac output are detectable at 4 d[10,13]. The main drawback of the model is that portal hypertension developes acutely. Thus, contrary to the majority of situations found in clinical practice, the degree of portal hypertension is maximal at 24 h, and decreases afterwards due to the development of portal-systemic collaterals[10].

### *Models of intrahepatic portal hypertension*

Intrahepatic portal hypertension can be classified as presinusoidal, sinusoidal and postsinusoidal. Models of cirrhosis, the most common cause of portal hypertension in western countries, have a double component, which are the pre- and post-sinusoidal components, but for practical purposes they will be discussed with the models of sinusoidal portal hypertension.

**Presinusoidal intrahepatic portal hypertension:** (1)Schistosomiasis Experimental infection with Schistosoma mansoni has been characterized in mice and hamsters. This model is achieved by injecting cercariae of the parasite in the abdominal wall. Portal hypertension develops 5 to 7 wk after inoculation[18,19]. An important feature of this model is that portal hypertension develops progressively. In the hamster Schistosoma infection does not induce the development of portal-systemic shunting despite the presence of portal hypertension[20]. On the contrary, mice infected with Schistosoma mansoni develop portal

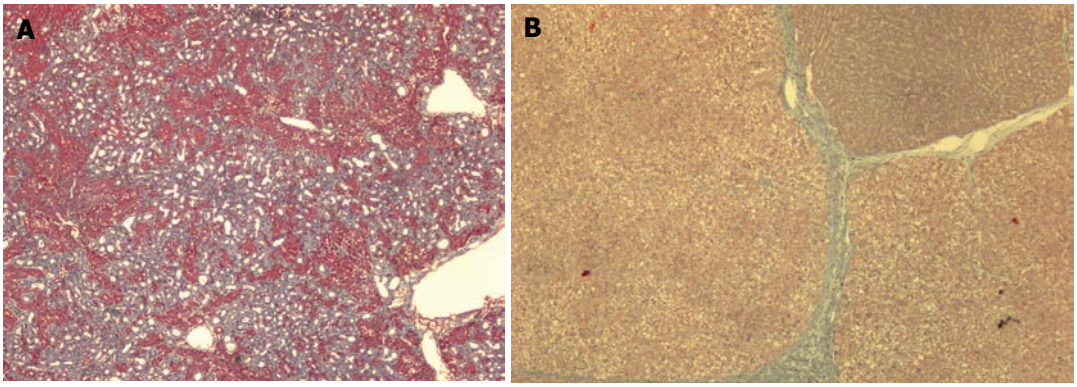


**Figure 2** A comparison of microscopic aspect of a liver 6 wk after CBDL (**A**) and 12 wk after TAA (**B**) administration.

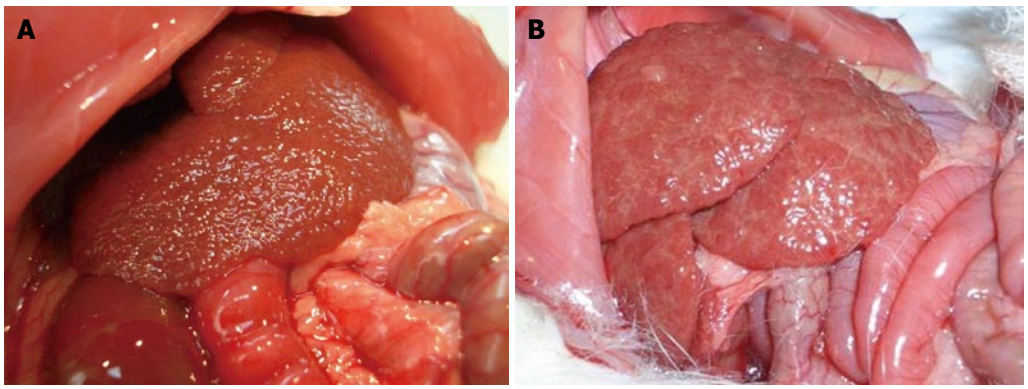


**Figure 3** Macroscopical images of livers from $CCl_4$ (**A**) and TAA (**B**) models.

hypertension with portal-systemic shunting. Shunting is detectable from wk 9 and reaches 15% at wk 11[18,19]. Currently this model is seldom used, and no studies have been published in the last decade.

**Sinusoidal portal hypertension:** A number of models of cirrhosis have been described. We will limit our discussion to those that have been used for the study of portal hypertension.

(1) Common bile duct ligation (CBDL) CBDL is a model of secondary biliary cirrhosis. It has been mainly developed in rats[21], which are especially appropriate due to the lack of a gallbladder, but it has also been developed in rabbits[22] and mice[23]. Mice, however, develop a marked dilation of the gallbladder after bile duct ligation, which may lead to perforation and choleperitoneum. The intervention consists of the isolation of the common bile duct followed by a double ligature. The first ligature is made below the junction of the hepatic ducts. The second is made above the entrance of the pancreatic ducts. The portion of the bile duct between the two ligatures is resected to avoid repermeabilization. Mortality is high after the 5$^{th}$ wk (20%). The use of prophylactic antibiotics (Ampicilin 100 mg/Kg s.c. or similars) before surgery and weekly administration of vit K (50 mcg s.c.)[24] notably improve the survival of CBDL rats. One of the drawbacks of this model is the potential formation of a biliary cyst, which may compress the portal vein at the hilum. This problem can be solved by gently injecting 10% formalin (120 μL/100 g) through a P10 catheter in the bile duct before ligation[14,25]. Other authors have prevented cyst formation by injecting Ethibloc®[26], a substance developed for vessel embolization, or by ligating the biliary duct of each lobule[27,28].

This model develops biliary fibrosis-cirrhosis in 4-6 wk. Histology shows marked cholangiolar proliferation and expansive portal fibrosis (Figure 2A), but the architectural disturbances typical of cirrhosis are seldom found[29]. At 2 wk rats develop mild portal hypertension[30] and at 4 wk severe portal hypertension, hyperdynamic circulation and portal-systemic shunting of 30%-60%[21,31,32]. Approximately, 60% of the rats develop ascites. Portal hypertension in this model has a presinusoidal component[30]. A major drawback of this model is that it is not adequate for pharmacological studies with drugs that are eliminated through the biliary route.

(2) Carbon tetrachloride induced cirrhosis ($CCl_4$) Acute administration of carbon tetrachloride induces acute hepatitis of primary perivenular localization. Continuous administration induces chronic liver injury that leads to cirrhosis. This methodology to induce cirrhosis has been used in rats[33,34], mice[35] and rabbits[22]. Route of administration varies among laboratories, but the most effective are oral[36,37], intraperitoneal[38,39] or inhalatory[34,40,41]. Subcutaneous route is not recommended due to its low yield of cirrhosis. The use of different administration schedules, even using the same route of administration, could explain the variability in the yield and time to cirrhosis in different laboratories. In our unit, Phenobarbital (0.3 g/L) is added to drinking water to increase the yield of cirrhosis, starting one week before first $CCl_4$ administration. Hemodynamic studies are performed 5-7 d after stopping $CCl_4$ and Phenobarbital.

Twelve to 15 wk after $CCl_4$ administration the rats develop micronodular cirrhosis (Figure 3A), portal

hypertension, portal-systemic shunting (30%-60%) and hyperdynamic circulation[2]. If maintained for 12 to 20 wk, most rats develop ascites. A major complexity of this model is the different sensitivity of the rats to $CCl_4$, which makes it difficult to obtain a homogeneous group of cirrhotic rats. Proctor *et al*[36] proposed a solution that consists of the individualization of the dose according to weight gain/loss of the animal in response to the previous dose.

(3) Cirrhosis induced by thioacetamide (TAA) This is another widely used model of toxic cirrhosis. The toxin affects both perivenular and periportal areas. It has been used in rats[42] and mice[43]. TAA can be administered in drinking water[42] or by i.p. injection[44,45]. I.p. injection offers much more consistent results[45].

This model develops macronodular cirrhosis with portal hypertension in 12 wk (Figures 2B and 3B)[42,45,46]. Longer periods of induction might be required for the instauration of overt hyperdynamic circulation[46]. Approximately 40% develop ascites[42]. One particular feature of this model is that, contrary to what occurs with the $CCl_4$ model, fibrosis remains stable for weeks after TAA withdrawal[45]. After 18 wk of TAA administration the rats might develop cholangiocarcinoma[47].

(4) Dimethylnitrosamine induced cirrhosis (DMNA) DMNA is another hepatotoxin that induces hepatocellular necrosis. After continuous administration (generally i.p.) the rats develop fibrosis with portal hypertension, already present at 5 wk, but at this time the animals do not have cirrhosis nor features of hyperdynamic circulation[48]. Overt cirrhosis with ascites develops in 13 wk[49,50]. This model has been seldom used for the study of the pathophysiology of portal hypertension, probably due to restrictions in the use of DMNA due to its high carcinogenetic potential.

(5) Diet induced cirrhosis A diet deficient in choline and methionin, or a diet with low protein and choline and enriched with fat, induces liver steatosis associated with marked oxidative stress that induces inflammation and fibrosis[49,51]. Cirrhosis is developed after 12-24 wk. These models have not been well-characterized from the hemodynamic point of view and have not been used for the study of portal hypertension.

**Postsinusoidal portal hypertension:** Recently, a model that reproduces the pathological and clinical characteristics of veno-occlusive disease has been developed[52]. This is achieved by the administration of monocrotalin by oral gavage. Rats develop hyperbilirrubinemia, hepatomegaly and ascites at 4-5 d. This model is useful for the study of the pathophysiology of veno-occlusive disease, but has not been characterized from the hemodynamic point of view.

### *Posthepatic portal hypertension*

The aim of these models is to reproduce the features of the Budd-Chiari syndrome; i.e. liver injury derived from hepatic venous outflow obstruction. This has been achieved by placing an ameroid, which is a stainless steel device that allows slow expansion inside upon contact with the wet tissue, in the hepatic veins inducing a progressive occlusion of hepatic venous outflow. This model has been developed in dogs[53]. However, in the rat it is almost impossible to dissect the hepatic veins, so hepatic venous outflow occlusion has been induced by occluding the inferior vena cava cranially to the hepatic veins[54]. This is not a pure model of Budd-Chiari. These models have been very seldom used, and therefore it is uncertain whether they have any utility in the study of the hemodynamics of Budd-Chiari syndrome.

**Table 2 Utility of the most commonly used animal models of portal hipertensión**

| Type of study | PVL | CBDL | $CCl_4$ | TAA |
|---|---|---|---|---|
| Abnormalities of the intrahepatic microcirculation in cirrhosis | - | ** | *** | *** |
| Pathophysiology of the hyperdynamic circulation | *** | *** | *** | *** |
| Temporary sequence of alterations leading to the hyperdynamic circulation | *** | ** | * | * |
| Pathophysiology of ascites | ** | ** | *** | *** |
| Mesenteric circulation | *** | ** | *** | ? No experience |
| Collateral circulation | *** | *** | *** | ? No experience |
| Portal hypertensive gastropathy | *** | *** | *** | ? No experience |
| Hepato-pulmonary syndrome | - | *** | - | - |
| Portal hypertension-related bleeding models | *** | *** | ? No experience | ? No experience |

Utility of the most commonly used animal models of portal hypertension for the study of the different manifestations of the syndrome. $CCl_4$ model has been the most widely used model for the study of the intrahepatic circulation. In the study of the splanchnic and systemic circulation the PVL model is particulary useful for hypothesis generation, that might be later confirmed in the cirrhosis models. Only the CBDL model develops features of hepatopulmonary syndrome (-: Not useful; *: Low utility; **: Moderately useful; ***: Very useful).

## SELECTION OF A MODEL FOR THE STUDY OF THE PATHOPHYSIOLOGY OF PORTAL HYPERTENSION AND ITS COMPLICATIONS (TABLE 2)

### *Abnormalities in the intrahepatic circulation in cirrhosis*

The most frequent cause of portal hypertension in western countries is liver cirrhosis. The primary factor leading to portal hypertension is an increased resistance to portal blood flow. This is not only the result of the disruption of the liver architecture, but is also due to an increased hepatic vascular tone. This concept has been demonstrated in isolated liver perfusion[55], which allows evaluation of the hepatic vascular tone and its response to vasoconstrictors and vasodilators. This is problematic in *in vivo* studies because it is very difficult to discern the effects of a particular vasoactive substance that depend on changes in systemic, splanchnic and collateral circulation

from those derived from changes on hepatic resistance. Another way of evaluating intrahepatic microcirculation is by intravital microscopy[56].

The model most frequently used for isolated perfusion has been $CCl_4$ induced cirrhosis. In these livers, it has been demonstrated that there is an increased hepatic vascular tone, hyperresponse to vasoconstrictors and hyporresponse to vasodilators. The main mechanism mediating these abnormal vascular responses is endothelial dysfunction with insufficient NO production and an increased production of vasoconstrictive eicosanoids[57,58]. Vascular responses have also been studied in other models of cirrhosis, such as CBDL[59,60] and TAA[46,61], but data are still scarce and less consistent than that obtained with the $CCl_4$ model. Moreover, it must be stressed that the CBDL model has an important presinusoidal component in the increase in hepatic resistance[30].

### Abnormalities in the systemic, mesenteric and collateral circulation in portal hypertension

These studies include *in vivo* hemodynamic studies and *ex vivo* perfusion of the mesenteric vascular bed and the portal-systemic collaterals.

The *in vivo* studies are very useful for the study of the pathophysiology of the hyperdynamic circulation associated with portal hypertension. The hyperdynamic circulation has been described in the PVL model[1,13] and in the models of cirrhosis induced by $CCl_4$[2], CBDL[21] and TAA[46,62]. The *in vivo* studies, on the other hand, globally evaluate the effects of a drug on portal pressure, which is the result of the integrated effects of the drug on portal blood inflow, collateral resistance and hepatic resistance.

Any model of portal hypertension is valid, in theory, for the study of mesenteric circulation. This has been evaluated by the isolated perfusion of the mesenteric vascular bed (McGregor's preparation[63]), by the study of vascular responses in isolated mesenteric vessels[64] or by the perfusion of intestinal microvasculature[65]. The characteristic hyporesponse to vasoconstrictors of the mesenteric circulation has only been demonstrated so far in the PVL and the $CCl_4$ models[40,64-66].

Collateral circulation has been studied with different methodologies. The most common has been the evaluation of portal-systemic shunting by the injection of radioactive, colored or fluorescent microspheres[67,68]. If different isotopes or colored or fluorescent markers are used, changes in portal-systemic shunting after different interventions can be evaluated in the same animal[9]. Another way of studying the collateral circulation is the measurement, with transit-time flow probes, of the blood flow of the spontaneous splenorenal shunt, a major collateral developed after portal hypertension[48]. Mosca et al developed a system for *ex vivo* perfusion of collaterals[69], in which the vascular responses of the collateral vascular bed to different vasoactive substances can be tested. The interpretation of the results obtained with this methodology might differ among models, because in the PVL model shunting is about 100%, while in the cirrhosis models shunting does not go beyond 60%.

The availability of the PVL model has shown great advantage for the study of the abnormalities of systemic, mesenteric and collateral circulation, because it is a very rapid model and much less expensive than models of cirrhosis. In the last 20 years, most investigators have chosen to generate and test hypothesis first in the PVL model, and subsequently confirm those hypothesis in the more laborious and expensive models of cirrhosis.

### Ascites and renal dysfunction

The vast majority of studies in this field have been performed using the $CCl_4$ cirrhosis model[70-72]. $CCl_4$ administration is maintained until the animal develops ascites at physical examination, which occurs between 12-20 wk. The PVL model, akin to what happens in patients with prehepatic portal hypertension, does not develop ascites. Even though, since this model is ideal for sequential studies, it has been instrumental in the description of hyperdynamic circulation[13] and in the validation of the peripheral arterial vasodilation hypothesis as the trigger for sodium and water retention in cirrhosis[13,73-75]. CBDL and $CCl_4$ models have been also used for longitudinal studies aimed at demonstrating the sequence vasodilation-sodium retention-ascites[76-78], but the temporal evolution of these models (especially for $CCl_4$) is less consistent.

### Portal hypertensive gastropathy

Several studies have demonstrated that PVL and cirrhotic rats show abnormalities of the gastric microvasculature comparable to those observed in portal hypertensive gastropathy in humans. Studies in these models have been useful to test therapeutic interventions that subsequently showed some efficacy in patients bleeding from portal hypertensive gastropathy[79,80].

### Hepatopulmonary syndrome

CBDL rats develop alterations analogous to those of hepatopulmonary syndrome seen in humans, such as intrapulmonary vascular dilatations and an increased alveolar to arterial oxygen gradient[81]. These alterations are obvious from the 2nd wk after bile duct ligation[44]. Other models of portal hypertension, such as the PVL and the TAA, do not develop hepatopulmonary syndrome[44,81].

### Models of portal hypertension-related bleeding

Our laboratory has recently described a portal hypertension-related bleeding model that has been useful in evaluating therapeutic interventions in acute variceal bleeding, such as for determining the best policy for volume replacement and the effects of vasoactive drugs on the outcome of bleeding[14,25,82]. This model consists of the isolation and section of a branch of the ileocolic vein (Figure 4). The severity of hemorrhage depends on the degree of portal hypertension and the size of the sectioned branch[14]. This model has been characterized in PVL and CBDL rats[14,25,82]. A section of the first order branch of the ileocolic vein results in 50% mortality in CBDL rats, whereas mortality is 0% in PVL rats. Subsequently, a modification of the model was developed, in which 2 successive sections of a first order branch of ileocolic

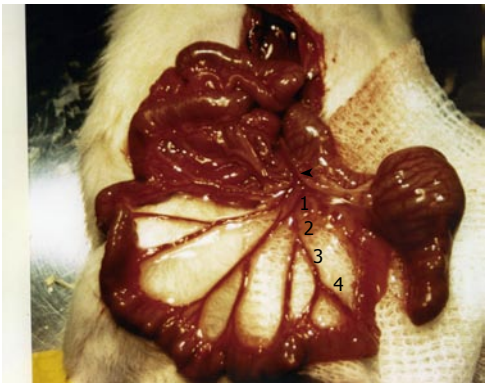


**Figure 4** Model of portal hypertension-related bleeding. In these model a section of a first (1), second (2), third (3) or fourth order branch of the ileocolic vein is isolated and sectioned[14,25,82].

vein are performed in PVL rats. In this way the second bleeding is induced when the rat is already hypovolemic. This modification increases mortality to 50%, and allows testing of the vasoactive drugs in hypovolemic conditions, a situation that better reproduces the clinical context in which these drugs are applied[82].

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*EDITORIAL*

# Celiac disease

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## Abstract

Celiac disease (CD) is a common autoimmune disorder, induced by the intake of gluten proteins present in wheat, barley and rye. Contrary to common belief, this disorder is a protean systemic disease, rather than merely a pure digestive alteration. CD is closely associated with genes that code HLA-II antigens, mainly of DQ2 and DQ8 classes. Previously, it was considered to be a rare childhood disorder, but is actually considered a frequent condition, present at any age, which may have multiple complications. Tissue transglutaminase-2 (tTG), appears to be an important component of this disease, both, in its pathogenesis and diagnosis. Active CD is characterized by intestinal and/or extra-intestinal symptoms, villous atrophy and crypt hyperplasia, and strongly positive tTG auto-antibodies. The duodenal biopsy is considered to be the "gold standard" for diagnosis, but its practice has significant limitations in its interpretation, especially in adults. Occasionally, it results in a false-negative because of patchy mucosal changes and the presence of mucosal villous atrophy is often more severe in the proximal jejunum, usually not reached by endoscopic biopsies. CD is associated with increased rates of several diseases, such as iron deficiency anemia, osteoporosis, dermatitis herpetiformis, several neurologic and endocrine diseases, persistent chronic hypertransami-nasemia of unknown origin, various types of cancer and other autoimmune disorders. Treatment of CD dictates a strict, life-long gluten-free diet, which results in remission for most individuals, although its effect on some associated extraintestinal manifestations remains to be established.



**Key words:** Celiac disease; Tissue transglutaminase antibodies; Autoimmune disorders; Gluten-free diet

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## INTRODUCTION

Celiac disease (CD) is an immune-mediated disorder, the only one with a well-established origin, resulting from a permanent gluten intolerance, which primarily involves the gastrointestinal tract.

It is characterized by the presence of chronic inflammation of the small bowel's mucosa and submucosa, and is clinically characterized by the presence of diverse systemic manifestations.

It may start at any age, both during childhood and adolescence, and is also relatively common in adulthood. It is being increasingly diagnosed even in elderly patients (up to 20% of patients are older than 60 years, at the time of diagnosis)[1-3].

The causal agent, gluten, is well established; a mixture of proteins present exclusively in cereals –mainly wheat, barley, rye and oats- but not corn. It affects only predisposed individuals, whose most susceptible genetic features are related to human leukocytes antigens from class two (HLA-Ⅱ), predominantly HLA-DQ2 (90%), while much less frequency HLA-DQ8 (5%-10%). However, these genetic markers are a necessary, but not a sufficient condition, since a significant frequency of CD patients (5%-10%) are DQ2 and DQ8-negative. This means that other, still not well-established, genotypes may exist that probably correspond to the class one HLA system (HLA-Ⅰ), including MICA, MICB and others[4].

A strong genetic susceptibility is present with about 75% concordance rate, among monozygotic twins. Certain populations have an increased prevalence of CD. For instance, the first-degree relatives of individuals with biopsy-proven CD, have a prevalence between 4%-12% of those suffering from this disease. Second-degree relatives also appear to have an increased prevalence. Patients with type 1 diabetes mellitus (IDDM) have a prevalence of CD ranging to 3%-8%. In Down´s syndrome, the presence of CD is found between 5%-12%. Also, CD is associated with Turner's and William's syndromes, IgA deficiency and several autoimmune disorders[5-7]. Most affected individuals show sustained clinical remission when they are put strictly on a gluten-free diet (GFD), which must be maintained indefinitely due to this illness' genetic background.

## EPIDEMIOLOGY

Until the last decade, CD was considered to be a rare disease, but today it is known to be universally distributed, to involve all races, and it is one of the most commonly known genetic diseases, with a mean prevalence of 1%-2% in the general population, being clearly underestimated and

underdiagnosed worldwide.

This would represent an estimated 3 million people affected in Europe and at least another 3 million in the United States. Selected ethnic groups may have a lower prevalence than that suggested for the Caucasian race, but not far from them, as the worldwide distribution of CD is pretty homogeneous, the only exception being the Saharawui population, which lives in refugee camps. This population in North Africa has a mean estimate of 5%, the highest found all over the world[8-10].

In Southern Asia there are still no data available of CD prevalence in the general population. This fact may be due to partly, because in past decades the reports of gluten intolerance were only of sporadic cases. Nowadays, several recent studies suggest that CD is also common in South Asia. For instance, 26%-49% of Indian children presenting with chronic diarrhea, are diagnosed with CD in tertiary care hospitals[11,12].

If we assume that China has approximately 1 billion inhabitants, we can extrapolate the same world proportion of CD (around 1% of the general population), then the approximate number of possible celiac patients in this country would be around ten million people. There are few reports of CD in Far East countries, yet recently, three adult cases have been reported in Canada among descendents of Japanese and Chinese immigrants[13].

These findings suggest that genetic susceptibility for CD also exists among people of the Far East, where the real incidence of the disease is clearly underestimated because their dietary habits are different (more rice than wheat, in the staple diet), and possibly for the interest of doctors to recognize CD. These concerns pose important issues, which require further studies of CD in the different countries of this important and extensive area of the world.

CD is also very prevalent in people from the Middle East and these data are not surprising as they inhabit countries included in the "Fertile Crescent" such as Anatolia (South of Turkey), Lebanon, Syria, Palestine and Iraq, where some 10000 years ago, some tribes changed from a nomadic lifestyle, to one of stable settlement, started the agriculture and probably propagated the spread of this disease throughout the world[14].

Thus, gluten intolerance appears to be a widespread public health problem and an increased level of awareness and clinical suspicion are needed to diagnose CD all over the world. Moreover, the GFD possess a big problem in developing countries, since commercial gluten-free products are not available, while in others they are very expensive and are difficult to acquire.

## CLINICAL PRESENTATIONS

The clinical presentation of CD varies greatly, ranging from asymptomatic to severely malnourished patients. The most common clinical manifestations of CD include abdominal cramping pain with moderate to severe abdominal distension, frequently associated with relapsing or permanent dyspepsia, presence of gastro-esophageal reflux (GERD) and recurrent episodes of altered bowel habits (diarrhea and/or constipation), weight loss, bone disease, anemia and weakness.

While diarrhea was almost considered a persistent symptom, this is not the case in adults, and up to 50% of patients predominantly have constipation, which on many occasions becomes refractory to all types of therapy. It should be noted, that up to 30% of celiac patients have increased body mass index (BMI) and obvious obesity at diagnosis.

CD is sometimes divided into clinical subtypes. The terms "symptomatic or classic" apply to cases that meet the typical features described above. By contrast, in the "atypical forms" of the disease, the gastrointestinal symptoms may be absent or less pronounced, and in this case the extra-intestinal features predominate, such as chronic iron deficiency anemia, osteoporosis, short stature or failure to thrive, infertility and increased number of abortions.

Since atypical presentations are found more frequently in later decades, CD is now considered to be a multisystemic disorder, rather than a sole gastrointestinal process (Table 1).

## PATHOGENESIS

The triggers for CD are specific immunogenic peptides that are present only and exclusively in the dietary gluten proteins, from wheat and similar structural cereals such as rye and barley. These peptides are resistant to digestion by gastric and pancreatic enzymes and find their way into the lamina propria of the small bowel, presumably after some changes occur in the intercellular tight junctions with an increase in the intestinal permeability. One such peptide is a 33-amino acid sequence, which is a potent activator of specific T-cell lines from patients with CD[15].

The subsequent infiltration by CD4 (+) T lymphocytes into the lamina propria and CD8 (+) into the intestinal epithelium, are a hallmark of active CD. The recognition of HLA-bound gluten peptides by T cells, leads to their activation and clonal expansion of B cells that produce antibodies. Other cytokines released by activated CD4 T cells that involve the adaptive immune response, promote various inflammatory mechanisms and produce the intestinal lesion.

Less information is available on the activation and mode of action of intraepithelial T cells, which are mediated by the innate immune system. The expression of the interleukin-15 cytokine appears to play a central role in driving various processes that lead to the increased number of intraepithelial lymphocytes (IELs) as well as in the destruction process of the epithelial cells and the mucosal damage[16].

Tissue transglutaminase 2 (tTG), plays an important role in the immune response and is present in several tissues in the body. The cross-linking activity of tTG is involved in several functions, such as wound healing, formation of cell envelopes in apoptosis and stabilization of the extra-cellular matrix. In addition, this enzyme can deaminate glutamine residues. Glutamine-rich gluten peptides are, therefore, excellent substrates for tTG. The resulting deaminated and thus, negatively-charged peptides, have much higher affinity for the HLA-DQ2 and DQ8

**Table 1 Risk groups and associated disorders**

- First degree relatives
- Down´s and turner´s syndromes
- IgA selective deficiency
- Endocrine diseases
  - Type 1 diabetes mellitus
  - Autoimmune thyroid diseases
  - Alopecia areata
- Neurologic diseases
  - Cerebellar ataxia
  - Epilepsy
  - Peripheral neuropathy
  - Multiple sclerosis
- Liver diseases
  - Primary biliary cirrhosis
  - Autoimmune hepatitis
  - Autoimmune cholangitis
  - Idiopathic hypertransaminasemia
- Rheumatologic diseases
  - Rheumatoid arthritis
  - Sjögren´s syndrome
- Heart diseases
  - Idiopathic dilated cardiomyopathy
  - Autoimmune myocarditis
- Cutaneous diseases
  - Dermatitis herpetiformis
  - Psoriasis
  - Vitiligo
- Others
  - Iron-deficiency anemia
  - Osteoporosis
  - Increased risk of fractures
  - Infertility
  - Amenorrhea
  - Dental enamel defects
  - Depression and anxiety
  - Chronic asthenia

molecules, and have a key step in the immune response in CD[17].

In summary, we must say that CD is a complex disorder that results from an interplay of several genetic, immunological and environmental factors, with many aspects for which the final pathogenetic mechanisms remains to be solved.

## SEROLOGICAL TESTS

Among the serological tests needed to diagnose CD, the measurement of anti-gliadin IgA antibodies (AGA), has completely fallen into disuse and probably justifiably abandoned, as its sensitivity and specificity is very low (around 50%). In 1997, tTG was established by Dieterich *et al* to be the auto-antigen for anti-endomysial antibodies[18] and since then, is preferred for clinical use, because it show good sensitivity, greater than 90%, and a high specificity, around 95%, although it displays small variations between the different commercial kits employed[19].

The presence of these antibodies correlates with the degree of villous atrophy and various studies have clearly shown that the sensitivity of testing tTG is decreased in patients with normal duodenal biopsies or with mild histological changes[20].

Measurement of tTG antibodies of the IgA isotype is usually determined in the clinical practice. Nevertheless, IgA deficiency occurs in 1.7%-2.6% of CD patients, which represents 10-15 times increase, over that in the general population. If IgA deficiency is found, measuring the IgG class tTG is recommended[21]. Diagnosis of CD based solely on serologic markers is not accepted and the identification of the characteristics changes at the duodenal mucosa is required before starting on a GFD.

In conclusion, in clinical practice, serologic tests for CD are frequently used to identify both symptomatic and asymptomatic at-risk individuals who require an intestinal biopsy examination to confirm the diagnosis.

## HISTOLOGICAL FINDINGS

The findings on a duodenal biopsy must be interpreted in detail by a pathologist experienced and interested in this condition and according to Marsh's criteria (modified), which stratify this disease into four types or stages. Biopsy samples are usually collected during an upper GI endoscopy in the second portion of the duodenum close to the Vater ampulla; at least 4-6 well-oriented samples should be submitted, as the CD lesions are often irregular and patchily distributed[22].

CD may be diagnosed with a normal duodenal biopsy (Stage 0), with an increased percentage of intraepithelial lymphocytes (IELs) above 30% (Stage 1), the presence of crypt hyperplasia and inflammatory infiltrates in the lamina propria (Stage 2), and all of them with well-preserved villi. It is not until Stage 3, that villous atrophy develops, which is divided into three different categories: Mild (A), Moderate (B), and Total or Subtotal (C). Finally, Stage 4, or total hypoplasia of the mucosa, is now rarely seen.

Routine use of Marsh's classification in the study of duodenal biopsies, is of great help, when recognizing occult, doubtful cases, and must always be used when performing a diagnostic upper endoscopy in patients belonging to so-called "at risk groups" regarding CD, including those with chronic diarrhea, refractory ferropenic anemia, or both[23].

Duodenal biopsy is still considered by most authors the "gold standard" in the diagnostic process, albeit its usefulness in the adult is still slightly controversial. If results of the histological study are negative but serologic tests are positive and CD is strongly suspected, the results of the biopsy should be reviewed with an expert gastrointestinal pathologist before additional biopsies are considered. In addition, if the histological exam yields equivocal results, it is useful to proceed with HLA typing. Although almost 40% of the general population has the HLA-DQ2 or HLA-DQ8 markers, nearly 90%-95% of CD patients have them[24].

## CLINICAL DIAGNOSIS

The main diagnostic criteria still rest on duodenal biopsy findings while the patient is following a normal diet and whether significant clinical and analytical improvement is seen while the patient is on a strict GFD. Patients usually undergo hematological and biochemical tests along with the determination of serological CD markers (usually

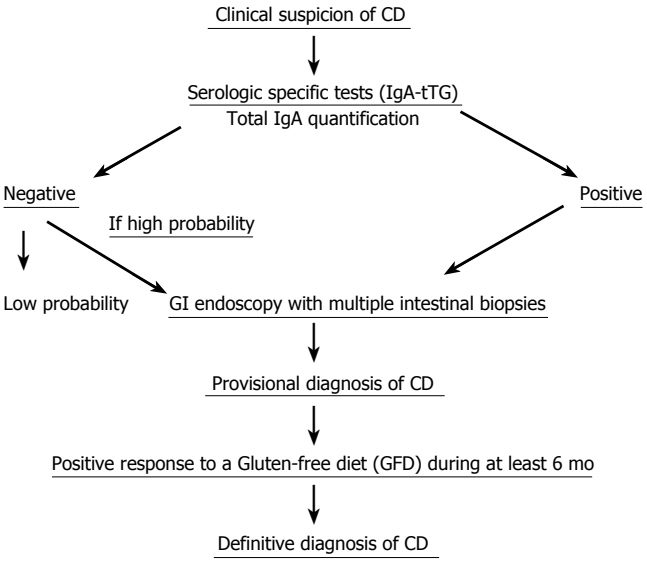


**Figure 1** Algorithm for evaluation of patients suspected of CD.

tTG-IgA class) by ELISA, when this disease is suspected. If serology results are negative, but clinical suspicion is high, intestinal biopsy should be performed. These biopsies must be oriented correctly in order to determine the degree of villous atrophy and to assess the presence and quantify the number of IELs, epithelial disarray, crypts hyperplasia and the degree of chronic inflammation present at the lamina propria.

A clear clinical improvement while the patient is following the GFD yields a definitive diagnosis. The serum antibodies generally disappear by 6 to 12 mo, although they are not necessarily a reliable indication of the mucosal response[25].

When patients do not present with the classic clinical symptoms of CD, a second biopsy that shows histologic improvement will confirm the diagnosis. An algorithm for a possible diagnosis of celiac disease, consisting of several consecutive steps, is shown (Figure 1).

For diagnostic confirmation in uncertain cases, several potential strategies may be agreed upon according to patient and family preferences. Amongst them, we shall quote the following: (a) repeat serology and laboratory tests periodically (b) perform a full family exploration, searching the presence of new potential cases (c) perform a new endoscopy with biopsy samples (less accepted) (d) try a GFD for 6 mo, monitoring the clinical response as well as the serologic and analytical changes. Gluten challenge is not considered necessary for diagnosis, except in patients for whom no initial diagnostic biopsy was done, or results of biopsy are unclear or uncharacteristic of CD. Patients should be informed that they may have a severe reaction with the gluten challenge.

## TREATMENT

The mainstay of treatment is a strict lifelong adherence to a GFD, in which the patient must avoid permanently all kinds of food products containing some wheat, rye, barley and oat. Commonly substituted grains, including rice and corn taken directly, or in the form of flour derived from them.

GFD is a very healthy and complete diet, very well balanced in all the immediate principles and rich enough in all kind of vitamins and minerals; it is the only type of treatment required for these patients. Compliance is a difficult task, at any age, because the wheat flour is present in a great part of foods or as an additive product. Patients whose disease does not respond to dietary treatment should undergo a systematic review[26,27].

The only gluten-free cereal is corn and is, therefore, allowed in the celiac patient's diet whether raw or roasted, in salads, *etc*. Corn flour, if pure in its composition (100%) regarding preparation, processing and manufacture, may be used for bread, cookies, baby food and sweets, which may render a celiac patient's diet more bearable.

Dairy products must be avoided when starting on a GFD, as secondary lactase deficiency is often associated with celiac disease. After 1-2 mo on a GFD, milk derivatives may be gradually reintroduced as long as the patient has no complaints following ingestion, since lactose intolerance is secondary to gluten, and usually regresses within three months with strict GFD adherence. On the other hand, dietary calcium and proteins are essential to correct existing mineral deficiencies, given the high prevalence of osteoporosis seen in celiac patients[28].

Beer must be completely avoided, even from alcohol-free brands, as its manufacture involves the fermentation of various gluten-containing cereals, including barley and rye. The same can be said of all kinds of whisky, obtained from malt distillation procedures. Approximately 70% of patients experience symptoms improvement after 2 wk on a GFD.

The rapidity and extent of histological regression are unpredictable, but there is invariably a delay against clinical improvement, which may not be apparent in repeated biopsies until after three to six months. While histological findings usually regress in children, one half of adults only achieve a partial histological resolution.

When there is severe iron-deficiency anemia, the administration of iron preparations through the intravenous or intramuscular route is recommended for a few months (2 or 3), in order to shorten the recovery time. The two most important questions to answer are if the patient truly has CD and whether he/she is following a strict GFD. The evaluation requires a review of the original biopsies and a complete assessment by an expert dietician. Several associated conditions must be ruled out including the concomitant presence of pancreatic insufficiency, bacterial overgrowth, lymphocytic colitis and true refractory sprue with a clonal T-cell population[29,30].

## DERMATITIS HERPETIFORMIS

Gluten sensitivity is relatively associated with several skin alterations but the most common presentation is in a form of chronic pruritic disease characterized by the presence of several symmetrical papulo-vesicular rash that evolves to crusting lesions broadly distributed over the body, but especially on the forearms, knees, buttocks, wrists and

scalp. It is well known as dermatitis herpetiformis (DH) and the skin biopsy shows the characteristic lineal, granular deposits of IgA in the dermal papillae. This condition affects about 15%-25% of patients with CD and its presence is known as the "card presentation" of some CD patients[31].

A GFD is the treatment of choice, although at the start of the diet, drug therapy may be added, usually dapsone, to effectively and quickly resolve the itching and the rash. This drug suppresses the inflammation of the skin but obviously has no any influence on the intestinal abnormalities. The resolution of the cutaneous lesions may be slow, and some patients must wait 1-2 years after starting on a GFD, for the complete disappearance of DH. The intestinal biopsies show identical changes to CD, but predominate with mild lesions and a patchy distribution. tTG positivity is also similar, although at lower levels, possible reflecting a milder enteropathy. One study has shown the presence of antibodies exclusively against tTG-3 (also known as epidermal transglutaminase), a cytosolic enzyme involved in cell envelope formation during keratinocyte differentiation[32]. Although these findings remain to be confirmed, these may offer some clues to understanding the difference in clinical presentation between CD patients with, or without, associated skin lesions.

## ENDOCRINE DISORDERS

CD patients can exhibit some immune-mediated endocrine disorders alongside their clinical evolution, the most common being type 1 diabetes mellitus (T1DM) and thyroid disease. Each of these conditions affects 5%-10% of CD patients throughout their lives[33].

The prevalence of CD in the T1DM patients is substantially higher than expected in the general population. Approximately one half of subjects do not have gastrointestinal (GI) symptoms and the rest have only mild digestive disturbances. Indeed, many diabetic patients undergo endoscopy to investigate the frequent GI symptoms that afflict those with T1DM. It would require little extra effort or cost, to obtain duodenal biopsies, at least once to identify CD, and the biopsy result may explain the GI procedure has been done. It is not clear what impact that discovery has, if any, on diabetic control or complications, although GI symptoms seem to improve on a GFD. The GFD influence on the control or management of thyroid disease is limited at best, and additional studies are clearly needed to reach firm conclusions[34,35].

One interesting study performed in Italian children found a high incidence of autoimmune thyroid disease in 90 of 343 (26.2%) patients with CD (62 on a GFD) and in 20 (10%) of the control subjects ($P = 0.001$). Fifty-four (15.7%) patients with CD and autoimmune markers had normal thyroid function (euthyroidism) as did 12 (6.0%) of the control subjects. Hypothyroidism was observed in 28 (8.1%) patients with CD and in 7 (3.5%) of the control subjects. Hyperthyroidism was diagnosed in four patients with CD and in none of the control subjects with autoimmune markers. An abnormal echographic pattern was seen in 37 patients with CD (16.8%) and only in one (1.6%) of the control subjects ($P = 0.002$). The high frequency of autoimmune thyroid disease found among patients with CD, even those on a GFD, may justify a thyroid status assessment at diagnosis and at follow-up evaluation of children and adults with CD[36].

## IRON-DEFICIENCY ANEMIA

Diet iron is absorbed by the proximal small intestine, the site of greatest damage in CD. It is not surprising therefore, that iron-deficiency anemia is a common finding in newly diagnosed CD. It also usually resolves with the institution of a GFD[37].

Several studies from Europe and North America have suggested that iron-deficiency anemia may be the sole manifestation of CD in the absence of diarrhea. This association may be especially high in those unresponsive to oral iron therapy[38].

Iron deficiency is common in the general population. If it occurs in young women, it is often ascribed to excess menstrual loss, an empiric therapy with oral iron supplementation is started. However, older patients or those with anemia that is refractory to treatment are often investigated further. Similarly, the persistence of anemia after menopause may be an important clue that leads to the detection of CD[39].

Indeed, female patients have undergone hysterectomies to treat the iron deficiency that persisted until the correct diagnosis was made. Anemia is rarely sought or diagnosed in children; in fact, hemoglobin is not routinely measured in children. Nevertheless, iron-deficiency anemia is a very common illness in primary care and often does not spur investigation in the younger patients. The prevalence of CD in patients referred to GI endoscopy for investigation of iron-deficiency anemia varies from 3% to 12%[40,41].

Clinicians should consider CD as a possible, although not common, cause of unexplained anemia, and gastroenterologists should biopsy the duodenum when an endoscopy is performed in patients with iron-deficiency anemia, even if biopsies are not specifically required.

## OSTEOPOROSIS

Low bone mass, is common in patients with newly diagnosed CD. The mechanism for this effect may be due to malabsorption of vitamin D and calcium and decreased intake of calcium, because of lactose intolerance. Other factors such as sex, malnutrition and physical activity, also contribute to the risk of low bone density in CD[42,43].

Decrease in bone mineral density (BMD) associated with CD responds to a GFD, with a gradual restoration to normal, over two years. The earlier in life that treatment is started, the better is the response[44]. A limited number of screening studies for CD, among patients with low bone mass (LBM), have been performed in Europe. CD was found in 3.4% in adults with LBM[45].

However, a carefully performed Canadian study in predominantly postmenopausal women has not identified an increased prevalence of CD. One likely explanation

is the way the low bone mineral density is defined. Individuals with BMD more than 2.5 standard deviations below the sex-specific peak bone mass are presumed to have osteoporosis. Therefore, it seems that screening those patients with simple postmenopausal osteoporosis, as defined by World Health Organization (WHO) criteria, is unhelpful[46].

Recently, the risk of fractures due to osteoporosis has become a major subject of interest. CD has been associated with an increase in fracture risk. What effect silent, undiagnosed CD, has on lifelong risk fracture risk, is not well known[47].

A large population-based cohort study performed in Great Britain showed that the overall hazard ratio in CD patients for any fracture was 1.30 and 1.90 for hip fracture[48].

## HYPERTRANSAMINASEMIA

The prevalence of hypertransaminasemia (HT) in children is increased in CD. In one study a total of 114 consecutive pediatric CD patients were studied (60% with classical and 40% with atypical forms). The authors found HT in 32% of patients, at the time of diagnosis. In five patients, it was the only manifestation of CD (4.3%). Patients with HT were younger (2.9 ± 0.4 year) than patients with normal aminotransferases (5.1 ± 0.5 year) ($P$ = 0.007). A higher percentage of patients with classical CD tends to have abnormal aminotransferases (73%; 95% CI = 65-81%) than do patients with atypical CD (27%; 95% CI = 19-35%) ($P$ = 0.068). A younger age was significantly associated with HT ($P$ = 0.039; OR = 0.8; 95% CI = 0.71-0.99). The aminotransferases normalized with a GFD in all 35 patients, who were followed-up for an average time of one year[49].

A significant percentage of adult patients with non-alcoholic fatty liver disease (NAFLD) have no metabolic risk factors and may be related with the concomitant presence of CD. Bardella *et al* found in a series of 59 patients that tissue transglutaminase antibodies were positive in six (10%) patients and the anti-endomysium in two (3.4%); only two (3.4%), positive for both anti-endomysium and anti-transglutaminase, demonstrated CD on histological findings. After 6 mo of a GFD, liver enzymes normalised[50].

Mild liver abnormalities are common in adult patients with celiac disease and usually resolve with a GFD. Four patients with untreated celiac disease and severe liver disease are described by Kaukinnen *et al* of Finland. Further, the occurrence of celiac disease was studied in 185 adults with previous liver transplantation using tTG and endomysium antibodies (EMA) testing. Of the four patients with severe liver disease and celiac disease, one had congenital liver fibrosis, one had massive hepatic steatosis and two had progressive hepatitis without any apparent origin. Three patients were even remitted for consideration for liver transplantation. Hepatic dysfunction reversed in all cases when a gluten-free diet was adopted. In the transplantation group, 8 patients (4.3%) had celiac disease. Six cases were detected before the operation: 3 had primary biliary cirrhosis, one had autoimmune hepatitis, one had primary sclerosing cholangitis and one had congenital liver fibrosis. Only one patient had maintained a long-term, strict gluten-free diet. The serological screening found 2 cases of celiac disease, one with autoimmune hepatitis and the other with secondary sclerosing cholangitis. The possible presence of celiac disease should be investigated in patients with severe liver disease. Dietary treatment may prevent progression to hepatic failure, even in cases in which liver transplantation is considered[51].

## NEUROLOGIC DISORDERS

Among the most common neurology problems associated with CD are peripheral neuropathy, cerebellar ataxia, epilepsy, multiple sclerosis and migraine. In a recent study of 26 patients with CD, 31% had abnormalities in neurophysiologic studies, compared with 4% of controls with reflux disease[52].

Nutritional factors have been suspected in association with neurological defects but are rarely found and their correction does not seem to influence in the prognosis. Some reports show certain neurologic symptoms that respond to a GFD, especially if it is started in the first few months after their appearance[53].

It is now evident that the link between CD and neurologic disorders results, in part, from common genetic background, most importantly, the HLA region on chromosome 6, and other markers. In addition to genetic predisposition, immunologic factors probably also play a role[54,55].

One way that this may occur is by antibody or T-cell cross-reactivity, a mechanism that is suspected of triggering the immune response in some autoimmune diseases. Alternatively, it may result from the involvement of additional autoantigens through epitope spreading.

The GFD effect on epilepsy control has been variable. In most patients, some beneficial effects have been reported such as better seizure control and a decrease in dosing of anti-epileptic medications, but without achieving a complete resolution of seizures with the diet alone[56].

Depression and other psychiatric symptoms have been reported as common complications of CD, occurring in about one third of patients. Common symptoms include apathy, excessive anxiety and irritability. All of them clearly improve after few months of adherence to a GFD[57].

## CANCER

The incidence of certain types of cancer is increased among patients with CD. These include non-Hodgkin's lymphomas (NHL) at any site, enteropathy-associated T-cell lymphoma (EATL) (a rare, high-grade T-cell non-Hodgkin's lymphoma of the small intestine), for which the outlook is poor, small intestine adenocarcinoma and esophageal and oropharyngeal carcinomas[58-60]. In one large cohort study performed on 4732 CD patients compared with 23 620 matched controls, the authors found in CD, an increased RR factor for mortality of 1.39 with a 95% CI (1.13-1.51) and for malignancy of 1.29,

CI-95% (1.06-1.55)[61]. The mechanisms responsible for the development of malignancies in CD patients are not known. The following explanations have been suggested: increased intestinal permeability of environmental carcinogens, chronic inflammation, chronic antigen stimulation, release of pro-inflammatory cytokines, immune surveillance problems and nutritional deficiencies caused by the disease or the GFD[62].

CD is clearly associated with a definite increase in the risk of developing cancer, especially EATL and other gastrointestinal cancers that are partially responsible for the overall increased mortality reported in these patients. However, the magnitude of the overall risk for NHL is much lower than previously thought, with a relative risk most probably ranging between 2 and 4[63,64]. Strict adherence to a GFD seems to protect against the development of some types of cancer[65,66].

## CONCLUSIONS

Several issues may be of help, as listed in the following decalogue: (1) Clinicians must have a better understanding of CD, have good knowledge of this common disease (1%-2% in the general population), and consider it in the differential diagnosis of multiple gastrointestinal and extraintestinal conditions. (2) It is very important to do a careful clinical history dating back to childhood, looking for a relation of periodic complaints to food ingestion, exploring the family's history of CD and searching for associated disease such as recurrent rhinitis, pharyngo-amigdalitis, otitis, sinusitis, asthma, and other immuno-allergic conditions. (3) To perform a systematic screening of CD in high risk patients such as those presenting with iron deficiency anemia, especially if it is refractory, and taking routine duodenal biopsies during upper gastrointestinal endoscopies in these patients. (4) In the presence of sustained hypertransaminasemia, in the absence of a history of liver disease, and when viral markers are negative, screen for CD, which is associated with the former conditions in around 10% of cases. (5) If endocrine disturbances are present, such as hypo- or hyperthyroidism, or positive anti-thyroidal antibodies with normal function are present, and also in the presence of associated type 1 diabetes mellitus, consider a potential case of CD. (6) Remember that screening serology, particularly the measurement of anti-transglutaminase antibodies, can sometimes be negative in adult patients. (7) Duodenal biopsy may be normal or show only minimal changes. An experienced pathologist deeply interested in the diagnosis of CD, and routinely using Marsh's classification in his or her reports, is needed. (8) Bear in mind that being DQ2 (+) is a necessary but insufficient condition, and that increasingly more cases are being diagnosed in DQ2 (-) individuals. (9) In doubtful cases, the introduction of a GFD may be suggested for a minimum of six months, after which the clinical and laboratory response is observed ("ex-iuvantibus" diagnosis). (10) Finally, the Gastroenterology Units in the tertiary hospitals should include monographic "Small Bowel Sections" for the routine study of these patients, and should be fitted with modern functional techniques and endoscopic procedures for their study, such as video-capsule and double-balloon enteroscopy.

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*EDITORIAL*

# Current status and prospects of clinical proteomics studies on detection of colorectal cancer: Hopes and fears

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## Abstract

Colorectal adenocarcinoma (CRC) is the third most common type of cancer and the fourth most frequent cause of death due to cancer worldwide. Given the natural history of CRC, early diagnosis appears to be the most appropriate tool to reduce disease-related mortality. A field of recent interest is clinical proteomics, which was reported to lead to high sensitivity and specificities for early detection of several solid tumors. This emerging field uses mass spectrometry-based protein profiles/patterns of easy accessible body fluids to distinguish cancer from non-cancer patients. These discrepancies may be a result of: (1) proteins being abnormally produced or shed and added to the serum proteome, (2) proteins clipped or modified as a consequence of the disease process, or (3) proteins subtracted from the proteome owing to disease-related proteolytic degradation pathways. Therefore, protein pattern diagnostics would provide easy and reliable tools for detection of cancer. This paper focuses on the current status of clinical proteomics research in oncology and in colorectal cancer especially, and will reflect on pitfalls and fears in this relatively new area of clinical medicine, which are reproducibility issues and pre-analytical factors, statistical issues, and identification and nature of discriminating proteins/peptides.



**Key words:** Proteomics; Colorectal cancer; Protein profiling; Diagnosis; Biomarkers; Statistical issues

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## BACKGROUND

In order to improve early detection, monitor disease outcome and find targets for more individualised therapy, there is an urgent demand for new biomarkers in oncology. A field of recent interest is clinical proteomics, which has been reported to lead to high sensitivity and specificities for early detection of several solid tumors[1,2]. This emerging field uses mass spectrometry-based protein profiles/patterns of easy accessible body fluids to distinguish cancer from non-cancer patients. This would offer a solution to the problem of cancer often being diagnosed in late stages, when curative resection of the diseased organ is no longer possible and the disease has already metastasized, thus dropping survival rates dramatically. However, after the initial positive announcements in early 2002, criticisms were raised about several aspects of serum proteomics. In this paper we describe the hopes and fears for the introduction of clinical proteomics for early detection of Colorectal adenocarcinoma (CRC).

## COLORECTAL CANCER

Colorectal adenocarcinoma is the third most common type of cancer and the fourth most frequent cause of death due to cancer worldwide. Worldwide almost one million new cases occur annually, amounting to 492 000 related deaths[3]. In developed countries it is the second most common type of tumor, with a lifetime risk of 5%, but its incidence and mortality is now decreasing[4,5]. Surgery is the cornerstone of therapy when the disease is confined to the bowel wall. This results in 70%-80% of patients who have tumors that, when diagnosed, can be resected with curative intent[6]. After curative surgery the five-year survival rate for patients with localised disease is 90%, decreasing to 65% in the case of metastasized disease to the lymph nodes. Adjuvant radiation therapy, chemotherapy, or both are useful in selected patients. Classification of tumors into pathogenetical subtypes with distinct clinical courses enables clinicians to target therapy. For CRC, the TNM staging system remains the gold standard and relies entirely on the morphological appearance of the tumor. However, tumors with similar histopathological characteristics may have different clinical outcomes and responsiveness to therapy[7]. Therefore, more individualised treatment would benefit the patient and may avoid unnecessary morbidity. Nonetheless, early detection of CRC will increase survival the most, in view of the fact that it is well recognized that CRC arises from a multistep sequence of genetic alterations that result

in the transformation of normal mucosa to a precursor adenoma and ultimately to carcinoma. Given the natural history of CRC, early diagnosis appears to be the most appropriate tool to reduce disease-related mortality[8-10].

## BIOMARKERS

In cancer research, biomarkers are molecules that indicate the presence of cancer in the body. Most biomarkers are based on mutations in genes or abnormal changes in RNA, proteins and metabolites. Since the molecular changes that occur during tumour development can take place over a number of years, some biomarkers can potentially be used to detect early colorectal cancer. Furthermore, they might be used to predict prognosis, monitor disease progression and therapeutic response. Gion *et al* classified different circulating biomarkers according to their clinical application[11]. These candidate biomarkers, however, are frequently found in relatively low concentrations amid a sea of other biomolecules. Therefore biomarker research and possible diagnostic tests depend critically on the ability to make highly sensitive and accurate biochemical measurements. Ideally, such biomarkers should be specific to the disease and easy accessible, such as in serum, plasma or urine, increasing their clinical applicability.

Carcinoembryonic antigen (CEA) is the best-characterised serologic tumor marker for CRC. However, its use as a population-based screening tool for early detection and diagnosis of CRC is hindered by its low sensitivity and specificity. Fletcher demonstrated that for screening purposes in a normal population, a cut-off concentration of 2.5 μg/L CEA would yield a sensitivity of 30%-40%. Based on these data, he calculated that there would be 250 false positive tests for every true positive test; i.e. a patient with cancer. Furthermore, 60% of the cancers would not be detected. The same poor sensitivity applies for diagnosis of CRC. In addition, as CEA can be elevated in the absence of malignancy, specificity is also impaired [12-15].

Faecal occult-blood testing (FOBT) is another biomarker for which clinical trials have shown evidence for a decreased risk of death. This approach is a non-invasive option that limits the need for follow-up colonoscopy to patients with evidence of bleeding. However, neoplasms bleed intermittently, allowing many to escape detection with faecal occult-blood testing. Annual retesting is therefore necessary but is still insufficient, detecting only 25 to 50% of colorectal cancers and 10% of adenomas. The specificity of FOBT is also limited by frequent false positive reactions to dietary compounds, medications, and gastrointestinal bleeding from causes other than colorectal cancer[16-18].

## A NEW DIAGNOSTIC PARADIGM: CLINICAL PROTEOMICS

In 2002, several studies demonstrated discrimination of patients with various cancers from healthy subjects on the basis of presence/absence of multiple low-molecular-weight serum proteins using SELDI-TOF mass spectrometry technologies[19-22]. The authors hypothesised that proteomic patterns are correlated to biological events occurring in the entire organism and are likely to change in the presence of disease. New types of bioinformatic pattern recognition algorithms were used to identify patterns of protein changes in order to discriminate cancer patients from healthy individuals with promising results.

Petricoin and his co-workers stated that finding a single disease-related biomarker is like searching for a needle in a haystack as each entity has to be separated and identified individually[23,24]. Moreover, they postulated that the blood proteome constantly changes as a consequence of the perfusion of the diseased organ adding, subtracting, or modifying the circulating proteome. These differences might be the result of proteins being abnormally produced or shed and added to the serum proteome, proteins being clipped or modified as a consequence of the disease process, or proteins being subtracted from the proteome owing to disease-related proteolytic degradation pathways. Therefore, protein pattern diagnostics would provide easier and more reliable tools for detection of cancer. The advantages of the SELDI proteomic pattern approach were stressed in several papers. In addition to the high sensitivity and specificity, cost-effectiveness, easy accessibility of body fluid and especially the high-throughput, ultimately allowing application in future screening studies, were discussed[20,25]. After these hopeful voices, soon critical notes were made on analytical reproducibility and the use of the so-called black box approach, lacking identification of discriminating proteins.

In the next paragraphs, this paper will focus on the current status of clinical proteomics research in oncology and will reflect on pitfalls and fears in this relatively new area of clinical medicine, including reproducibility issues and pre-analytical factors, statistical issues, and the identification and nature of discriminating proteins/peptides.

## REPRODUCIBILITY ISSUES AND PRE-ANALYTICAL FACTORS

Boguski and McIntosh were among the first to argue that serum proteomics may be susceptible to observational biases. They stated that any confounding factor could conceivably cause a phenotypic response that might be confused with a specific characteristic of the disease process under study[26]. Confounding factors, which not only include smoking, diet and preoperative stress, but also logistic factors such as sample collection and sample quality, make a reliable and clear differentiation between a normal or malignant status hazy. Another cause for concern, mentioned in this study, is the sample quality and number. The authors favoured use of homogeneous groups with sufficient sample size and stringent standard procedures for serum collection, which is an aspect that is also advocated in other studies[27,28]. Another critical study questioned the reliability of the presence of statistically significant signals at M/Z values less than 500, as used in one of the first studies. Sorace *et al* claimed that the presence of statistically significant bands of low M/Z includes degradation products of higher molecular weight macromolecules or

**Table 1 Recommendations of various pre-analytical variations from three MALDI-TOF based reproducibility studies**

| | Blood component | Peptide isolation | Temperature before sample handling (℃) | Time before centrifugation | Storage of serum | Freeze/ thaw cycles | Circadian rhythm effect |
|---|---|---|---|---|---|---|---|
| Baumann *et al* | Serum plasma | C3, C8, C18 beads | 21 | < 30 min | -80 ℃ | 1 | NA[1] |
| de Noo *et al* | Serum | C8 beads | 21 | Ideally < 30 min, practically < 2-4 h | NA[1] | 2 | No effect |
| West-Nielsen *et al* | Serum plasma | C8 beads | 21 | < 8 h | -20/-80℃ | 1 | NA[1] |

[1]Not applicable for this study.

a matrix effect. Furthermore, this study raised caution regarding poor reproducibility of experimental conditions of chip-based mass spectrometry[29]. This is also reported by another group, which described the poor reproducibility of the SELDI-TOF ovarian cancer data. Baggerly and colleagues postulated that this could partly be contributed to baseline correction, poor sample features in noise regions and even a change of protocol mid-experiment[30]. Most importantly, the promising results that were reported earlier could not be reproduced, therefore stressing the importance of standardised approaches and stringent experimental design. Furthermore, their study pointed out that strong pre-processing of the protein spectra is required in order to obtain reliable classification results in the search for new biomarkers.

Possible confounding factors can be categorized into three sources of variation and bias: biological variation, pre-analytical variation and analytical reproducibility. Biological variation, consisting of both environmental and individual factors, such as race, age, diet, smoking, stress, general physical condition and the use of drugs, may also influence serum protein profiles. However, at the present time no data have been published on this source of variation. Nevertheless, in a previous study our group analyzed pre-analytical and reproducibility issues of our MALDI-TOF approach[31]. The pre-analytical variations corresponded to the logistical conditions in the routine clinical setting; i.e. the effects of sample handling and storage. So far, only a few other studies have reported on the effects of different serum sample preparations and the use of a magnetic-beads-based approach to capture and concentrate serum proteins for MALDI-TOF mass spectrometry[32-34]. Villanueva *et al* mostly focused on influences of different magnetic bead capturing and automation on the reproducibility of serum protein profiles, while Baumann and co-workers mainly studied pre-analytical variation of sample handling.

In Table 1, results for sample handling experiments of the above mentioned studies are summarized. For clinical studies, the use of two freeze/thaw cycles is recommended in three manuscripts. This is mainly due to logistical reasons, such as the ´standard´ for centralized sample collection in large hospitals. The point all authors agreed on is the influence of sample handling; i.e. the time venous blood is left to stand before serum centrifugation. This aspect appears to account for the largest effect on serum or plasma protein profiles. Consequently, standardized sample collection and a well-documented population are recommended in all studies. Standardized protocols should be used from the point of sample collection, sample handling, and storage and freezing of the samples. Although the importance of homogeneity and uniformity within sample groups must once again be stressed, variation of such factors cannot totally be excluded in a clinical setting. In all, when these recommendations are strictly followed and both clinical and analytical factors are controlled, we are confident that the methodology can be standardized to a level that allows application as a tool in biomarker discovery.

## STATISTICAL ISSUES

As in all research with high dimensional data, two practical realities constrain the analysis of mass spectra in proteomics. The first is the 'curse of dimensionality', which means the number of features characterizing these data is in the thousands or tens of thousands. The second is the 'curse of dataset sparsity', which means the number of samples is limited. Somorjai *et al* showed the influences of these two curses on classification outcomes. Both the sample per feature ratio, which should ideally be 5 to 10, and feature selection are of pivotal importance for reliable classification and biological optimal relevance[35,36].

Previous to any feature selection or classification, raw mass spectra have to be submitted to so-called pre-processing. During pre-processing, the noise of protein/peptide mass spectra is reduced and the spectra are normalised. Furthermore, smoothing, binning and baseline correction are also performed during pre-processing of the data. Currently, there is a lot of discussion among several groups on how to establish the best method, because data pre-processing is extremely important. There are complex interactions between baseline subtraction, normalization, noise estimation, and peak identification, and therefore these steps should not be considered in isolation[31,37-40].

Another recurring topic for debate is the bioinformatic approach and statistical analysis of protein spectra. Clinically, the most relevant is the issue of an independent validation set for the classification of diseased versus healthy individuals. This is primarily based on a specific problem in the discovery-based research field of clinical proteomics, namely overfitting. Overfitting may occur in the analysis of large datasets when multivariate models show apparent discrimination that is actually caused by data over-interpretation, and hence give rise to results that are not reproducible[30,41,42]. The chance of overfitting, however, can be reduced by appropriate application of validatory estimation and assessment, such as through application of double cross-validation, when properly implemented[43]. Although we have shown

this in a previous study, the general opinion is in favor of performing a classification study with independent validation. In addition, feature selection is also given a lot of attention by statisticians in the field. Several experimental investigations have been made with different peak-feature selection methods. A common approach so far is analysing the data in two phases. First, the peaks in the spectra are extracted and quantified. Secondly, a resulting matrix of peak quantifications is created. For more detailed information on this statistical matter, we refer the reader to the literature[37,44-46].

## IDENTIFICATION AND NATURE OF DISCRIMINATING PROTEINS

The controversy regarding the use of protein profiles as a pattern diagnostic, without identification of the individual diagnostic biomarkers, remains to be solved before its clinical application. Whereas the first clinical proteomics studies published their classification method mainly as a black box study, at present identification of the most discriminating proteins or peptides is required for publication in most scientific journals. Identification and functional analysis of these discriminating proteins/peptides might render new insights on tumour development and environmental responsiveness, which could eventually be translated into new diagnostic and prognostic insights for the clinician. Unfortunately, little success has been achieved to date in assigning reproducible discriminating biomarkers[35,42].

Furthermore, several studies have identified their discriminating peaks as components of the coagulation cascade or complement system[47-51]. Therefore, in contrast to the original reflection that discriminating proteomic patterns would identify cancer-specific proteins, it appears that these potential markers belong to the normal serum and plasma proteome. Consequently, some investigators have argued that low molecular weight proteins in serum and the serum peptidome are biologically aspecific and therefore do not yield any reliable biomarkers in the currently technically available mass range[29,52]. Others have proposed that the discriminatory protein peaks represent acute phase reactants that are present in serum in extremely high concentrations[49,53]. Conversely, a study recently reported that although discriminating peptides do indeed belong to the well-known coagulation and complement pathways, their patterns or signatures can nevertheless indicate the presence of cancer. Villanueva *et al* showed that most of the cancer-type specific biomarker fragments were generated in patient serum by enzymatic cleavage at previously known endoprotease cleavage sites after the blood sample was collected[54,55]. They postulated that the discriminating peptides originated after *ex vivo* proteolysis by tumor specific proteases of high abundance protein fragments primarily generated by the coagulation and complement enzymatic cascades. In view of this, they consider these cancer-specific low molecular weight proteins in the serum peptidome to be an indirect snapshot of the enzyme activity in tumor cells. We support their hypothesis that proteolytic process profiles in the serum peptidome hold important information that may have direct clinical utility as a surrogate marker for the detection and classification of certain types of tumors. Unique proteases may be shed by tumor cells or reflect activity of the host immune response, which may contribute to new proteins such as chemokines and lymphokines. These processes result in subtle changes in low molecular proteomic signatures, which may ultimately be used for classification methods in various cancers and diseases in the future[54]. Proteases have been extensively implicated in the development and progression of cancer[56,57]. Song *et al* recently stated that proteolytic processing of high abundance host-response proteins actually amplifies the signal of potentially low-abundance biologically active disease markers such as proteases. Therefore, it might be expected that more convenient and reliable blood proteins and peptides simply serve as an endogenous substrate pool for proteases as surrogate markers for the detection and classification of cancer[58].

Another recurrent topic of debate concerns the type of blood component that is best for protein profiling and peptidome analysis. Some investigators favour the use of plasma because they presume that, in serum, ongoing enzymatic activity occurring during clotting is likely to cleave even proteins that are not involved in biologically relevant pathways[53,59]. Others, however, advocate the use of serum. We support the hypothesis that since the kidneys rapidly clear peptides smaller than 4 kDa, which are generated *in vivo* in the circulation, the majority of peptides in blood samples exist from *ex vivo* proteolysis. This explains the possibility that low abundance proteins, including possible tumor markers, may be totally obscured and not retraceable during direct mass spectrometry. However, it has recently been shown that exogenous proteases are functionally measurable in serum, however in higher concentrations than in plasma[54].

Functional proteomics studies allow the investigation of environmental factors over time, rendering the monitoring of metabolic responses to various stimuli. Hence, post-translational modifications can be studied, whereas they cannot be detected by genomic studies. Post-translational modification changes, like glycosylation of proteins and lipids, are a common feature in colorectal cancer influencing cancer cell behaviour and can be detected using mass spectrometry due to characteristic mass shifts[60]. We expect that both phosphoproteomics and/or glycoproteomics, enabling study of crucial post-translational modifications of proteins in the cancer pathway, will revolutionize our understanding of the function of these proteins and hence render new insights for monitoring and therapy.

## CLINICAL PROTEOMICS IN CRC

Until present, few protein profiling studies have been published on the detection of CRC, two being based on SELDI-TOF and one on MALDI-TOF mass spectrometry. The first SELDI-TOF study showed seven potential biomarkers that could differentiate CRC patients from those with colorectal adenoma with a sensitivity of 89% and specificity of 83%. The seven potential biomarkers have a large range in mass values, differing from 4654 to 21 742 Da[61]. A more recently published study found 5 possible bi-

omarkers to differentiate between healthy control subjects and CRC patients. The study consisted of a training set of samples from 40 patients with colorectal cancer (all Dukes' D) and 49 healthy controls. The second set included samples from 37 patients with colorectal cancer (1 Dukes' A, 2 Dukes' B, 12 Dukes' C, 17 Dukes' D, 5 unknown) and 31 healthy controls. For three of these potential markers, they found a sensitivity and specificity between 65% and 90%. They reported that m/z 3.100, 3.300, 4.500, 6.600 and 28.000 were the most important biomarkers. The total sample set showed that 1 of 1 Dukes' A, 1 of 2 Dukes' B, 11 of 12 (91.7%) Dukes' C, and 47 of 57 (82.5%) Dukes' D were correctly classified. Stratification by Dukes' stages showed a significantly better sensitivity of the classification trees (91.7%, 11/12) compared to CEA (25.0%, 3/12) in Dukes' C colorectal cancer, although at stage D CEA performed better. No conclusions can be drawn on the performance of our classification trees at earlier stages of colorectal cancer due to limited samples, but 2 of 3 patient samples from stage A and B were correctly classified by the trees and none when using the clinical cut-off for CEA[59]. Our group used MALDI-TOF mass spectrometry to differentiate CRC patients from healthy controls. In a randomized block design, pre-operative serum samples obtained from 66 colorectal cancer patients and 50 controls were used to generate high-resolution MALDI-TOF protein profiles[42]. After pre-processing of the spectra, linear discriminant analysis with double cross-validation, based on principal component analysis, was used to classify the protein profiles. Thirty-four patients out of thirty-seven with early stage disease (stage 1 and 2) and all patients with stage 3 or 4 disease were correctly classified as having cancer. For the misclassified control subjects, it was not possible to retrieve the current physical state as it concerned anonymous healthy controls.

A total recognition rate of 92.6%, a sensitivity of 95.2% and a specificity of 90.0% for the detection of CRC were shown. In our study two first principal components accounted for most of the between-group separation, both with a m/z between 1 and 2 kDa.

Although much research has been done using 2D gel electrophoresis to detect possible biomarkers and targets for CRC, this falls outside the scope of this review since this technique cannot be scaled up to a directly applicable diagnostic test. On the other hand, a screening assay based on an APC protein truncation test has recently been proposed and other studies mention the potential use of protein microarrays[2,60,62,63]. However, studies linking large protein expression patterns with clinical outcome in colorectal cancer are still in their infancy. To be able to predict occurrence of disease and treatment outcome, more studies on genotype-phenotype correlations are needed both in sporadic and in hereditary colorectal cancer.

## FUTURE PERSPECTIVES

The best anticancer strategies still rely on early detection followed by close monitoring for early relapse so that therapies can be appropriately adjusted[64]. In addition, new targets for therapy are a constant subject of study in oncology. In fact, increased understanding of the molecular mechanisms of cancer progression may refine treatment and management of patients. Advances in genomics and proteomics may lead to earlier detection of cancer thus enabling a more precise classification of (smaller subsets of) patients based on their predicted response to individual therapies. Conceptually, proteomics is more suitable than genomics for novel targeted therapies, since most protein biomarkers are based on aberrant protein signalling circuits represented by post-translational modifications. The dynamic range of the proteome allows more insight in the functional state of a cell, tissue or organ over a period of time. Besides, protein profiling and classification of several components of multiple aberrant cell signalling cascades would be expected to predict disease behaviour better than just single pathways in isolation[62]. Therefore, proteomics could be expected to render better insight in pathogenetic mechanisms, disease progression and treatment response. This is of paramount importance as cancer advances dynamically and affects heterogeneous cell populations, either as a part of cancer or as a part of a tumor-host reaction[49,65].

Further refinement of serum protein profiles is needed before these mass spectrometry-based techniques become part of clinical routines. Currently, several studies have carefully evaluated reproducibility, automation, sample throughput and sensitivity of serum proteomic techniques. The first problems related to these factors seem to have been overcome due to stringent standardised approaches as described earlier. However, proteomics studies still have several drawbacks: (a) current tools only allow narrow-range analyses, (b) identification of proteins of interest remains cumbersome, (c) protein studies address mixtures of high complexity. Hence, due to the dynamic ranges of the human proteome and the lack of amplification methods in protein studies, targeted proteomics techniques for (quantitive) identification of low-abundant proteins have to be investigated further[66]. Another approach to studying proteins at a functional level might be the use of array-based proteomics platforms. This technique offers the potential for highly multiplex and sensitive analysis of serum or tumor proteins[62]. Using this direct approach to study the proteomic circuitry would theoretically allow for the creation of functional signalling maps of cancers, even at the level of the individual patient. Regarding identification of potential biomarkers, limitations of direct MS/MS have been stressed previously as well as the fact that antibody-approaches may yield higher sensitivity[53,54].

In the next era, research in oncology will drift to more individualised medicine. In this view, molecular profiling forms a welcome addition to the pathology report of cancer. Until now, histopathological staging and demographics have been used to predict disease outcome. However, we believe that protein profiling and other proteomics techniques may lead to more individualised medicine and tailor made therapy[67,68]. At first, both approaches should be used complementarily instead of competitively.

It is unlikely that in the next decade, serum protein profiles will replace the current gold standard, which is colonoscopy, for the diagnosis of CRC. Nevertheless, we hypothesise that MALDI-TOF based serum protein profiles, once validated in independent studies, could be used

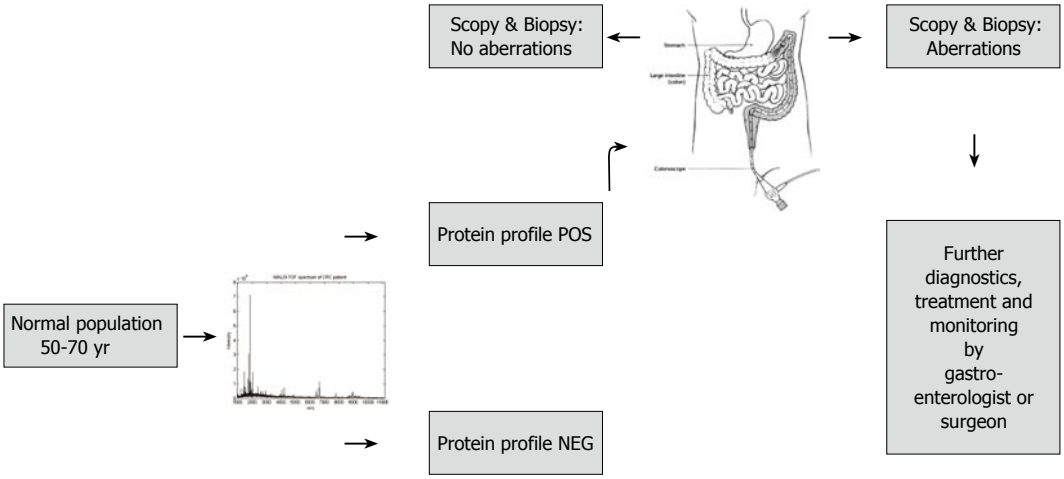


**Figure 1** Flow chart of possible clinical application of MALDI-TOF.

as selection criteria for the more invasive and time-consuming diagnostic colonoscopy (Figure 1). Eventually, with the present debate on screening programs for colorectal cancer in several countries, clinical proteomics may replace and surpass the use of faecal occult-blood testing (FOBT). When used in independent validation studies, sensitivity and specificity remain about 90%. Protein profiling might even replace FOBT, since this approach has a lower specificity and a number of disadvantages. Non-bleeding tumors and, more relevantly, polyps and adenomas cannot be detected using FOBT, whereas we expect to realise this detection with serum protein profiling within the next decade[17,18].

Although the current reality may not have kept pace with previous expectations and the translation from bench to bedside is more laborious than initially thought, there is supporting evidence for the potential vast use of clinical proteomics in oncology. Particularly, this potential will be realized when technical innovations to further increase sensitivity and specificity of proteomic techniques are implemented and more sensitive methods for protein identification on alternations are developed. In combination with the use and set-up of well-defined cases together with well-documented serum banks, including not only for CRC samples but also inflammatory disease and polyps, serum protein profiling may propel diagnostic research in CRC in the right direction.

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*REVIEW*

# Surgical solutions to the problem of massive weight loss

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## Abstract

In response to the global rise in obesity, bariatric surgery has become increasingly more popular and successful. As a result, the demand for body contouring following massive weight loss is rapidly growing. Although bariatric procedures may produce impressive weight loss, people who achieve massive weight loss are often unhappy with the hanging folds of skin and subcutaneous tissue that remain. This review examines the nature of the post-bariatric deformity in each body region and briefly reviews common approaches to their treatment.



**Key words**: Weight loss; Bariatric surgery; Body contour surgery

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## INTRODUCTION

As we enter the 21st century, a new health crisis has dawned upon affluent western societies, the plague of obesity. In the United States, the situation is particularly acute with recent estimates showing that nearly 65% of adult Americans (127 million) are over their ideal body weight, 30% (48 million) are obese [Body mass index (BMI) $\geqslant$ 30], and nearly 5% (9 million) are morbidly or severely obese (BMI $\geqslant$ 40)[1]. Further, the obesity epidemic has expanded globally, with approximately one-fifth of the one billion overweight or obese people in the world being Chinese[2]. Although the ultimate impact of mild obesity on survival has recently been challenged, there is no doubt that morbid or severe obesity is detrimental to both the physical and psychological well being of the afflicted patient[3,4]. Morbidly obese patients have often reached a point of no return in which they are too large and physically unable to exercise.

With some exceptions, the feed-forward cycle of weight gain in the morbidly obese can be interrupted only by physically restricting oral intake. This surgical subspecialty, known as bariatric surgery, has exploded in popularity in the last decade largely because of improved outcomes, enhanced patient safety and superior techniques and devices[5,6]. Although early bariatric procedures produced impressive weight loss, they also resulted in significant morbidity both initially because of wound healing problems and anastomotic leakage, and ultimately as a result of their malabsorptive effects[7]. Modern laparoscopic surgical approaches have minimized wound healing morbidity, while the improvement of more restrictive procedures, such as the minimally invasive LAP-Band®, have made weight loss surgery in morbidly obese patients a reliable and relatively safe procedure[8-12]. According to the most recent available data, approximately 170 000 bariatric surgeries were performed in the United States in 2005[13].

However, for the morbidly obese patient, the massive weight loss that follows successful bariatric surgery is only the first step in the process[14]. After massive weight loss, patients are left "deflated". The excess skin that hangs from the torso, abdomen and extremities is not only extremely unsightly, but can be painful and susceptible to recurrent intertriginous infections[15,16]. In order for these patients to complete their transformation to a formerly morbidly obese patient, a plastic surgeon must address the deformities resulting from the massive weight loss[17].

This type of extreme body contouring has grown concomitantly with the popularity of bariatric surgery, with 55 927 procedures performed in 2004[18]. More than just excising excess skin and subcutaneous tissue, post-bariatric body contouring requires unique insights into the nature of the post-bariatric deformities[19]. In addition, there must be an appreciation of the fact that massive weight loss patients, because they are often relatively malnourished or anemic, constitute a distinct surgical cohort that must be carefully evaluated pre-operatively[20,21]. In the following sections, we will examine the nature of the post-bariatric deformity in each body region and briefly review common approaches to their treatment.

## TORSO/ABDOMEN

In formulating a strategic approach to the massive weight loss patient, the abdomen is usually the first body territory

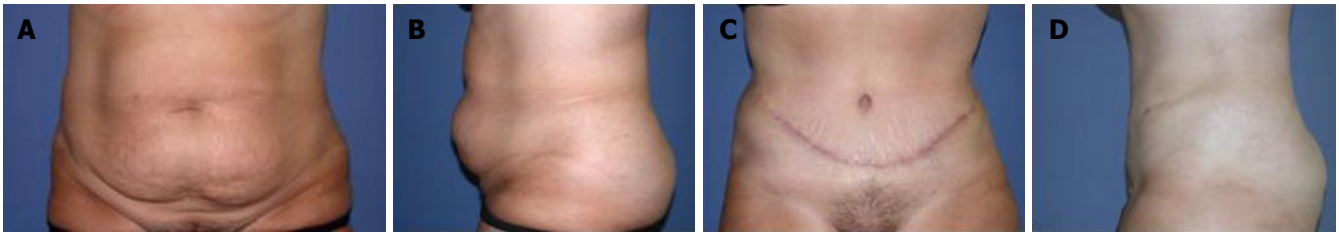


**Figure 1** Typical frontal (**A**) and lateral (**B**) appearance of the anterior abdominal wall of a (non-weight loss) post-partum female. In addition to excess skin and subcutaneous tissue, there is significant laxity of the anterior abdominal wall of this 50-year-old female; **C** and **D**: Post-operative appearance several months after standard abdominoplasty.

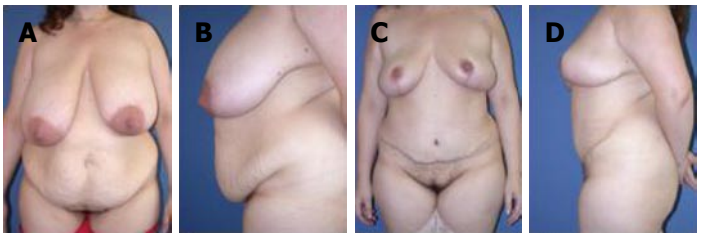


**Figure 2** Typical frontal (**A**) and lateral (**B**) pre-operative appearance of a panniculectomy candidate; This 41-year-old patient is status post a 60-lb weight loss. In addition to the unsightly appearance, the hanging pannus can be the source of pain and recurrent infection. Removal of the pannus (in this case with umbilical translocation) even without plication of the underlying abdominal wall resulted in significant improvement in contour and relief of symptoms (**C** and **D**). The patient also underwent a concurrent bilateral mastopexy.

addressed. The appropriate surgical intervention depends upon the amount and distribution of excess skin and subcutaneous tissue as well as the degree of abdominal wall laxity/integrity. In general, as the amount of weight loss increases, so does the aggressiveness of the surgical intervention.

For skin excess limited to the anterior abdomen, as might occur after moderate weight loss or pregnancy, a traditional abdominoplasty will suffice. In this procedure, the skin and subcutaneous tissue between the umbilicus and pubis is excised in an elliptical pattern, leaving a scar concealed within the underwear/bikini line[22]. Since there is usually an associated abdominal wall laxity, prior to closing the incision, the anterior abdominal fascia is plicated, with particular emphasis on re-creation of an appropriate waist in females[23] (Figure 1A-D).

In some weight loss patients, the resulting abdominal pannus consisting of loose hanging skin and subcutaneous tissue can be the source of significant discomfort, recurrent infection and difficulty with personal hygiene[15]. A simple elliptical excision of the pannus, which can weigh 25 kg or more, can provide immediate relief of the aforementioned symptoms, with minimal morbidity[24]. In addition, a panniculectomy is often the only post weight loss surgery that may be covered by a third party payer[25] (Figure 2A-D).

In most massive weight loss patients, however, the excess skin and subcutaneous tissue is not confined solely to the anterior abdomen[26,27]. In order to excise this "cone-like" circumferential excess, a circumferential excision or "belt lipectomy" is required[28,29]. The amount of tissue removed is tailored to the individual patient's needs. An optimal excision will not only tighten and flatten the lower abdomen but will also give a beneficial lift to the buttocks, mons pubis and lateral thighs[29] (Figure 3A-D).

In some patients (usually those with the most significant excess), the circumferential excision alone is not sufficient to address the horizontal dimension of the excess tissue. In these patients, an anterior ellipse or fleur-de-lis pattern of excision is required[30,31]. For patients who have had previous "open" bariatric surgery (and therefore already have a vertical midline scar), the additional scar burden is minimal. Even for patients without a pre-existing midline scar, the trade-off for optimal abdominal contour is usually acceptable.

Massive weight loss patients undergoing abdominal contouring procedures often have associated abdominal hernias which are not always evident on pre-operative physical exam[32,33]. The surgeon should be prepared to repair these in the course of the abdominal contouring. General complications of abdominal contouring include skin necrosis, persistent paresthesias of the abdominal wall, seroma, infection and wound dehiscence[16,34-36]. Patients are encouraged to refrain from smoking for at least 3 wk prior to surgery, as tobacco use has been shown to significantly increase the morbidity of body contouring procedures[37].

## UPPER TORSO/BREAST

Following massive weight loss, in both males and females, excess rolls (sometimes three or more per side) of skin and subcutaneous tissue remain. Because of firm underlying fascial attachments, though these may be improved somewhat by belt lipectomy[29,38], they can only be completely ablated by direct excision[26]. Usually, the deformity caused by these folds is significant enough that patients are willing to trade a horizontal scar across the upper back (hidden in the bra line in females). Furthermore, in women this excess tissue may be transferred anteriorly and used for autologous breast augmentation (see breast discussion below)[39,40].

The major deformity of the breast after massive weight loss in both men and women is ptosis or sagging of the breast which leaves the nipple areola complex (NAC) below its appropriate anatomic position. In males, the aim is to excise the excess skin and subcutaneous tissue while maintaining a flat breast profile[41]. Various patterns of

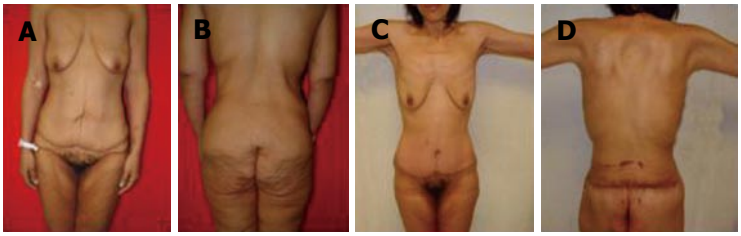


**Figure 3** A 34-year-old female status post 120-lb weight loss. Note the deflated, empty breasts and excess anterior abdominal skin and subcutaneous tissue anteriorly (**A**) and the excess skin and subcutaneous tissue posteriorly as well as the sagging buttocks (**B**); One month after circumferential abdominoplasty (and bilateral brachioplasty), there is significantly improved contour with re-creation of a feminine waistline (**C**); In addition, the buttocks have been lifted as a result of the circumferential excision (**D**). The patient is awaiting bilateral breast augmentation and bilateral medial thigh lift.

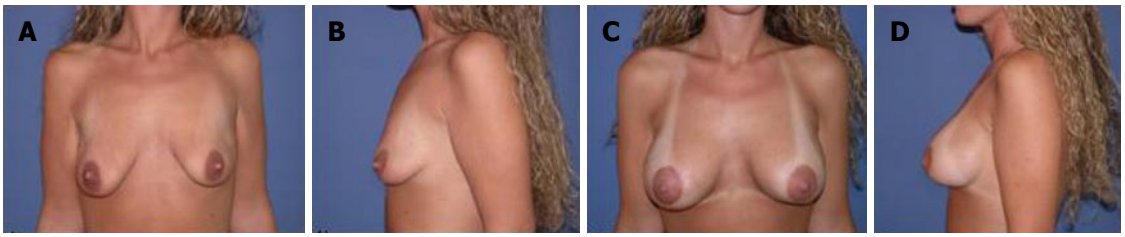


**Figure 4** Pre-operative appearance of a 23-year-old post-partum female after 60 lb weight loss. The breasts are deflated and ptotic (**A** and **B**). Status post bilateral breast augmentation (with saline prostheses) and right sided circumareolar mastopexy (**C** and **D**). Note the improved projection, especially in the upper pole. Additionally, the pre-operative asymmetry has been corrected.

excision exist to achieve this goal. In extreme cases where the NAC must be moved a long distance superiorly (more commonly in females), it can be removed and replaced as a full thickness skin graft[42]. Patients must be warned that this will cause the NAC to become insensate and may lead to de-pigmentation[43].

Although the NAC can be returned to its normal anatomic position by various means (at the level of the inframammary fold for women), a major challenge in female weight loss patients is recreation of breast fullness and projection. In most cases of massive weight loss, women are left with deflated empty "pancake" breasts. For female patients, the goal is to excise excess breast envelope skin while preserving as much volume as possible.

For most massive weight loss patients, satisfactory volume and projection can only be achieved with breast implants placed in combination with a breast lifting skin excision or mastopexy[19,32,39,44] (Figure 4A-D). The choice of implant used for breast augmentation is currently limited to saline-filled, silicone-shelled devices or (for surgeons involved in certain manufacturer study protocols) silicone-filled, silicone-shelled devices (since 1992 the Food and Drug Administration (FDA) has banned the routine use of silicone-filled implants in the United States, although they remain largely the implants of choice in Europe and the rest of the world and are available to a handful of surgeons in the United States who are part of ongoing clinical trials concerning their use).

The most common complication of these devices (regardless of the implant filler material used) is capsular contracture, in which excessive fibrous scar tissue forms around the implant leading to firmness, distortion of the implant and pain[45,46]. Implant removal or revision of excessive scar tissue may be required in 15%-35% of cases[46,47]. Other known but less frequent complications of breast implants are infection, malposition and rupture[45]. Breast implants have not been shown to cause autoimmune disorders[48,49] or increase the risk of (delayed detection) of breast cancer, although special mammographic views may be required[50-52].

In some patients, autologous volume augmentation can be achieved by recruiting local folds of tissue that might be otherwise excised in the course of body contouring. Most commonly the excess folds of the upper lateral chest or back tissue are de-epithelialized and rotated anteriorly into the breast[39,40]. Other surgeons utilize excess tissue on the upper anterior abdomen from a "reverse abdominoplasty". Common complications of breast lift with or without autologous augmentation are hematoma, infection, seroma and changes in NAC appearance and sensitivity (although many massive weight loss patients have little or no NAC sensitivity pre-operatively)[53-55]. In rare instances, the NAC may be partially or totally lost secondary to ischemic changes caused by surgery and patients should be fully informed pre-operatively[56] of this unfortunate possibility.

## EXTREMITIES

Contour deformities of the extremities after massive weight loss can be quite significant. Large amounts of excess skin draped from the proximal half of the arm gives the appearance of a "bat wing", while excess skin hanging from the thigh is unsightly and prevents patients from wearing bathing suits and shorts[19]. The unsightly appearance of their extremities will often prompt patients to wear long sleeves and pants at all times. To correct these deformities, the excess tissue must be directly excised. The pattern of excision used is similar for both the arms and the legs, since the deformity is essentially the same (a circumferential excess)[26,57,58].

For patients with a mild excess of tissue, the incision can usually be hidden in either the axilla (for a brachioplasty or arm lift)[59] or the groin (for a thigh lift)[60-62] using an elliptical pattern of excision perpendicular to the long axis of the extremity. This type of excision ("mini" brachioplasty or thigh lift) is usually insufficient to

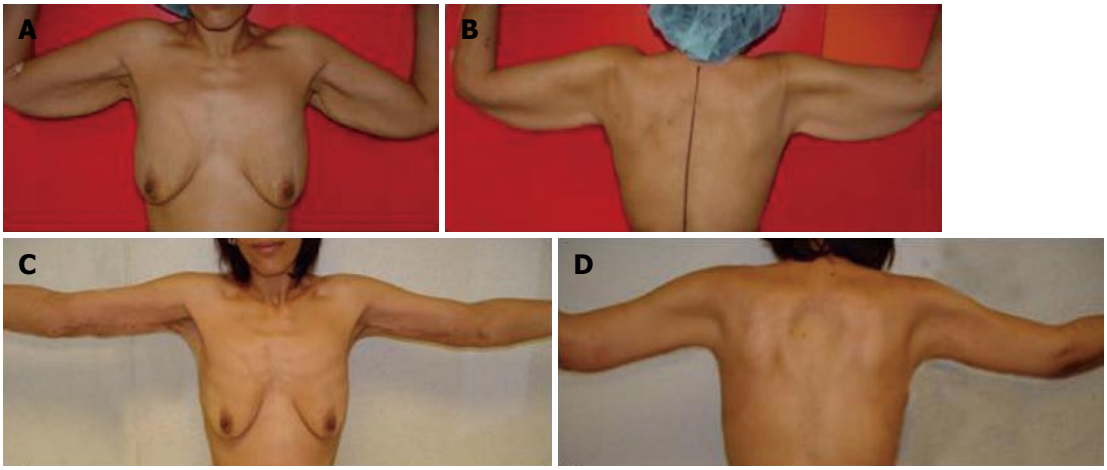


**Figure 5** Typical appearance of upper extremity deformity after massive weight loss. Note the "bat-wing" appearance of the proximal arm (**A** and **B**); One month after brachioplasty, there is significant improvement in contour (**C** and **D**). The incision is designed to keep the resultant scar on the medial surface of the upper extremity so that it is hidden when the patient stands at rest with her arms at her side.

correct the amount of excess skin present in most massive weight loss patients[63].

For more significant deformities, an ellipse parallel to the longitudinal axis of the extremity is utilized, with or without the aforementioned perpendicular ellipse[58] (Figure 5A-D). Although this approach can provide remarkable improvement, brachioplasty scars are long and tend to hypertrophy[64,65]. Furthermore, contractures across the axilla may occur despite use of "z-plasty" designs. For brachioplasty, the longitudinal scar is best placed along the medial surface of the upper arm where it is not visible while the patient's arms are at rest[32,66,67]. Similarly, for thigh lifts a scar is best placed medially along the thigh, although some surgeons advocate use of an anteriorly or laterally based excision[62,68,69]. Patients must be thoroughly counseled regarding the inevitable scars as well as other common complications, such as persistent edema, parasthesias and dysesthesias as well as delayed wound healing, particularly in the groin area[62].

## SUCTION-ASSISTED LIPECTOMY (LIPOSUCTION, SAL)

Although some patients may experience an evenly distributed massive weight loss resulting in a relatively thin layer of subcutaneous tissue throughout their body, for most patient this is not the case. In order to address these recalcitrant adipose deposits which are common in the upper abdomen, back, flanks, arms and legs, plastic surgeons utilize suction-assisted lipectomy (SAL), more commonly known as liposuction[70]. Liposuction is not for weight loss, although as much as 6-8 liters or more of fat may be removed safely in a single session[71]. Liposuction is used to "sculpt" areas of the body that need further refinement in their contour. SAL, like most procedures, is effective and safe when performed by a board certified plastic surgeon in an appropriately monitored setting. Although SAL alone is commonly performed as an ambulatory procedure, when more than 5 liters of lipoaspirate is removed in one session, the procedure is considered "large volume" liposuction and overnight monitoring is required[71]. SAL may be used concurrently with any of the above mentioned contouring procedures, or may be used afterwards to fine tune the results. Although not very painful, liposuction can prolong swelling and edema when used in conjunction with other body contouring procedures. A less frequent complication of SAL is persistent seroma. Transient paresthesias and bruising may also occur after SAL[72].

## SEQUENCE OF SURGERY

Body contouring after massive weight loss is a significant undertaking on the part of both the surgeon and the patient. As stated earlier, many of these patients are chronically malnourished which predisposes them to increased surgical morbidity[21,73]. The surgery itself is laborious and sometimes involves considerable blood loss. For these reasons, most surgeons stage the procedures[19]. Usually the abdomen and torso are treated first and, in less severe cases, may be combined with brachioplasty, thigh lift and or breast lift. The number of procedures performed at one time depends upon the health of the patient and the number of surgeons available. In centers where a "total body lift" is performed, two teams operating simultaneously are mandatory to limit time under anesthesia, although such procedures may still take eight or more hours[39].

As mentioned previously, special consideration must be given to the massive weight loss patient, since they are often relatively malnourished and even anemic secondary to their weight loss. Although severe complications such as pulmonary embolism or even death are relatively rare (0.02% or less)[74], minor complications such as seroma, infection or delayed wound healing are common with some surgeons reporting a greater than 70% total complication rate, especially in refractory smokers[16]. However, most patients will gladly trade these minor complications for the significant improvements in their body contour and by direct extension, their self image and confidence.

## MOLECULAR ADVANCES IN WEIGHT LOSS

Few drugs remain approved for weight loss. The dearth of pharmaceutical solutions is not for a lack of effort on the part of medical researches, but rather the complex nature of obesity. Most promising advances in weight loss technology surround the hormones leptin and ghrelin,

and the peptide known as PYY. These molecules all help regulate mechanisms of obesity. Currently, pharmaceutical companies are developing a variety of compounds to modulate the effects of these compounds.

## CONCLUSION

Body contouring after bariatric surgery is currently the fastest growing field within plastic surgery[75]. At the same time, body contouring after massive weight loss continues to evolve as surgeons refine patient selection, technique, and improve safety. However, the surge in weight loss-related surgeries has resulted in ethical questions, such as who should be treated, which operation is best, and who should be performing the surgeries. For these reasons, national consortiums have been founded to develop consensus responses to some of these questions. Meanwhile, though post-bariatric surgery is associated with a greater frequency of complications than "traditional" body contouring, the major transformation imparted by these procedures results in a high degree of patient satisfaction.

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*REVIEW*

# Surgical management of esophagogastric junction tumors

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## Abstract

Surgical resection with lymphadenectomy is the mainstay of treatment for all resectable esophagogastric junction tumors, prior to systemic generalization of the disease. This makes accurate pre-treatment staging and classification of the tumors most demanding. A well-established and internationally accepted classification for adenocarcinomas of the esophagogastric junction (AEG) helps to choose the appropriate surgical approach and to make results from different institutions comparable. Distal esophageal adenocarcinomas (AEG Ⅰ) are distinguished from true cardia carcinomas (AEG Ⅱ) and subcardiac gastric cancers (AEG Ⅲ). Substantial advancements in this surgical field during the preceding decades have clearly revealed that individualization of the surgical strategy is the key to successfully approaching these entities. In this review we discuss the surgical management of esophagogastric junction tumors with a tailored surgical strategy.



**Key words:** Adenocarcinoma of the esophagogastric junction; Esophageal cancer; Gastric cancer; Surgical resection

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## INTRODUCTION

Different tumor entities arise in the vicinity of the esophagogastric junction. The appropriate classification of these entities is essential for choosing the appropriate surgical approach and making results from different instutions comparable. Surgical resection is the mainstay of treatment for esophagogastric junction tumors of all resectable tumor stages prior to systemic generalization of the disease. Therefore a meticulous pretreatment staging is mandatory for planning the therapeutic approach. The major goal of surgical resection is complete removal of primary tumor together with its lymphatic drainage, because R0-resection (microscopic complete removal of the tumor) as well as nodal status and lymph node ratio (number of infiltrated nodes per node removed) are major prognostic factors. In locally advanced tumors, with low chances for R0 resection by means of primary surgery, multimodality treatment is attempted, aiming at downsizing the primary tumor and possibly downstaging the disease.

## CLASSIFICATION

The appropriate and uniform classification of carcinomas arising within the vicinity of the esophagogastric junction is essential both for planning therapeutic/surgical approaches and for making results from different institutions comparable. Well-established and meanwhile increasingly used world-wide is the classification of "adenocarcinomas of the esophagogastric junction" (AEG)[1,2]. Adenocarcinomas of the distal esophagus (AEG type Ⅰ) are distinguished from carcinomas arising at the level of the anatomical cardia (AEG type Ⅱ) and subcardiac gastric cancers (AEG Ⅲ). The classification has been introduced 17 years before from a surgical viewpoint[1,2]. But meanwhile it becomes more and more evident, that this classification also reflects the pathophysiology of different entities very well[3].

The center of the main tumor mass in relation to the anatomical cardia comprises the basis for the AEG-classification. For clinical usage, a definition of the anatomical cardia from the endoscopist's viewpoint is required. The cardia is localized, where the gastric folds end. The Z-line (correlative to the squamocolumnar junction) is shifted proximally in Barrett's esophagus but not at the level of the cardia, like it is under physiologic conditions[4,5]. The AEG classification is recommended by the consensus conference of the International Society for Diseases of the Esophagus[2] and increasingly accepted and used worldwide[6-11].

## PATHOPYSIOLOGY AND EPIDEMIOLOGY

AEG type I tumors are found in the majority of patients with Barrett's cancers, arising within the precancerous

Barrett's esophagus[12]. In patients with no evidence of Barrett's metaplasia on initial work-up, this can be due to an advanced primary tumor "overgrowing" the intestinal metaplasia. It has been shown that Barrett's metaplasia can get "unmasked" by preoperative chemotherapy in a substantial number of cases[13]. In the series by Theisen *et al*[13] over 97% of the patients with AEG I tumors are associated with Barrett's esophagus.

Carcinogenesis within the specialized intestinal metaplasia follows a metaplasia-intraepithelial neoplasia-carcinoma sequence. Initiation and progression of the disease process are promoted by the chronically damaging effect of gastroesophageal reflux[4,5]. Gastroesophageal reflux disease and its complication Barrett's esophagus are associated with a compromised lower esophageal sphincter. Patients have a high prevalence of hiatal hernia, which has been evaluated as a risk factor for esophageal adenocarcinoma[14,15]. Furthermore, patients are often obese (pathologically elevated body mass index), and only occasional alcohol drinkers in sharp contrast to patients with squamous cell cancers of the esophagus. It has been clearly demonstrated that the histological tumor types (esophageal adenocarcinomas and squamous cell cancers) comprise two entirely different entities[16] occurring in completely different types of patients.

The association of hiatal hernias and obesity with cancer development decreases in patients with tumors localized more distally. In patients with AEG Ⅱ/Ⅲ tumors long-lasting GERD and correlated morbidity (hiatal hernias, obesity) have diminished importance as risk factors[14,17]. AEG Ⅱ and Ⅲ tumors also have only a weak or no association with specialized intestinal metaplasia of the esophagus in the majority of patients[3]. Regarding the pathophysiology, these tumors seem to have more similarities with gastric cancers. A strong association with *H pylori* and intestinal metaplasia at or below the gastric cardia has been demonstrated[18].

Nevertheless, there are numerous hints supporting the concept of distinguishing these entities from gastric cancers, and regarding them as own entities. Especially striking is the fact that their incidence is increasing, compared to the decreasing incidence of gastric cancers.

## PRE-TREATMENT STAGING

Accurate pre-treatment staging is most demanding, because therapy for esophagogastric junction tumors must be performed by adjusting stage of the disease. Tailored surgical strategies are based on accurate localization of the primary tumor and its classifcation.

For all upper gastrointestinal tumors, endoscopy is the basic staging modality, allowing direct visualization of the primary tumor, exact localization and establishment of the diagnosis by means of biopsy. An up-to-date practice guideline by the American Society for Gastrointestinal Endoscopy deals with the use of endoscopy for esophageal cancer, and addresses the broad spectrum of endoscopy. New developments in the field of endoluminal diagnostics make recognition of early lesions more and more precise, especially by introduction and evaluation of new technologies, i.e. high resolution devices[19].

The depth of tumor invasion defines the T-category according to the TNM-classification system of the UICC, which is commonly used for staging[20]. For defining depth of invasion of a primary tumor in the clinical setting, endoscopic ultrasound (EUS) is the best staging modality. It has a major impact on choosing the appropriate treatment strategy (retrospective, but blinded evaluation)[21]. Furthermore, definition of T- and N-categories by EUS has been demonstrated to be of value for predicting long-term survival (prospective evaluation of 150 patients)[22].

CT scan remains the preferred staging method for exclusion of systemic tumor spread[23], providing anatomical information. The more functional positron emission tomography with fluorodeoxyglucose (FDG-PET), visualizing the regional glucose metabolism, is increasingly used for staging[23,24] and response evaluation during multimodality treatment[24,25]. Many studies have assessed the value of PET as a staging method and the former is meanwhile decreasing to a more realistic view. By critically reviewing their data, Kneist *et al* have demonstrated that the use of PET does neither lead to a different therapeutic approach nor provide new information on the indication for surgery.

In our experience, the use of FDG-PET as a staging method should be limited to early tumor stages, namely early cancers (T1) with very low prevalence of lymphatic or systemic tumor spread, and systemically disseminated disease. This is the clue to make usage of FDG-PET cost-effective.

## MULTITREATMENT MODALITY

In the Western world, neoadjuvant treatment concepts for administering systemic antineoplastic therapy (i.e. chemotherapy or chemoradiation prior to subsequent surgical resection) are preferred over adjuvant chemotherapy. Studies on treating adenocarcinomas of the esophagogastric junction are scarce, due to non-uniform classification. These tumors are either included in esophageal or gastric cancer trials. Furthermore some studies on esophageal cancer still have not distinguished adenocarcinoma from squamous cell cancers, although this is essential, because these histological tumor types comprise two entirely different entities[16].

Only two studies on neoadjuvant treatment of esophageal cancer have been able to show a survival benefit with this concept[26,27]. Meta-analyses including 9 and 11 randomized trials[28,29] have demonstrated decent survival benefits. But these studies deal with esophageal cancer, and not exclusively adenocarcinoma. Nevertheless, the major message of these trials is probably true for carcinomas of the esophagogastric junction. Only a subgroup of patients undergoing neoadjuvant treatment experiences a survival benefit. These are the 'responders'. 'Non-responders' by contrast do progress or deteriorate during preoperative treatment. In our experience the response frequency to the preoperative antineoplastic regimens accounts for 30%-60%[26,27].

An amazing concept for assessing the early response

during the course of the antineoplastic regimen is response evaluation with FDG-PET. This has been demonstrated in esophageal squamous cell cancers[24,30] and also in AEG tumors and gastric cancers[25,31]. It has been recognized that tumors responding to chemotherapy show an early decrease of glucose uptake. This tool has been used and intensively studied concerning the selective usage of neoadjuvant protocols.

## SURGICAL STRATEGIES

Complete removal of the primary tumor (R0 resection) is one of the major prognostic factors in adenocarcinomas of the esophagogastric junction[12]. Its predictive value is strong as demonstrated by univariate analysis[32] (Figure 1). Multivariate analysis demonstrates that R0 resection is independent of other strong predictors of survival, like T, N and M[12]. Thus, the primary goal of surgical resection of esophagogastric junction tumors is complete removal of the primary tumor, together with its lymphatic drainage. Which surgical approach best suits this purpose is still controversial. A vast variety of approaches for surgical resection of tumors of the esophagogastric junction has been proposed, including abdominothoracic *en bloc* esophagogastrectomy, subtotal esophagectomy with resection of the proximal stomach, total gastrectomy with transhiatal resection of the distal esophagus, limited resection of the esophagogastric junction.

In the past 20 years we have operated on more than 1500 patients with AEG tumors. A variety of approaches have been assessed[13]. Based on this surgical experience tailored surgical strategies have been developed, with respect to distinct requirements of the different AEG tumor types and different stages of the disease. Although still practiced[33], complete esophagogastrectomy has been abandoned as a procedure for carcinomas within the esophagogastric junction.

### *AEG Ⅰ tumors (distal esophageal adenocarcinomas)*

It is beyond dispute that AEG I tumors require an esophagectomy. Resection must include complete removel of the precancerous Barrett's esophagus[1]. For this purpose, preoperative clipping of the oral margin (level of the squamocolumnar junction) by the endoscopist is recommended. These tumors, which are mostly Barrett's cancers, have been shown to metastasize predominantly to the mediastinal lymph nodes[34]. But lymphatic spread occurs later than in esophageal squamous cell cancers and the prevalence of lymphatic metastases is lower in distal esophageal adenocarcinomas[12].

The transthoracic or transhiatal resection of Barrett's cancers is the best approach which is a topic of intensive research. Transthoracic esophagectomy with *en bloc* removal of esophagus and adjacent lymph nodes is the optimal approach in respect to radical resection of tumors. These lymph nodes are left behind with transhiatal (transmediastinal) esophagectomy, because a formal lymphadenectomy is not performed with this technique. The transhiatal approach can result in a reduced postoperative morbidity and mortality, because

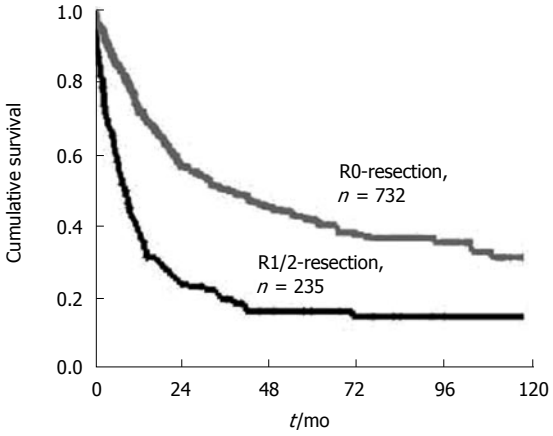


**Figure 1** Overall 10-year survival rate of patients with resected adenocarcinoma of the esophagogastric junction. Patients with complete macroscopic and microscopic tumor resection (R0 resection) versus patients with resection (R1/R2 resection) (Date of the Chirurgische Klinik und Poliklinik, Klinikum rechts der Isar der TU Munchen 1982-1999).

thoracotomy is avoided. This view is not supported by a recent multicenter trial from the USA[35]. In this large scale investigation the reported differences between transhiatal and transthoracic approaches in respect to morbidity and mortality are not statistically significant.

Another prospective trial, a recent single center study from Amsterdam/The Netherlands, comparing the two approaches in a series of patients with distal esophageal adenocarcinomas[36] showed that patients may benefit from transthoracic resection, thus having a longer survival. A clear superiority of either procedure has not been demonstrated, requiring individualized strategy. For all patients who are likely to benefit from the complete nodal clearance, transthoracic *en bloc* esophagectomy appears to be the procedure of first choice. In terms of radical resection, transhiatal esophagectomy is appropriate for earlier distal esophageal adenocarcinomas (with low propability of lymphatic involvement) and patients with substantial co-morbidity (who may benefit from avoiding the thoracotomy)[3,34].

### *AEG Ⅱ/Ⅲ tumors (cardia carcinomas and subcardiac gastric cancers)*

In our experience, total gastrectomy with transhiatal resection of the distal esophagus (transhiatally extended gastrectomy) is the best approach for AEG Ⅱ tumors[37]. Short-term postoperative results, i.e. morbidity and mortlity, are better with this approach, compared to transhiatal esophagectomy (e.g. 5.6% *vs* 1.9% mortality in a consecutive patient series of 46 transmediastinal esophagectomies and 103 extended total gastrectomies). Multivariate analysis has shown that R0 resection is the single most important prognostic factor. Regression analysis of the subgroup of R0-resected patients has demonstrated that absence of lymph node metastases and extended gastrectomy are two independet predictors of long-term survival[37] (Figure 2). This approach (extended gastrectomy superior to transhiatal esophagectomy for AEG Ⅱ) is also supported by data from other institutions[38,39].

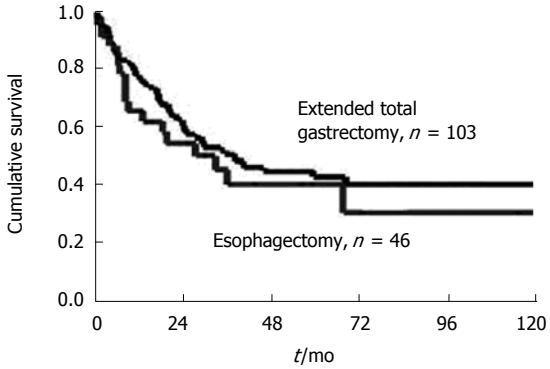


**Figure 2** Ten-year survival rate of patients with R0-resected true carcinoma of the gastric cardia (AEG Type-Ⅱ). Radical transmediastinal esophagectomy versus extend total gastrectomy (Date of the Chirurgische Klinik und Poliklinik, Klinikum rechts der Isar der TU Munchen 1982-1999).

## EXTENT OF LYMPHADENECTOMY AND SPLENECTOMY

The required extent of lymphadenectomy for AEG tumors has never been studied systematically. Similar to the findings of Japanese institutions, we have demonstrated that patients with tumors limited to the mucosa (pT1a) have virtually no lymph node involvement. Furthermore lymph node metastases are uncommon in carcinomas invading only the submucosa (pT1b). This holds also true, when more sensitive methods (immunohistochemistry/PCR techniques) are used for detection of micrometastases. Patients with more advanced AEG Ⅱ carcinomas harbor metastases in lymph nodes of paracardial region, lesser and greater curvatures, left gastric artery towards celiac axis, splenic artery, superior border of the pancreas towards the splenic hilum, lower posterior mediastinum, left adrenal gland and left renal vein[34,27,40,41].

This comprises the basis for the current concept of standard lymphadenectomy for AEG Ⅱ and Ⅲ tumors. Lymphadenectomy (in addition to lymph nodes adjacent to the gastrectomy specimen) starts with removal of the lymph nodes along the splenic artery towards the splenic hilum. Lymph nodes around the left renal vein are included. Formerly a retroperitoneal lymphadenectomy with left-sided pancreatic resection plus splenectomy is frequently performed in addition. Although the number of resected lymph nodes is increased with this procedure, the negative side-effects are predominant. A substantial number of septic complications, pancreatic fistulae and abscess formation have been observed[42-44].

## LIMITED RESECTION

Extended gastrectomies, especially esophagectomies, are associated with a considerable morbidity and mortality. Although these indicators for short-term postoperative outcome have been markedly improved during recent years[2,45,46], the remaining risk is nevertheless substantial. Furthermore, the quality of life after esophagectomy and gastrectomy is compromised. This fact has led to limited resections of adenocarcinomas of the esophagogastric junction. Resection of the distal esophagus and esophagogastric junction, with regional lymphadenectomy and jejunal interposition for reconstruction, has been described as a suitable surgical alternative[5,6]. Reconstruction is done by jejunal interposition and the short- and long-term results are excellent and the quality of life is improved as expected.

## THERAPEUTIC STRATEGIES

Esophagectomy is the appropriate approach for surgical resection of AEG Ⅰ tumors, whereas transhiatally extended gastrectomy is recommended for AEG Ⅱ and Ⅲ tumors. In our experience it is not necessary to perform more extended procedures in most cases, like esophagogastrectomy. Limited resection of the esophagogastric junction and reconstruction with interposition of a jejunal loop can be successfully applied to early cancers arising in the vicinity of the EGJ[6]. The value of neoadjuvant treatment concepts is not entirely clear as yet. Although a subset of patients benefits from chemotherapy preoperatively, the effective tool for response prediction is positron emission tomography (PET).

## FUTURE PERSPECTIVES

Further individualization of surgical strategies can be expected. The most striking problem with surgery of esophagogastric junction tumors is that the subset of patients who benefit from neoadjuvant protocols is unknown. Therefore the patients who do not respond to this aggressive regimen would suffer from the side-effects of this therapy. Response prediction for defining subsets of patients benefiting most from neoadjuvant treatment regimens would become a matter of molecular characterization. It might become possible to distinguish responders from non-responders before initiating the treatment according to their genetic profiles. The molecular characterization by genomic profiling perhaps can predict lymph node status and survival, as in gastric cancer[47] and Barrett's cancer[48] and other entities. Regarding the surgical technique, progress can be achieved with the technique of sentinel lymph node biopsy[49]. This technique helps us to individualize the extent of required lymphadenectomy, which is important because it is a major factor contributing to postoperative morbidity and mortality.

In the near future, new technical devices like a new FDG-PET hand device, help to identify metastatic lymph node intraoperatively. Further development in the field of nuclear medicine with new tracers and more sensitive detection systems is perhaps helpful regarding pre-treatment staging and intra-operative identification of metastatic disease. The combination of functional (PET/scintigraphy) and antomical (CT) information helps to summarize information of the major staging methods for esophagogastric junction tumors. Although at its very beginning of clinical application[50], PET/CT is an amazing tool.

## KEY ISSUES

Surgical resection is the mainstay of treatment for esophagogastric junction tumor prior to its systemic generalization.

A meticulous preoperative staging based on appropriate classification is required for choosing the appropriate therapeutic approach and surgical strategy.

Endoscopy, endoscopic ultrasound, pharyngoesophagography and CT scan are the basic staging modalities. The functional FDG-PET scan, visualizing areas of increased glucose uptake, is increasingly used for staging and response evaluation during multi treatment modality.

Uniform classification of tumors within the vicinity of the esophagogastric junction is important for choosing the appropriate surgical approach and making results from different institutions comparable. The classification of adenocarcinomas of the esophagogastric junction (AEG), can distinguish distal esophageal adenocarcinomas (AEG I) from true cardia carcinomas (AEG Ⅱ) and subcardiac gastric cancers (AEG Ⅲ).

AEG I tumors are usually Barrett's cancers arising in the precancerous Barrett's esophagus under the chronically damaging effect of acid. AEG Ⅱ and Ⅲ tumors share more properties with gastric cancers, but comprise nevertheless distinct entities

Esophagectomy is the appropriate surgical procedure for AEG I tumors, whereas transhiatally extended gastrectomy (with resection of an esophageal sleeve) is the best procedure for AEG Ⅱ and Ⅲ tumors. Esophagogastrectomy has been abandoned in most institutions, because it is associated with a substantial morbidity and mortality as well as a bad quality of life of the patients. Although its value has not been fully defined yet, a substantial number of patients with locally advanced tumors seem to benefit from neoadjuvant treatment concepts, aiming at down staging and down sizing of the primary tumor.

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*LIVER CANCER*

# Development and characterization of multidrug resistant human hepatocarcinoma cell line in nude mice

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## Abstract

**AIM:** To establish a multidrug resistant (MDR) cell subline from the human hepatocarcinoma cell line (HepG2) in nude mice.

**METHODS:** HepG2 cell cultures were incubated with increasing concentrations of adriamycin (ADM) to develop an ADM-resistant cell subline (HepG2/ADM) with cross-resistance to other chemotherapeutic agents. Twenty male athymic BALB/c-nu/nu mice were randomized into HepG2/nude and HepG2/ADM/nude groups (10 in each group). A cell suspension (either HepG2 or HepG2/ADM) was injected subcutaneously into mice in each group. Tumor growth was recorded, and animals were sacrificed 4-5 wk after cell implantation. Tumors were prepared for histology, and viable tumor was dispersed into a single-cell suspension. The $IC_{50}$ values for a number of chemotherapeutic agents were determined by 2, 3-bis (2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide inner salt (MTT) assay. Rhodamine-123 retention/efflux and the level of resistance-associated proteins were determined by flow cytometry. The mRNA expression of *mdr1*, *mrp* and *lrp* genes was detected using reverse transcriptase polymerase chain reaction (RT-PCR) in HepG2/nude and HepG2/ADM/nude groups.

**RESULTS:** The appearances of HepG2/nude cells were slightly different from those of HepG2/ADM/nude cells. Similar tumor growth curves were determined in both groups. A cross-resistance to ADM, vincristine, cisplatin and 5-fluorouracil was seen in HepG2/ADM/nude group. The levels of P-glycoprotein and multidrug resistance-associated proteins were significantly increased. The mRNA expression levels of *mdr1*, *mrp* and *lrp* were higher in HepG2/ADM/nude cells.

**CONCLUSION:** ADM-resistant HepG2 subline in nude mice has a cross resistance to chemotherapeutic drugs. It may be used as an *in vivo* model to investigate the mechanisms of MDR, and explore the targeted approaches to overcoming MDR.



**Key words:** Hepatocarcinoma; Multidrug resistance; Adriamycin; Model; Nude mouse

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## INTRODUCTION

Tumor cell drug resistance represents a significant obstacle to successful chemotherapy. Cells which have acquired resistance to one anti-tumor drug usually show resistance to other anti-tumor drugs[1]. This cellular resistance is known as multidrug resistance (MDR). Overexpression of P-glycoprotein (P-gp), multidrug resistance-associated protein (MRP), and lung resistance-related protein (LRP) is associated with development of MDR in cancer cells[2]. P-gp acts as an energy-dependent outward transport pump and can decrease intracellular drug accumulation by removing chemotherapeutic drugs from the cytoplasm[3]. A correlation between *mdr1* mRNA expression and drug resistance has been demonstrated using human cancer cell lines[4]. MRP is an ATP-dependent transmembrane protein related to MDR[5], and LRP is also expressed in a MDR lung cancer line[6]. The *mrp* and *lrp* genes encoding MRP and LRP, respectively, have been cloned in MDR cell lines that do not exhibit P-gp overexpression.

Several cell lines from resistant carcinomas have been established to elucidate the mechanisms of MDR. MDR cell lines and the expression of three resistance-associated markers (P-gp, MRP and LRP) have been extensively studied *in vitro*[7]. These resistant tumor cell lines have been established *in vitro* from P-gp negative cell lines *via* the exposure of chemotherapeutic drugs such as adriamycin (ADM), and their acquired resistance is mainly dependent on P-gp expression levels[8]. Unfortunately, it seems that the predictive value of MDR in established cell lines *in vitro* for determining the responsiveness of a relative tumor in clinical practice is poor. This misinterpreted role of

MDR may explain the failure of *in vivo* strategies to reverse MDR by using modulators in solid tumors[9,10]. The aim of this study was to establish a multidrug resistant subline from a human hepatocellular carcinoma line (HepG2) in nude mice, and to acquire more insights into *in vivo* drug resistance.

## MATERIALS AND METHODS

### Drugs and chemicals

2, 3-bis (2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide inner salt (MTT), ADM, vincristine (VCR), cisplatin, (CDDP), 5-fluorouracil (5-Fu), 2-(6-amino-3-imino-3H-xanthen-9-yl)-benzoic acid methyl ester (rhodamine123, Rh123), and dimethyl sulphoxide (DMSO) were obtained from Sigma (St Louis, Missouri, USA). Mouse monoclonal antibody MRK16 was supplied by Santa Cruz Biotechnology (Santa Cruz, California, USA). Rat monoclonal antibody MRPr1 and mouse monoclonal antibody LRP56 were obtained from Caltag Laboratories (Burlingame, California, USA). Fluorescein isothiocynate (FITC)-labeled goat anti-mouse immunoglobulin G (IgG) was from Tago Immunologicals (Camarillo, California, USA), and mouse monoclonal IgG was from Chemicon (Temecula, California, USA). TRIzol, RT-PCR kit, and primers were supplied by Life Technologies Inc. (Rockville, Minnesota, USA).

### Cell lines

HepG2 was provided by the Institute of Biochemistry and Cell Biology (Chinese Academy of Sciences, Shanghai, China). Cells were grown as monolayers in RPMI 1640 medium, supplemented with 10% (v/v) heat-inactivated fetal calf serum, 1.0 mmol/L sodium pyruvate at 37℃ in a humidified atmosphere containing 50 mL $CO_2$. ADM was added to HepG2 cell cultures in stepwise increasing concentrations (starting at 0.001 mg/L and ending up to 1.0 mg/L) to develop a drug resistant cell subline (HepG2/ADM). After removal of dead cells, the remaining viable cells were identified as being drug resistant, and then cultured in a higher concentration of anticancer drug again. With the gradual increase in drug concentration, cells could finally be maintained in a culture medium containing 1000 μg/L ADM. The MDR characteristics of these HepG2/ADM cells were tested at various concentrations of anticancer drugs including ADM, VCR, CDDP and 5-Fu.

### Animal experiments

The animal study was approved by the Experimental Animal Committee of Chongqing University of Medical Sciences, and all animal experiments adhered to the Animal Welfare Committee guidelines. Male athymic BALB/c nu/nu mice (4-6 wk old) were obtained from the Institute of Materia Medica (Chinese Academy of Sciences, Shanghai, China) and housed in laminar-flow cabinets under specific pathogen-free (SPF) conditions.

Twenty male athymic BALB/c-nu/nu mice were randomized into HepG2/nude and HepG2/ADM/nude groups, 10 in each group. A suspension of either HepG2 or HepG2/ADM cells ($10^7$ cells in 0.15 mL of Hanks' solution) was injected into the right subaxillary region of mice in each group. After implantation, the tumor growth was detected daily by measuring its diameter with Vernier caliper. Tumor weight (TW) was calculated using the following formula: TW (mg) = tumor volume ($mm^3$) = $d^2 \times D/2$, where d and D are the shortest and longest diameters, respectively. Animals were sacrificed 4-5 wk after implantation when the subcutaneous tumor reached the size of 1.5 cm in diameter, and samples were harvested. Tumor tissue blocks were created for histological examinations. Viable tumor tissue was ground into a single-cell suspension in a loose-fitting ground glass homogenizer. Cell suspensions were washed twice with DMEM, and cultured in RPMI 1640 medium supplemented with 10% fetal calf serum.

### MTT assay

MTT cell proliferation assay was performed to determine the percentage of viable HepG2 or HepG2/ADM cells *in vitro,* or HepG2/nude and HepG2/ADM/nude cells were derived from viable tumor tissues *in vivo* after incubation with various concentrations of anti-tumor drugs. In brief, cell suspensions were seeded in 96-well culture plates at a density of $1 \times 10^5$ cells/well. After 24 h incubation, the cells were exposed to the drug under study for 72 h, followed by 4 h incubation with the tetrazolium staining. Using a multiwell spectrophotometer reader (Molecular Devices, Menlo Park, California, USA), the optical intensity at 570 nm was measured, and cell viability was assessed in each well from the level of a dark blue formazan crystal created. Each assay was performed in triplicate, and RPMI 1640 medium was used as a blank control. The $IC_{50}$ values were defined as the concentration resulting in 50% cell survival. After the dose-response curve was plotted, the $IC_{50}$ for each anti-tumor drug was determined. The relative resistance to anti-tumor drugs was determined by dividing the $IC_{50}$ values obtained for HepG2/ADM cells by those of HepG2 cells.

### Rhodamine 123 assay

A total of $1 \times 10^6$ HepG2 or HepG2/ADM cells were seeded into each well of a 12-well culture plate, and incubated for 24 h. After the culture medium was replaced by Hanks' solution supplemented with 10 mmol/L HEPES buffer and 10% fetal calf serum (FCS), the cells were incubated for 15 min at 37℃. Rhodamine 123 (Rh123) was then added to give a final concentration of 10 g/L. The cells were further incubated for 30 min, washed twice with ice-cold PBS, harvested with trypsin, and suspended in PBS. The cells were further incubated in Rh123-free medium for 30 min to determine the Rh123 efflux. Cell surface-associated fluorescence was measured after addition of ice-cold Rh123 to the cells. Using the FACS caliver system (Elite ESP, Coulter Electronics, Miami, USA), the fluorescence intensity of Rh123 was measured at excitation and emission wavelength of 485 nm and 530 nm, respectively.

### Flow cytometry

The protein levels of P-gp, MRP and LRP were determined by flow cytometry using the monoclonal

antibodies MRK16, MRPr1 and LRP-56. A total of 1 × $10^6$ HepG2 or HepG2/ADM cells were incubated in suspension with 10 g/L of the primary antibodies and an isotype-matched mouse IgG2a, respectively, for 1 h at 37℃. After washing, the cells were incubated with 0.175 g/L FITC-labeled goat anti-mouse IgG (for P-gp and LRP-56) or FITC-labeled goat anti-rat IgG (for MRPr1) for 1 h at 37℃. The fluorescence intensity (excitation wavelength 488 nm) was determined using an Epics ESP flow cytometer (Coulter Electronics, Miami, USA). The ratio of the specific fluorescence intensity of the HepG2/ADM cells to that of the HepG2 cells indicated the relative level of resistance-associated proteins.

### RT-PCR

Reverse transcriptase polymerase chain reaction (RT-PCR) was used to measure the mRNA expression of *mdr1*, *mrp* and *lrp* genes. The primers of *mdr1*, *mrp* and *lrp* are as follows: *mdr1*: 5'-GGC TCC GAT ACA TGG TTT TCC-3', 3'-TTC AGT GCG ATC TTC CCA GC-5'; *mrp*: 5'-TGA AGG ACT TCG TGT CAG CC-3', 5' GTC CAT GAT GGT GTT GAG CC-3'; *lrp*: 5'-CCT CGA GAT CCA TTG TGC TGG-3', 5'-CAC AGG GTT GGC CAC TGT GCA-3'. β-actin expression was used as a control for the amount of RNA used, and its primer was 5'-ACC CCC ACT GAA AAA GAT GA-3', and 5'-ATC TTC AAA CCT CCA TGA TG-3'. Total RNA was extracted from cells using TRIzol. *mdr1*, *mrp*, *lrp* and β-*actin* RNA transcripts were detected using RT-PCR. An aliquot of each reaction mixture was then analyzed by electrophoresis using 1.5% agarose gel. Densitometry was performed with an UVP gel image analysis system (BIO-RAD, Melville, NY, USA) and the ratio between the target and control PCR products was determined by dividing the densitometric volume of the target band by that of the control band.

The PCR cycle included heat denaturation at 94℃ for 45 s, annealing at 60℃ for 45 s, and polymerization at 72℃ for 90 s. cDNA was prepared from 4.5 mg of total RNA using the GeneAmp RNA PCR kit. The reaction mixture was incubated at 42℃ for 30 min and then heated for 5 min at 99℃ to inactivate MMLV reverse transcriptase. The cDNA derived from 0.15 mg of total RNA was mixed with 20 mL reaction mixture (10 mmol/L Tris-HCl, pH 8.3, 50 mmol/L KCl, 1.25 mmol/L $MgCl_2$, 188 nmol/L gene-specific primers and 0.625 U AmpliTaq DNA polymerase) with β-actin (0.765 amol). Thirty-two cycles of PCR were performed, each consisting of heat denaturation at 94℃ for 45 s, annealing at 55℃ for 1 min, and polymerization at 72℃ for 2 min.

### Statistical analysis

All observed data were expressed as mean ± SD. The statistical significance of any observed difference between the mean values of HepG2 and HepG2/ADM groups was evaluated using the Student's *t* test. $P < 0.05$ was considered statistically significant.

## RESULTS

### Histology

Both HepG2/nude and HepG2/ADM/nude tumor cells demonstrated polygonal epithelial-like morphology, with firm attachment to the culture flask. They had conspicuous large nuclei. Electron microscopy showed abundant microvilli and projections on the cell surface in both cell lines. Some of the projections on HepG2/nude cells were long, terminated with swellings, while those of HepG2/ADM/nude cells were short and compact. Both cell types had many lysosomes in the cytoplasm and were usually concentrated on one side of the HepG2/nude cells, but scattered around the cytoplasm in HepG2/ADM/nude cells. No obvious desmosomes and tight junctions were observed in the cells.

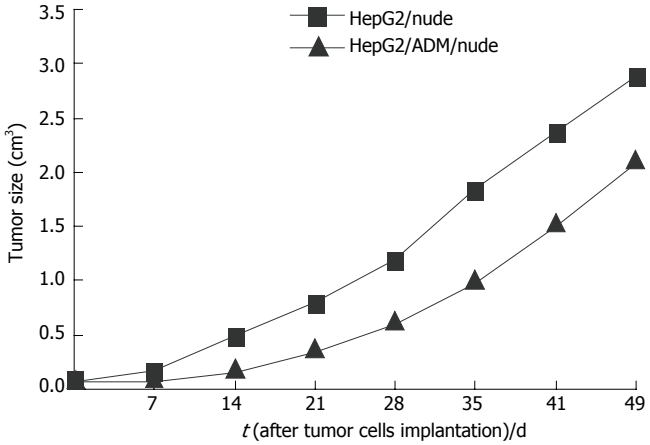


**Figure 1** Growth curves of tumors after implantation of HepG2 or HepG2/ADM cells in nude mice. Athymic mice were injected s.c. with 1 × $10^7$ (0.2 mL/mouse) HepG2 or HepG2/ADM cells on d 0. Symbols and bars denote the means and SD for 10 nude mice in the HepG2 group and 10 nude mice in the HepG2/ADM group.

### Tumor growth

Tumor growth was evaluated in nude mice after subcutaneous implantation of HepG2/nude and HepG2/ADM/nude cells. Mean tumor volumes measured with a Vernier caliper are shown in Figure 1.

### MDR characteristics

HepG2/ADM tumor cells became drug resistant following incubation of HepG2 cells with increasing concentrations of ADM (Table 1). In addition to direct resistance to ADM, these cells were also resistant to other chemotherapeutic agents including VCR, 5-FU and CDDP. The $IC_{50}$ values for the drugs were significantly greater in the HepG2/ADM group than in the HepG2 group (Table 1), indicating that HepG2/ADM tumors awarded MDR characteristics to nude mice. However, the resistant index for ADM and VCR was significantly lower in the HepG2/ADM/nude group than in the HepG2/ADM group (ADM: 26.31 *vs* 51; VCR: 6.17 *vs* 57.06), but that for CDDP and 5-Fu was higher (CDDP: 6.45 *vs* 2.33; 5-Fu: 4.09 *vs* 1.49).

### Rh123 retention and efflux

Compared to both HepG2 and HepG2/nude tumor cells, either decreased retention or increased efflux of Rh123 was observed in the HepG2/ADM and HepG2/ADM/nude tumor cells in nude mice. The pattern of altered retention or efflux of Rh123 in the ADM resistant cells is shown in Figure 2.

**Table 1 Sensitivity of *in vitro* and *in vivo* HepG2 and HepG2/ADM tumor cells to chemotherapeutic agents (mean ± SD)**

| Drug | IC50 values (mg/L) | | | | RIa | RIb |
|---|---|---|---|---|---|---|
| | HepG2 | HepG2/ADM | HepG2/nude | HepG2/ADM/nude | | |
| ADM | 0.02 ± 0.015 | 1.02 ± 0.326[b] | 0.16 ± 0.015 | 4.21 ± 0.012[d] | 51.00 | 26.31 |
| VCR | 0.18 ± 0.011 | 10.27 ± 2.415[b] | 0.18 ± 0.211 | 1.11 ± 0.075[d] | 57.06 | 6.17 |
| CDDP | 0.87 ± 0.023 | 2.03 ± 0.360 | 0.22 ± 0.023 | 1.42 ± 0.032[d] | 2.33 | 6.45 |
| 5-FU | 9.48 ± 0.112 | 14.16 ± 1.924 | 0.53 ± 0.112 | 2.17 ± 0.670 | 1.49 | 4.09 |

HepG2/nude: HepG2 tumor in nude mice; HepG2/ADM/nude: HepG2/ADM tumor in nude mice; RIa: Resistant index between HepG2 and HepG2/ADM; RIb: Resistant index between HepG2/nude and HepG2/ADM/nude. [b]$P < 0.01$ *vs* HepG2; [d]$P < 0.01$ *vs* HepG2/nude.

**Table 2 Levels of resistance-associated proteins in HepG2 and HepG2/ADM cells (mean ± SD)**

| Tumor cell | P-gp | MRP | LRP |
|---|---|---|---|
| HepG2 | 1.00 ± 0.04 | 0.16 ± 0.10 | 0.001 ± 0.00 |
| HepG2/ADM | 4.68 ± 0.63[b] | 1.40 ± 0.15[b] | 0.785 ± 0.04 |
| HepG2/nude | 1.00 ± 0.04 | 0.16 ± 0.08 | 0.001 ± 0.00 |
| HepG2/ADM/nude | 4.73 ± 0.68[d] | 1.43 ± 0.16[d] | 0.771 ± 0.02 |

[b]$P < 0.01$ *vs* HepG2; [d]$P < 0.01$ *vs* HepG2/nude.

### *Level of resistance-associated proteins*

The relative expressions of resistance-associated proteins were given as the ratios of the specific fluorescence intensity of HepG2/ADM and HepG2/ADM/nude cells to those of HepG2 and Hep2/nude cells respectively. As shown in Table 2, HepG2/ADM/nude tumors co-expressed the resistance-associated proteins, P-gp, MRP and LRP respectively. Compared to the HepG2/nude cells, the levels of P-gp and MRP were significantly increased in the HepG2/ADM/nude cells ($P < 0.01$), whereas no significant change in LRP expression was observed between the HepG2/nude and HepG2/ADM/nude groups ($P > 0.05$).

### *mRNA expression of mdr1, mrp and lrp genes*

Positive mRNA expression of the *mdr1* and *mrp* genes and negative mRNA expression of the *lrp* gene were seen in both HepG2 and HepG2/nude cells. However, mRNA expression *of the mdr1*, *mrp* and *lrp* gene*s* was greater in HepG2/ADM and HepG2/ADM/nude tumor cells than in HepG2 and HepG2/nude tumor cells (Figure 3).

## DISCUSSION

As drug resistance is one of the major barriers to the successful treatment of malignancies, investigation of the mechanisms of drug resistance and approaches to overcoming it has been widely performed in the past decades. Many MDR cell lines have been established *in vitro* as clinically relevant cancer models in these studies. However, compared to *in vitro* studies, the results *in vivo* are unsatisfactory in the treatment of solid tumors with modulators of Pg-p[9]. It appears that the predictive

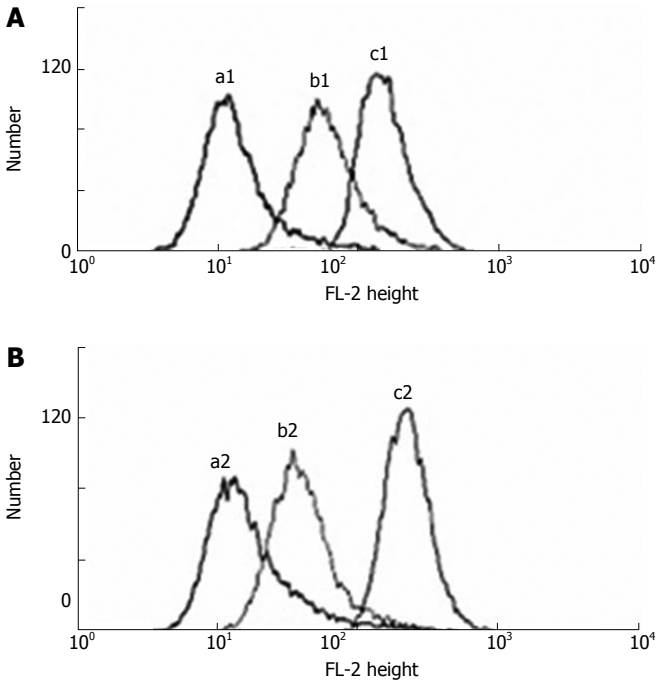


**Figure 2** Retention and efflux of rhodamine 123 in HepG2/ADM/nude cells measured by flow cytometry. **A**: Intracellular Rh123 retention. a1: HepG2/ADM/nude cells incubated in RPMI 1640 medium; b1: HepG2/ADM/nude cells incubated with Rh123; c1: HepG2/nude cells incubated with Rh123; **B**: Rh123 Efflux. a2: HepG2/ADM/nude cells incubated in RPMI 1640 medium; b2: HepG2/ADM/nude cells incubated with Rh123; C2: HepG2/nude cells incubated with Rh123.

value of MDR *in vitro* is not so good for determining the responsiveness of tumors in clinical practice[10]. In addition to tumor heterogeneity in the expression of MDR[11], several reasons are given for these negative results. The interface between MDR modulators and chemotherapeutic agents increases drug toxicity[12]. Furthermore, MDR represents the net effect of the expression of a variety of genes involved in the development of drug resistance[13,14]. Finally, some MDR results obtained in *ex vivo* studies have been overvalued or misunderstood[15]. Tumor cells grown in solid tumors *in vivo* are less vulnerable to drugs than the same cells grown in monolayer culture *in vitro* due to inadequate drug penetration, a reduced growth fraction and a decreased sensitivity mediated by cell-cell interactions. It is therefore important to establish a well-characterized and standardized MDR model *in vivo* for drug screening and prediction of clinical drug response.

In this study, we established an ADM-resistant subline *in vitro* from the human HepG2 cell line with increasing concentrations of ADM. This subline (HepG2/ADM) demonstrated cross resistance to other chemotherapeutic drugs. The MTT assay showed that HepG2/ADM cells not only presented direct resistance to MDR-related drugs such as ADM (51-fold) and VCR (57-fold), but also were sensitive to non-MDR-related compounds such as CDDP (2.33-fold) and 5-Fu (1.49-fold). Furthermore, it showed characteristics of MDR tumor cells, including expression of MDR-associated genes, and presence of resistance-associated proteins. However, it is important to confirm whether these resistant characteristics could be maintained

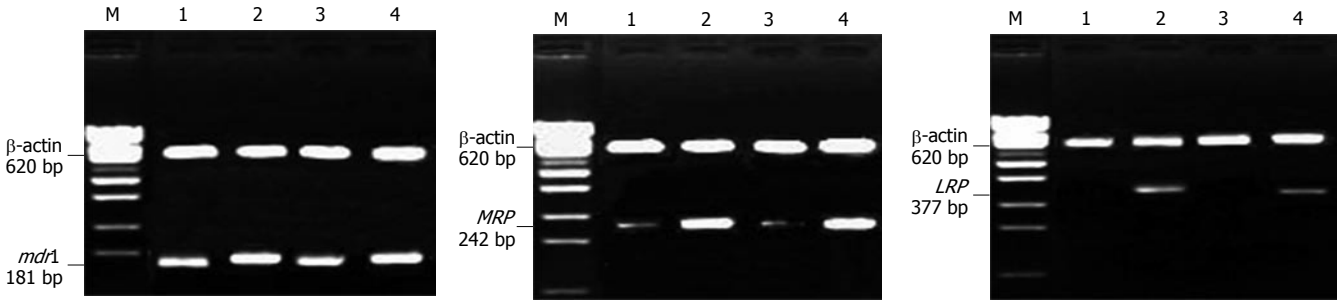


**Figure 3** mRNA expression of *mdr*1, *MRP* and *LRP* in HepG2/nude and HepG2/ADM/nude cells. Lane M: 100-bp DNA marker; lane 1: HepG2 cell line; lane 2: HepG2/ADM cell subline; lane 3: HepG2 cells in nude mice; lane 4: HepG2/ADM in nude mice.

*in vivo* following their implantation in nude mice.

Slight differences were observed under light microscope in HepG2/nude cells, and HepG2/ADM/nude tumor cells. These changes became more apparent under electron microscope, especially in the shape of cellular microvilli and the distribution of lysosomes in the cytoplasm. These changes had no significant effects on tumor growth between the two experimental groups. However, the *in vivo* tumor growth rates were almost similar between the two groups. Furthermore, the chemoresistance *in vitro* was confirmed *in vivo* after subcutaneous implantation of HepG2/ADM cells in nude mice. The $IC_{50}$ values for ADM and VCR were significantly higher in the HepG2/ADM group than in the HepG2/ADM/nude group, but those for CDDP and 5-Fu were lower in the HepG2/ADM group than in the HepG2/ADM/nude group, indicating that a partial transfer of resistance ability acquired *ex vivo* to the *in vivo* situation is possible.

In the present study, a number of proteins involved in the development of drug resistance were analyzed quantitively using flow cytometry to determine the level of MDR-associated proteins *in vivo*, including P-gP, MRP and LRP. The levels of P-gP, MRP and LRP were significantly higher in the HepG2/ADM/nude subline than in the HepG2/nude cells, but no statistical difference was seen between HepG2/ADM and HepG2/ADM/nude groups. Similar results were seen in the *in vivo* expression of MDR-associated genes (*mdr1*, *mrp* and *lrp*) on mRNA levels. Although RT-PCR used in this study was semi-quantitative, it showed the mRNA expression in HepG2/ADM/nude tumor cells induced by ADM. These findings confirmed quantitively by both immunoflow cytometry might be beneficial for protein and functional rhodamine assays.

However, it still remains unclear why the resistant index changed significantly in *ex vivo* conditions compared to *in vivo* conditions. This can be probably explained by the assumption that other MDR mechanisms are involved in the subline[16], such as the increase of ATP dependent glutathione S-conjugate transporter[17], and the decrease of DNA topoisomerase Ⅱ activity[14].

In conclusion, a MDR model of nude mice can be successfully established using human hepatocarcinoma cell line HepG2. This new model shows resistance to chemotherapeutic drugs, and mRNA expression of various MDR-associated genes on the protein levels. It can be used as an *in vivo* model to investigate the molecular mechanisms involved in MDR-related genes of hepatocarcinoma and to explore the targeted approaches tor overcoming MDR in tumor cells.

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*LIVER CANCER*

# Precore/basal core promoter mutants and hepatitis B viral DNA levels as predictors for liver deaths and hepatocellular carcinoma

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## Abstract

**AIM:** To conduct a retrospective study in 400 chronic hepatitis B patients in order to identify hepatitis B viral factors associated with complications of liver disease or development of hepatocellular carcinoma.

**METHODS:** The mean follow-up time was 83.6 ± 39.6 mo. Alpha-fetoprotein test and abdominal ultrasound were used for cancer surveillance. Hepatitis B basal core promoter mutants, precore mutants, genotypes, hepatitis B viral DNA (HBV DNA) level and hepatitis B e antigen (HBeAg) were measured. Univariate analysis and logistic regression were used to assess odds ratios for viral factors related to liver deaths and hepatocellular carcinoma development.

**RESULTS:** During follow-up, 38 patients had liver deaths not related to hepatocellular carcinoma. On multivariate analysis, older age [odds ratio: 95.74 (12.13-891.31); $P < 0.0001$], male sex [odds ratio: 7.61 (2.20-47.95); $P = 0.006$], and higher $\log_{10}$ HBV DNA [odds ratio: 4.69 (1.16-20.43); $P < 0.0001$] were independently predictive for these liver related deaths. Also, 31 patients developed hepatocellular carcinoma. Multivariate analysis showed that older age [odds ratio: 26.51 (2.36-381.47); $P = 0.007$], presence of precore mutants [odds ratio: 4.23 (1.53-19.58); $P = 0.02$] and presence of basal core promoter mutants [odds ratio: 2.93 (1.24-7.57); $P = 0.02$] were independent predictors for progression to hepatocellular carcinoma.

**CONCLUSION:** Our results show that high levels of baseline serum HBV DNA are associated with non-hepatocellular carcinoma-related deaths of liver failure, while genetic mutations in the basal core promoter and precore regions are predictive for development of hepatocellular carcinoma.



**Key words**: Basal core promoter mutants; Precore mutants; Hepatitis B viral genotypes; Hepatitis B viral DNA; Hepatitis B e antigen; Liver failure; Hepatocellular carcinoma

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## INTRODUCTION

Infection with hepatitis B virus (HBV) results in different clinical outcomes which are related to age of exposure. Previous studies have shown that up to 90% of infants born to hepatitis B surface antigen (HBsAg) positive, hepatitis B e antigen (HBeAg) positive mothers become chronic carriers, while acute HBV infection in adults leads to chronicity in 5% to 10% of individuals[1,2]. Thereafter, 15% to 40% of patients with chronic hepatitis B will progress to cirrhosis and hepatocellular carcinoma (HCC)[3]. In a previous report, we showed that 9.5% of our HBsAg positive patients died of non-HCC related liver complications and another 7.8% developed HCC over a mean seven year follow-up period[4]. Significant clinical and biochemical factors which predicted all deaths of HBV infection in this group of patients include decreased serum albumin, low platelet levels and presence of cirrhosis. However, the reasons for the differences in these clinical outcomes remain unknown.

Hepatitis B viral factors such as HBeAg, HBV DNA, HBV genotype, basal core promoter (BCP) mutants and precore (PC) mutants may either alone or in combination play a major role in determining the progression of disease

in patients with chronic hepatitis B. A report from Taiwan indicates that the relative risks for HCC is 9.6 among men who are positive for HBsAg alone but rise to 60 in those who are positive for both HBsAg and HBeAg[5]. Also, the risk for HCC is increased in patients with high circulating levels of serum hepatitis B viral DNA (HBV DNA)[5,6]. Other studies from Asia indicate that HBV genotypes B and C are associated with an increased risk for HCC[7-11]. A dual mutation in the BCP region of the HBV genome involving an A to T substitution at nucleotide 1762 and a G to A substitution at nucleotide 1764 has been associated with more severe liver damage and HCC development[12-16]. In addition, a mutation in the PC region of the HBV genome involving a G to A change at nucleotide 1896 has been described in patients with HBeAg negative chronic hepatitis[17]. However, its role in the pathogenesis of severe liver disease and HCC is less clear[14,18,19].

In the present report, we describe the baseline hepatitis B virologic profiles of 400 HBsAg positive patients who were followed up in our liver center. The clinical endpoints in this study were either death of non-HCC related liver disease or of progression to HCC. Odds ratios were used to identify significant hepatitis B viral factors associated with these serious and lethal complications.

## MATERIALS AND METHODS

### Patients

From January 1989 to March 1998, we followed up 400 HBsAg positive patients who presented to our clinic. Patients who were hepatitis C antibody positive, human immunodeficiency virus antibody positive, and had a history of chronic alcoholism with other chronic liver diseases were excluded. In this report, patients who received liver transplantation for non-HCC hepatic decompensation or for HCC were considered as deaths. The baseline demographic information, laboratory tests and outcomes have been reported recently[4].

### Laboratory tests

At baseline, HBeAg and hepatitis B e antibody (anti-HBe) were measured with commercially available kits (Abbott Laboratories, North Chicago, IL). Serum was also collected at the initial visit and stored at -70℃. In 2004, we collaborated with the Hepatitis Research Center at National Taiwan University Hospital in Taipei, Taiwan, and serum samples were sent for analysis of HBV DNA, HBV genotypes, PC and BCP mutants.

Serum HBV DNA was quantified by a real-time polymerase chain reaction assay in the linear range from $10^2$ to $10^{11}$ copies/mL[20]. For reporting purposes, the HBV DNA values were $\log_{10}$ transformed. The identification of HBV genotypes was performed by melting curve analysis[20], and supplemented by direct sequencing of the pre-S amplicon and phylogenetic analysis by comparing the nucleotide sequence with 33 reference HBV strains obtained from GenBank as previously described[21].

Amplification and sequencing of PC (nucleotides 1814-1900) and BCP (nucleotides 1742-1849) genes were performed as previously described[14,22]. Nucleotide sequences of the amplified products were directly determined by using fluorescence labeled primers with 3100 automatic sequencer (Applied Biosystems, Foster City, CA, USA). Sequencing conditions were specified in the protocol for the Taq DyeDeoxy terminator cycle sequencing kit (Applied Biosystems). The inner primer pair was used as sequencing primers for both directions of the gene.

### HCC surveillance

For HCC surveillance, abdominal ultrasound examinations and serum alpha-fetoprotein (AFP) testing were performed every 6 to 12 mo in non-cirrhotic patients and every six months in patients with cirrhosis. If the serum AFP was elevated or if a lesion was noted by abdominal ultrasound, further investigations using computerized tomography scan, magnetic resonance imaging or biopsy of the lesion were used to confirm the diagnosis of HCC.

### Statistical analysis

Categorical data were summarized by using frequencies and analyzed by chi-squared methods for assessment of differences. All continuous data were descriptively summarized with means and standard deviations, and further analyzed for assessment of differences by ANOVA with *post-hoc* pair-wise Student's *t*-tests. All variables found to be significant by univariate analysis were subjected to multivariate analysis utilizing step-wise logistic regression. The odds ratio was defined as previously described[23]. Analyses were conducted by using SAS software version 9.1 (Cary, NC). Statistical significance was defined as two-sided $P$ values $< 0.05$.

## RESULTS

### Patients

One hundred and thirty-nine of 400 HBsAg positive patients had liver biopsy confirmed cirrhosis and were classified in the Child-Pugh Class A category. The remaining 261 were non-cirrhotic patients with chronic hepatitis B. Seventy-eight percent of our patients were of Asian descent. The mean follow-up time was 83.6 ± 39.6 mo.

### Baseline virologic characteristics

**Basal core promoter 1762/1764 mutants:** Of 280 patients who had detectable BCP sequences, 124 (44.3%) had BCP 1762/1764 mutants and 156 (55.7%) had BCP 1762/1764 wild type sequences (Table 1). Patients with BCP 1762/1764 mutants were older than those with BCP 1762/1764 wild type sequences ($P = 0.01$). Seventy-four percent of patients with BCP 1762/1764 mutants had genotype C ($P < 0.0001$). Patients with BCP 1762/1764 wild type sequences had higher mean serum HBV DNA levels than those with BCP 1762/1764 mutants ($P = 0.003$). No concordance was noted between patients with BCP 1762/1764 mutants and PC 1896 mutants ($P = 0.8$, Kappa = 0.001). Also, there was no significant difference in the HBeAg status among patients with BCP 1762/1764 mutants.

**Precore 1896 mutants**: Of 382 patients who had detec-

**Table 1 Demographic and virologic characteristics of basal core promoter 1762/1764 mutants**

| Characteristics | Mutant | Wild type | *P* |
|---|---|---|---|
| Number | 124 | 156 | |
| Age (yr) (mean ± SD) | 50.9 ± 13.5 | 46.3 ± 15.9 | 0.01 |
| Male/Female | 93/31 | 112/44 | 0.5 |
| Asian/Non-Asian | 107/17 | 121/35 | 0.06 |
| Genotype, *n* (%) | | | < 0.0001 |
| A | 12/121 (9.9) | 28/147 (19.1) | |
| B | 18/121 (14.9) | 54/147 (36.7) | |
| C | 90/121 (74.4) | 62/147 (42.2) | |
| Baseline HBV-DNA level (mean ± SD, copies/mL) | 6.7 ± 1.6 $Log_{10}$ | 7.3 ± 1.9 $Log_{10}$ | 0.003 |
| PC[1] 1896 mutant, *n* (%) | | | 0.8 |
| Yes | 35/124 (28.2) | 46/156 (29.5) | |
| No | 89/124 (71.8) | 110/156 (70.5) | |
| HBeAg, *n* (%) | | | 0.07 |
| + | 68/124 (54.8) | 101/154 (65.6) | |
| − | 56/124 (45.2) | 53/154 (34.4) | |

[1]Precore.

**Table 2 Demographic and virologic characteristics of precore 1896 mutants**

| Characteristic | Mutant | Wild type | *P* |
|---|---|---|---|
| Number | 112 | 270 | |
| Age (yr) (mean ± SD) | 49.7 ± 14.8 | 47.3 ± 15.1 | 0.16 |
| Male/Female | 77/35 | 192/78 | 0.6 |
| Asian/Non-Asian | 99/13 | 201/69 | 0.002 |
| Genotype, *n* (%) | | | < 0.0001 |
| A | 2/93 (2.1) | 54/227 (23.8) | |
| B | 53/93 (56.9) | 39/227 (17.2) | |
| C | 38/93 (40.9) | 128/227 (56.4) | |
| Baseline HBV-DNA level (mean ± SD, copies/mL) | 5.5 ± 2.0 $Log_{10}$ | 6.5 ± 2.3 $Log_{10}$ | < 0.0001 |
| HBeAg, *n* (%) | | | < 0.0001 |
| + | 33/109 (30.1) | 161/268 (60.1) | |
| − | 76/109 (69.7) | 107/268 (39.9) | |

table PC sequences, 112 (29.3%) had PC 1896 mutants and 270 (70.7%) had PC 1896 wild type sequences (Table 2). More Asian patients had PC 1896 mutants ($P = 0.002$). Among the PC 1896 mutants, only 2.1% had genotype A ($P < 0.0001$). The mean serum HBV DNA levels were significantly higher in patients with PC 1896 wild type sequences ($P < 0.0001$). Patients with PC 1896 mutants were most often HBeAg negative ($P < 0.0001$).

**HBV genotypes**: In 332 patients who had detectable genotypes, genotype A was present in 56, genotype B in 92, genotype C in 166, genotype D in 10, genotype E in 2, and mixed HBV genotypes in 6 patients (Table 3). HBV genotype C followed by genotype B was detected more frequently in Asian patients, while genotype A was more frequent in non-Asian patients ($P < 0.001$). Patients with HBV genotype C had the highest serum HBV DNA levels compared to those with genotypes A and B ($P = 0.003$). A majority of genotype A and genotype C patients were HBeAg positive, and more genotype B patients were HBeAg negative ($P = 0.0002$). Genotype B patients had more PC 1896 mutants ($P < 0.0001$). Genotype C patients had more BCP 1762/1764 mutants ($P < 0.0001$).

**Serum HBV DNA level**: Baseline serum HBV DNA levels in 390 patients ranged from 2.1 $log_{10}$ to 11.5 $log_{10}$ copies/mL (median 6.1 ± 2.3 $log_{10}$ copies/mL). Males had higher mean HBV DNA levels than females (6.39 ± 2.30 $log_{10}$ copies/mL *vs* 5.52 ± 2.14 $log_{10}$ copies/mL; $P = 0.0007$, Table 4). HBeAg positive patients had significantly higher serum HBV DNA levels than HBeAg negative patients ($P < 0.0001$).

**HBeAg**: At baseline, 197 of 395 (49.9%) patients were HBeAg positive and 198 (50.1%) were HBeAg negative. HBeAg positive patients were younger than HBeAg negative patients (45.2 ± 15.9 years *vs* 49.9 ± 13.7 years, $P = 0.002$). Also, more males than females were HBeAg positive [150 of 278 (53.9%) *vs* 47 of 117 (40.2%), $P = 0.01$].

### *Non-HCC related deaths*

During follow-up, 38 patients died of non-HCC related deaths. Twenty-seven died of liver failure, seven of bleeding esophageal varices, and four of sepsis. Four other patients died of non-liver related disease. Univariate analysis showed that older age, male sex, presence of cirrhosis and high baseline serum HBV DNA levels were associated with non-HCC related liver deaths (Table 5). On multivariate analysis, old age (OR: 95.74; 95% CI: 12.13-891.31; $P < 0.0001$), male sex (OR: 7.61; 95% CI: 2.20-47.95; $P = 0.006$), and high baseline HBV DNA (OR: 4.69; 95% CI: 1.16-20.43; $P = 0.03$) were independently predictive of non-HCC related liver deaths. HBeAg, HBV genotype, PC 1896 mutants and BCP 1762/1764 mutants were not associated with non-HCC related liver deaths.

### *HCC development*

During follow-up, HCC developed in 31 (7.8%) patients. Twenty-two of 139 (15.8%) patients with cirrhosis and nine of 261 (3.4%) patients without cirrhosis progressed to HCC. Baseline tests in these 31 patients showed that 12 (38.7%) were HBeAg positive, 18 (58.1%) were anti-HBe positive, and one HCC patient was positive for both. In comparing the 31 patients who developed HCC to those who did not, univariate analysis of baseline variables showed that older age, male sex, cirrhosis, presence of PC 1896 mutants and BCP 1762/1764 mutants were associated with development of HCC (Table 6). Multivariate analysis showed that age (OR: 27.51; 95% CI: 2.36-381.47; $P = 0.007$), presence of PC 1896 mutants (OR: 4.23; 95% CI: 1.53-19.58; $P = 0.02$) and BCP 1762/1764 mutants (OR: 2.93; 95% CI: 1.24-7.57; $P = 0.02$) were independent predictors for HCC development. HBeAg, HBV genotype, and serum HBV DNA were not predictive for development of HCC.

## DISCUSSION

Our findings of death of non-HCC liver complications and development of HCC in hepatitis B patients in the United States are in accordance with natural history studies

**Table 3 Demographic and virologic characteristics of HBV genotypes**

| Characteristics | Genotype A | Genotype B | Genotype C | *P* |
|---|---|---|---|---|
| *n* | 56 | 92 | 166 | |
| Age (yr) (mean ± SD) | 51.0 ± 15.8 | 46.3 ± 17.7 | 47.4 ± 14.7 | 0.2 |
| Male/Female | 47/9 | 64/28 | 117/49 | 0.02 |
| Asian/Non-Asian | 16/40 | 84/8 | 157/9 | < 0.0001 |
| HBV-DNA (mean ± SD, copies/mL) | 6.68 ± 2.53 $Log_{10}$ | 6.12 ± 2.08 $Log_{10}$ | 6.94 ± 1.81 $Log_{10}$L | 0.003 |
| HBeAg, *n* (%) | | | | 0.0002 |
| + | 37/55 (67.3) | 35/91 (38.5) | 104/165 (63.0) | |
| - | 18/55 (32.7) | 56/91 (61.5) | 61/165 (37.0) | |

from Asia and Europe. The highest complication rate occurred in the 139 patients who presented with cirrhosis. During follow-up, 38 (27.3%) patients died either of liver failure, bleeding esophageal varices or of sepsis. Our annual rate of non-HCC related liver deaths was 3.9% per year and is similar to 2.4%-4% reported elsewhere[24,25]. Also, 9 (3.4%) of 261 patients without cirrhosis and 22 (15.8%) of 139 patients with cirrhosis developed HCC, resulting in annual rates of 0.5% and 2.3%, respectively. These also are similar to 1.5%-3.8% in Europe[26] and 0.7%-2.2% in Asia[27,28].

In the present report, BCP 1762/1764 mutants were detected in 44% of our HBsAg positive patients. The BCP 1762/1764 mutants were most often detected in our patients with genotype C compared to genotype A or B patients (59%, 30% and 35%, respectively). A recent report from Taiwan showed that genotype C has a significantly higher prevalence of BCP 1762/1764 mutants than genotype B. Also, BCP 1762/1764 mutants are more frequently detectable in patients with HCC than in chronic carriers or patients with chronic hepatitis[12,14]. In one report from Guangxi Zhuang Autonomous Region in China, BCP 1762/1764 mutants were detectable in all 11 HCC tissues tested[14]. Other studies indicated that BCP 1762/1764 mutants were most often present in patients who had more progressive liver disease and hepatic decompensation than in those with stable liver disease[13-16]. In the present study, the presence of BCP 1762/1764 mutants was closely associated with non-HCC liver deaths as well as HCC development.

The significance of PC 1896 mutants is less clear. A study from Taiwan showed that the frequency of PC 1896 mutants was similar in both HCC patients and inactive carriers[14]. Also, no difference has been found in the prevalence of PC 1896 mutants between Chinese patients with or without cirrhosis related complications[15]. Another report from Japan suggested that acquisition of mutation in the PC 1896 region may contribute to inactivation of chronic liver disease[13]. In our study, 29.3% of patients had the PC 1896 mutants, which were primarily HBeAg negative, but 30% were HBeAg positive. One study from China also showed that PC mutants were already present in HBeAg-positive patients who had not yet experienced seroconversion to anti-HBe[15]. These authors suggested that development of HCC may not be related to PC 1896 mutations but probably due to the persistence of significant viremia after HBeAg seroconversion. The low incidence of PC 1896 mutants in our genotype A patients is in accordance with other reports since genotype A patients have a C at nucleotide 1858 which destabilizes the stem loop region and precludes emergence of the PC 1896 mutants[29,30]. In our report, 42% of the patients with PC 1896 mutants had cirrhosis, and 45.2% of patients who developed HCC had PC 1896 mutants. The role of PC 1896 mutants in disease progression requires further investigation.

**Table 4 Demographic and virologic characteristics of baseline serum HBV-DNA Levels (mean ± SD, copies/mL)**

| Characteristics | Mean HBV-DNA levels | *P* |
|---|---|---|
| Sex | | 0.0007 |
| Male | 6.39 ± 2.30 $Log_{10}$ | |
| Female | 5.52 ± 2.14 $Log_{10}$ | |
| Race | | 0.24 |
| Asian | 6.05 ± 2.21 $Log_{10}$ | |
| Non-Asian | 6.39 ± 2.57 $Log_{10}$ | |
| HBeAg | | < 0.0001 |
| + (*n* = 194) | 7.42 ± 1.97 $Log_{10}$ | |
| − (*n* = 191) | 4.86 ± 1.81 $Log_{10}$ | |

The distribution of genotypes A, B, C, and D (16%, 27%, 49%, and 3% respectively) in our patients is consistent with the results of a recent survey of HBV genotypes in patients with hepatitis B from the western part of the United States where there is a predominant Asian population (18%, 34%, 41%, and 5% respectively)[29]. In the latter study, the ethnic distribution in the southern part of the United States was predominantly Caucasians, and in this area, 63% of the patients have genotype A. HBV genotypes A and D appear to be more common in Caucasians, while HBV genotypes B and C are predominantly found in Asians[7,8,10,31]. However, no HBV genotypes were found to be predictive for either non-HCC liver deaths or progression to HCC in our study. Studies from Asia showed that the prevalence of genotypes B and C is similar in patients with cirrhosis and HCC, but progression to cirrhosis might be slower in genotype B patients[10]. Another report indicated that genotype B may be responsible for HCC development in children and young non-cirrhotic adult males in Taiwan[11,32]. These conflicting findings indicate that the role of genotypes in the natural history of HBV is still unclear.

Recently, the role of HBV DNA in predicting

**Table 5 Univariate analysis of factors associated with non-HCC related liver deaths**

| Factor | Alive | Expired | *P* |
|---|---|---|---|
| *n* | 326 | 43 | - |
| Age (yr) (mean ± SD) | 45.5 ± 14.7 | 57.2 ± 12.2 | < 0.0001 |
| Male/Female | 213/113 | 41/2 | < 0.0001 |
| Asian/Non-Asian | 264/62 | 24/19 | 0.0005 |
| Cirrhosis | | | < 0.0001 |
| Yes | 77/139 | 38/139 | |
| No[1] | 257/261 | 4/261 | |
| Baseline HBV-DNA level (mean ± SD) | 6.0 ± 2.38 $Log_{10}$ copies/mL | 6.92 ± 1.96 $Log_{10}$ copies/mL | 0.02 |
| BCP 1762/1764 mutant | | | 0.07 |
| Yes | 86 | 20 | |
| No | 132 | 16 | |
| PC 1896 mutant | | | 0.78 |
| Yes | 86 | 12 | |
| No | 223 | 30 | |
| Genotype | | | 0.43 |
| A | 46 | 8 | |
| B | 76 | 8 | |
| C | 126 | 19 | |
| HBeAg | | | 0.17 |
| + | 159 | 26 | |
| − | 163 | 17 | |

[1]Four other patients without cirrhosis died of non-liver related deaths.

**Table 6 Univariate analysis of factors associated with development of HCC**

| Factor | HCC | No HCC | *P* |
|---|---|---|---|
| *n* | 31 | 369 | |
| Age (yr) (mean ± SD) | 57.4 ± 2.7 | 46.9 ± 0.8 | 0.0002 |
| Male/Female | 28/3 | 254/115 | 0.006 |
| Asian/Non-Asian | 26/5 | 288/81 | 0.4 |
| Cirrhosis | | | < 0.0001 |
| Yes | 22/139 | 117/139 | |
| No | 9/261 | 252/261 | |
| BCP 1762/1764 mutant | | | 0.007 |
| Yes | 18 | 106 | |
| No | 6 | 148 | |
| PC 1896 mutant | | | 0.05 |
| Yes | 14 | 98 | |
| No | 17 | 253 | |
| Genotype | | | 0.2 |
| A | 2 | 54 | |
| B | 8 | 84 | |
| C | 19 | 147 | |
| HBeAg | | | 0.3 |
| + | 12 | 165 | |
| - | 18 | 180 | |
| Baseline HBV-DNA level (mean ± SD, copies/mL) | 6.3 ± 1.4 $Log_{10}$ | 6.1 ± 2.3 $Log_{10}$ | 0.6 |

progression to cirrhosis and HCC has been reported from Taiwan[6,33]. These authors showed that elevated levels of baseline HBV DNA were associated with increased deaths of cirrhosis[6,33]. Also, levels of HBV DNA ⩾ 10 000 copies/mL at baseline and at follow-up were a strong predictor of HCC development, which was independent of HBeAg, serum ALT and cirrhosis[6]. In our report herein, patients who died of non-HCC related liver complications had significantly higher baseline HBV DNA levels than those who remained alive. Thus, chronic inflammation caused by the host immune response to active viral replication appears to be more responsible for the non-HCC related liver deaths than mutations in the HBV genome[34]. However, the baseline levels of HBV DNA in our patients who developed HCC during follow-up were similar to patients who did not progress to HCC.

The role of HBeAg in predicting progression of liver disease is unclear. A recent study from Taiwan showed that the presence of HBeAg positivity significantly increased the risk of developing HCC. Also, another study from Europe showed that HBeAg positivity was associated with worse survival and that the risk of death decreased after HBeAg seroconversion. However, in our report 53% of our cirrhosis patients were HBeAg positive on presentation, and 47% were HBeAg negative, indicating that progression to cirrhosis occurs regardless of HBeAg status. Also, we noted that HBeAg positive patients had higher baseline HBV DNA levels than HBeAg negative patients, and our HBeAg negative patients had more PC 1896 mutants than HBeAg positive patients. However, during our analysis of deaths of non-HCC liver complications or development of HCC, HBeAg did not appear to play a predictive role in predicting either complication.

HCC developed in 31 of our patients during follow-up. In comparing patients who developed HCC to those who did not, both PC 1896 mutants and BCP 1762/1764 mutants were independent predictors for development of HCC. Our findings confirm studies from Asia which have

implicated BCP 1762/1764 mutants and HCC. In addition, our observations indicate that patients with PC 1896 mutations also have an increased risk of developing HCC. Since the majority of our HBsAg positive patients are from Asian countries, the risk of developing HCC persists in those with pre-existing BCP 1762/1764 mutants and PC 1896 mutants, even after years of immigration from their native countries to the USA.

There were the limitations in our study. The analyses of HBV genotypes and BCP 1762/1764 mutations were not possible in some of our patients because of low or undetectable levels of HBV DNA, which did not permit adequate amplifications of the serum samples. In addition, this study only described analysis of laboratory tests performed at the time of presentation, and no longitudinal analyses were completed. It is well known that patients with chronic hepatitis B experience exacerbations and remissions which are usually accompanied with abnormal liver tests and varying levels of HBV DNA[35]. However, our primary endpoints in this study were either death of liver disease or development of HCC, and our aim was to identify hepatitis B viral factors at presentation which may predict these serious outcomes.

In summary, the presence of high baseline serum HBV DNA levels can predict non-HCC related liver deaths, while mutations in the BCP and PC regions of the HBV genome are more predictive for HCC development. These findings will assist in the planning of treatment strategies to prevent these serious and lethal complications which occur in patients with chronic hepatitis B infection.

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# Chemokine expression in hepatocellular carcinoma versus colorectal liver metastases

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## Abstract

**AIM:** To evaluate and compare the expression profiles of CXCL12 (SDF-1), CCL19 (MIP-3β), CCL20 (MIP-3α) and CCL21 (6Ckine, Exodus2) and their receptors on RNA and protein levels in hepatocellular carcinoma (HCC) versus colorectal liver metastases (CRLM) and to elucidate their impact on the carcinogenesis and progression of malignant liver diseases.

**METHODS:** Chemokine expression was analyzed by RT-PCR and ELISA in 11 cases of HCC specimens and in 23 cases of CRLM and corresponding adjacent non-tumorous liver tissues, respectively. Expressions of their receptors CXCR4, CCR6 and CCR7 were analyzed by RT-PCR and Western blot analysis in the same cases of HCC and CRLM.

**RESULTS:** Significant up-regulation for CCL20/CCR6 was detected in both cancer types. Moreover, CCL20 demonstrated significant overexpression in CRLM in relation to the HCC tissues. Being significantly up-regulated only in CRLM, CXCR4 displayed an aberrant expression pattern with respect to the HCC tissues.

**CONCLUSION:** Correlation of CXCR4 expression with CRLM suggests CXCR4 as a potential predictive factor for CRLM. High level expression of CCL20 and its receptor CCR6 in HCC and CRLM with marked up-regulation of CCL20 in CRLM in relation to HCC tissues indicates involvement of the CCL20/CCR6 ligand-receptor pair in the carcinogenesis and progression of hepatic malignancies.



**Key words:** Chemokines; Chemokine receptors; Gene expression; Hepatocellular carcinoma; Colorectal liver metastases

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## INTRODUCTION

Chemokines represent a family of small chemotactic cytokines, initially identified as mediators of leucocyte trafficking and homing. In the last few years, chemokines have been shown to participate also in tumor growth and the lymphatic and even distant spread of malignant tumors[1,2]. Here we compare the expression profiles of several chemokine/chemokine receptor pairs, namely CXCL12/CXCR4, CCL20/CCR6 and CCL19/CCL21/CCR7 in hepatocellular carcinoma (HCC) versus colorectal liver metastases (CRLM). We chose to investigate this group of chemokines and their receptors because their roles in tumor growth and metastasis have recently gained increasing importance[3,4]. Like HCC, which is a highly malignant tumor with a poor prognosis due to its rapidly progressing and infiltrating growth, colorectal cancer (CRC) also still remains one of the leading causes of cancer-related death worldwide[5-8]. The mortality of CRC is principally attributable to the development of metastases, which primarily infest the liver, and CRLM are present in up to 95% of patients in the advanced disease stage.

The CC-chemokines CCL19 and CCL21 are highly expressed in lymph nodes and signal through a common receptor, the lymphocyte chemoattractant receptor CCR7[9-10]. While CCR7 was recently reported to predict lymph node metastasis in colorectal carcinoma and other cancer types[11,12], the cognate CXCL12 receptor CXCR4 has been suggested as a risk factor for the outgrowth of colon carcinoma micrometastases[13] and the invasion and spreading of several other cancers[15-17]. Also CCL20/CCR6 involvement in the neoplastic progression and cancer-specific metastasis of several tumor types is presently

reported, with major focus on the amplification of local necroinflammatory response in the liver[18-19], suggesting CCR6 as an important factor in the recruitment of lymphocytes from peripheral blood to HCC[20-21]. However, data concerning the expression profiles and clinical impact of CCL20/CCR6 in HCC and CRLM are still limited and no expression data are currently available concerning the pathophysiological relationship of chemokines in HCC and CRLM. While our group and others previously suggested an association between the CCL20/CCR6 expression in CRC and the promotion of CRLM[22-23], we now demonstrate a correlation between the CCL20/CCR6 expression profile in HCC and CRLM with a marked overexpression of the CCL20 gene product in relation to HCC tissues. Consequently, we hypothesize that the CCL20/CCR6 chemokine receptor pair may be of general importance in the development of hepatic malignancies of different origins.

## MATERIALS AND METHODS

### Patients

HCC and CRLM specimens and corresponding non-tumorous liver tissues were collected from 11 HCC and 23 CRLM patients who underwent resection in our department between 2002 and 2006. All patients provided informed consent for tissue procurement, which was approved by the ethics commission of the Ärztekammer of the Saarland. The clinical variables presented in Table 1 were obtained from clinical and pathological records according to the UICC TNM classification[24] system.

### Tissue preparation

Immediately after resection tissue samples were collected and processed under nucleic acid sterile conditions, snap frozen in liquid nitrogen and then stored at -80℃ until RNA and protein were extracted. For corresponding normal tissue we used adjacent healthy tissue from the same resected liver specimen. All tissues obtained were reviewed by an experienced pathologist and examined for the presence of tumor cells. As minimum criteria for usefulness for our studies we only chose tumor tissues in which tumor cells occupied a major component (> 60%) of the tumor biopsy.

### Single-strand cDNA synthesis

Total RNA was isolated using RNeasy columns from Qiagen (Hilden, Germany) following the manufacturer's instructions and RNA integrity was confirmed spectrophotometrically and by electrophoresis on 1% agarose gels. For cDNA synthesis, 5 μg of each patient's total RNA sample were reverse-transcribed in a final reaction volume of 50 μL containing 1 × TaqMan RT buffer, 2.5 μmol/L random hexamers, 500 μmol/L each dNTP, 5.5 mmol/L $MgCl_2$, 0.4 U/μL RNase inhibitor, and 1.25 U/μL Multiscribe RT. All RT-PCR reagents were purchased from Applied Biosystems (Applied Biosystems, Foster City, CA). The reaction conditions were 10 min at 25℃, 30 min at 48℃, and 5 min at 95℃.

**Table 1 Clinical characteristics of patients with HCC and CRLM**

| Factor | HCC[2] *n* = 11 | CRLM[3] *n* = 23 |
|---|---|---|
| Localization of primary tumor | | |
| Liver | 11 | - |
| Colon | - | 14 |
| Rectum | - | 9 |
| Gender | | |
| Male | 5 | 17 |
| Female | 6 | 6 |
| Age, yr[4] | 62.6 (29-82) | 65.0 (39-76) |
| Hepatitis (A, B or C) | | |
| Positive | 4 | 1 |
| Negative | 7 | 22 |
| Liver chirrhosis | | |
| Positive | 5 | 6 |
| Negative | 6 | 17 |
| Fibrosis | | |
| Positive | 1 | 3 |
| Negative | 10 | 20 |
| Largest tumor diameter (cm)[4] | 4.3 (1.2-6.8) | 10.3 (1.3-19) |
| TNM[1] stage of primary tumor | | |
| Ⅰ | 2 | 2 |
| Ⅱ | 4 | 2 |
| Ⅲ | 5 | 15 |
| Ⅳ | 0 | 3 |
| Grading | | |
| Ⅰ | 0 | 2 |
| Ⅱ | 7 | 16 |
| Ⅲ | 4 | 5 |
| Ⅳ | 0 | 0 |
| Lymphatic permeation | | |
| Positive | 1 | 16 |
| Negative | 10 | 7 |
| Vascular invasion | | |
| Positive | 4 | 1 |
| Negative | 7 | 22 |
| Chemotherapy before operation | 0 | 13 |
| Radiotherapy before operation | 0 | 3 |

[1]Tumor-node-metastasis; [2]Hepatocellular carcinoma; [3]Colorectal liver metastases; [4]Median with range in parentheses.

### Real-time PCR

All qRT assays containing the primers and probe mix were purchased from Applied Biosystems, (Applied Biosystems, Foster City, CA) and utilized according to the manufacturer's instructions. PCR reactions were carried out using 10 μL 2 × Taqman PCR Universal Master Mix No AmpErase® UNG and 1 μL gene assay (Applied Biosystems, Foster City, CA), 8 μL Rnase-free water and 1 μL cDNA template (50 mg/L). The theoretical basis of the qRT assays is described in detail elsewhere[25]. All reactions were run in duplicates along with no template controls and an additional reaction in which reverse transcriptase was omitted to allow for assessment of genomic DNA contamination in each RNA sample. For the signal detection, ABI Prism 7900 sequence detector was programmed to an initial step of 10 min at 95℃,

followed by 40 thermal cycles of 15 s at 95℃ and 10 min at 60℃ and the log-linear phase of amplification was monitored to obtain $C_T$ values for each RNA sample. Gene expression of all target genes was analyzed in relation to the levels of the slope matched housekeeping genes Cyclophilin C (CycC) and ß2-Microtubulin (B2M)[26]. Since reporting of data obtained from raw $C_T$ values falsely represent the variations, we converted the individual $C_T$ values to the linear form as follows:

$$\text{Fold difference} = 2^{-(\text{mean } C_T \text{ pathological tissue-mean } C_T \text{ calibrator})} = 2^{-\text{delta } C_T}$$

Hence, the liver of the tumor-neighboring tissue became the 1 × sample, and all other quantities were expressed as an n-fold difference relative to this tissue.

### Isolation of total protein

Protein lysates from frozen tissues were precipitated with the RIPA buffer. Protein quantitation was performed using the Pierce BCA protein assay reagent kit (Pierce, Rockford, USA).

### Western blot analysis

Chemokine receptors were detected with anti-CXCR4 (1:500, Serotec, AHP442 rabbit anti-human, Serotec, Oxford, UK; 1:5000, BioRad cat. 170-6515 goat anti-rabbit HRP, BioRad, Muenchen, Germany), anti CCR6 (1:500, Biomol, goat anti-human C2099-70B, Biomol, Hamburg, Germany; 1:5000 Santa Cruz, sc-2056 donkey anti-goat HRP) and anti-CCR7 (1:500, Santa Cruz, sc-9700 goat anti-human; 1:5000, Santa Cruz, sc-2056 donkey anti-goat HRP, Santa Cruz Biotechnology, Santa Cruz, CA USA), visualized by ECL Western blotting analysis system (Amersham Biosciences, Piscataway, NJ, USA), and quantified densitometrically. Human cell lysates HL-60 (sc-2209, Santa Cruz Biotechnology, Santa Cruz, CA, USA), Imgenex cell lysates A375, and HeLa (Imgenex, San Diego, USA) served as positive controls.

### Enzyme-linked immunosorbant assay

Chemokine protein levels in the different tissue lysates were determined by sandwich-type ELISA according to the manufacturer's protocol: R&D systems (R&D Systems Inc. Minneapolis, Minnesota, USA) for the quantification of CCL19, CCL20, CXCL12 or ProSci (ProSci, San Diego, USA) for CCL21**.** The absorbance was read at 450 nm.

### Laser capture microdissection

Laser microbeam microdissection (LMM) was employed for obtaining pure tumor cell and pure normal cell samples for subsequent genetic analysis. LMM was performed on three samples for each tissue type and each chemokine, respectively. Histochemical staining was used on cryo sections before microdissection. Specimen preparation, microdissection and catapulting were performed following a laser pressure catapulting protocol according to the manufacturer's instructions (P.A.L.M. Microlaser Technologies, Bernried, Germany). RNA was extracted using the P.A.L.M. RNA extraction kit and for reverse transcription the invitrogen reverse transcription kit (Invitrogen Life Technologies, Karlsruhe, Germany) was applied. Subsequently quantitative PCR analysis was performed as described earlier.

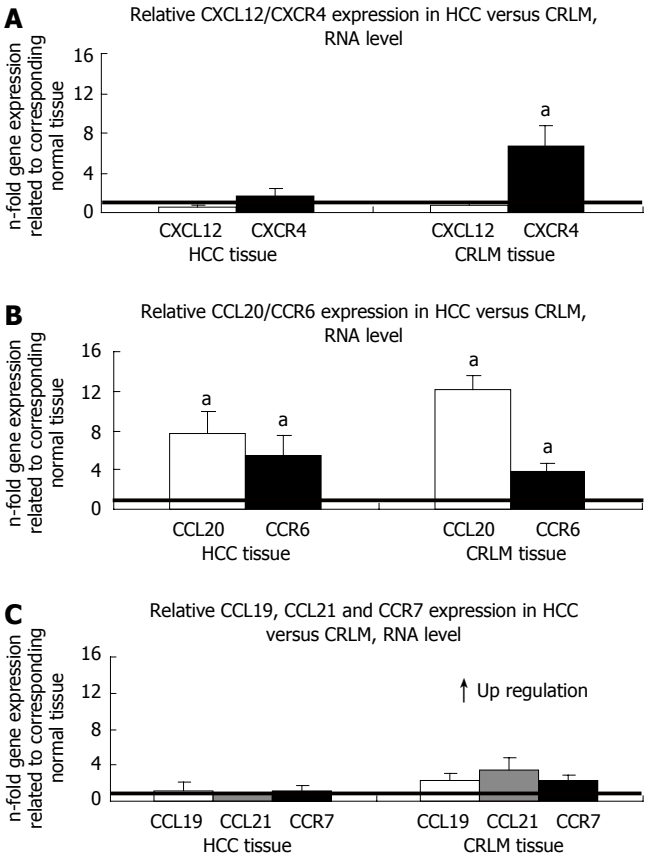


**Figure 1** Expression of chemokine/chemokine receptor pairs in HCC and CRLM as determined by Q-RT-PCR. **A**: CXCL12/CXCR4 expression; **B**: CCL20/CCR6 expression; **C**: CCL19/CCL21/CCR7 expression (mean ± SE, $^aP < 0.05$, $n = 11$ and 23, respectively).

### Statistical analysis

The statistical significance of differences in chemokine and chemokine receptor expression were summarized using mean and SEM (standard error of the mean). All statistical calculations were done with the MedCalc software package (MedCalc software, Mariakerke, Belgium)[27]. Where appropriate, either the Student's t-test or the Wilcoxon's rank sum test was applied to test for group differences of continuous variables. $P < 0.05$ was considered significant.

## RESULTS

### Chemokine/chemokine receptor expression

To assess potential differences in chemokine/chemokine receptor expression levels in HCC *vs* CRLM, we performed quantitative RT-PCR analysis in 11 HCC and 23 CRLM specimens and their corresponding tumor neighboring tissues, respectively. The adjacent, non tumor invaded liver tissues of the HCC and CRLM patients served as control groups. qRT analysis of the chemokine ligand CXCL12 displayed no significant difference in gene expression between the tumor and the tumor neighboring tissues in HCC or CRLM as shown in Figure 1A. In contrast to its ligand, the CXCL12 receptor CXCR4 demonstrated

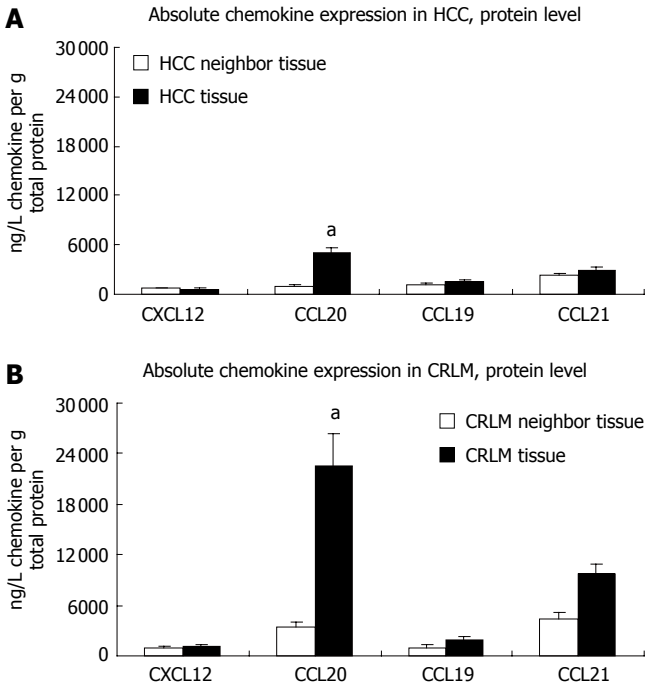


**Figure 2** Expression of CXCL12, CCL20, CCL19 and CCL21 in HCC and CRLM as determined by the enzyme-linked immunosorbant assay (ELISA). **A**: Chemokine expression in HCC; **B**: Chemokine expression in CRLM. (ng/L chemokine ligand related to 1 g total protein for the HCC and CRLM tissues. mean ± SE, $^{a}P < 0.05$; $n = 11$ and 23, respectively).

significant up-regulation in the CRLM compared with the tumor neighboring liver tissues ($P < 0.05$) thus demonstrating that CXCL12 and CXCR4 are inversely expressed in CRLM (Figure 1A). However, no significant difference in gene expression was detected for CXCR4 in the HCC tissues between the tumor and the tumor neighboring liver tissues thus indicating a clear difference in CXCR4 expression between HCC and CRLM. CC-chemokine CCL20 was found to be significantly up-regulated in the tumor tissue of patients with HCC and CRLM in comparison to their tumor neighboring tissues ($P < 0.05$), as shown in Figure 1B. Similarly, the CCL20 receptor CCR6 revealed a significantly higher mRNA expression in the tumor specimens of both HCC and CRLM patients in relation to the corresponding normal liver tissues, respectively ($P < 0.05$) (Figure 1B). In contrast, we observed no significant difference in CCL19 and CCL21 gene expression between the tumor and tumor neighboring tissues in either tissue type as demonstrated in Figure 1C. Likewise, no significant up- or down-regulation was detected for the corresponding receptor CCR7 in the HCC or CRLM tissues, respectively (Figure 1C). Analysing the differences between gene expressions from matched normal/cancer samples corresponded widely with the results presented in Figure 1 thus ensuring that averaging out the Ct values did not mask significant differences between individual paired samples. Sections of tumor and normal cells have been microdissected in three CRLM and HCC tissue specimens, respectively, followed by subsequent chemokine qRT gene expression analysis that corresponded well with the results presented in Figure 1.

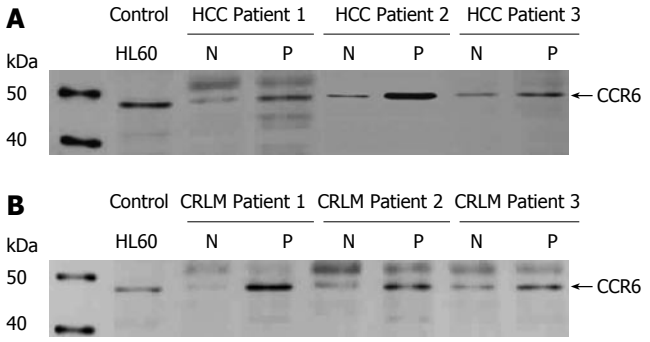


**Figure 3** Expression of chemokine receptor CCR6 in HCC and CRLM as determined by Western blot analysis. **A**: Chemokine expression in HCC; **B**: Chemokine expression in CRLM. Total cell lysates of tumor (P) and corresponding normal tissues (N) of three patients with HCC and CRLM, respectively, were immunoblotted with antibodies specifically recognizing chemokine receptor CCR6. Cell line HL60 served as a positive control for the detection of CCR6. ($n$ = 11 and 23, respectively).

### *Chemokine/chemokine receptor expression on the protein level*

Consistent with our RNA data, gene expression data for CXCL12, as assessed by enzyme-linked immunosorbant assay (ELISA), showed no significant difference in expression between the tumor and tumor neighboring tissues in either tissue type (Figure 2). Similarly, absolute CXCL12 protein quantities were largely the same in HCC and CRLM tissues. In contrast, we found statistically relevant up-regulation of CCL20 protein expression in both CRLM and HCC tissues compared with the respective tumor neighboring tissues ($P < 0.05$). These findings are again well in line with the RNA expression profiles. Comparative analysis of the absolute protein quantities between CRLM and HCC tissues revealed a significantly higher CCL20 expression of almost 24000 ng/L CCL20 in the CRLM tissues compared to approximately 6000 ng/L CCL20 in the HCC tissues ($P < 0.05$) as shown in Figure 2. In accordance with our qRT results we found no significant difference in protein expression for CCL19 and CCL21 between the tumor and tumor neighboring tissues in either tissue type (Figure 2). Despite a seemingly higher CCL21 protein expression level in the CRLM tissues compared with the HCC tissues, this difference was statistically not significant.

As assessed by western blot analysis and subsequent densitometric measurements, no significant difference in gene expression was observed for CXCR4 in the HCC tissues, whereas a statistically relevant 4-fold up-regulation of CXCR4 expression was detected in the CRLM tissues ($P < 0.05$), thus confirming the RNA transcript level analysis. Likewise, we found statistically relevant up-regulation of CCR6 expression in both tissue types ($P < 0.05$) as shown in Figure 3, but no significant difference in CCR7 expression between the HCC and CRLM tissues thus paralleling our qRT results.

## DISCUSSION

To date, various studies implicate chemokines CXCL12,

CCL20, CCL19 and CCL21 and their corresponding receptors not only in inflammatory cell recruitment but also in the tumorigenic process and metastatic homing of tumor cells. Recent data focus special emphasis on their roles in CRC, HCC and liver metastasis of different origins[21,28-31]. These findings prompted us to comparatively investigate their expression profiles in HCC and CRLM as the primary site of hematogenous metastases in CRC.

In recent years chemokine receptor CCR7 attracted considerable interest as a key receptor in determining lymph node metastasis in various malignant processes and tumor types such as leukemia, melanoma, gastric or non-small cell lung cancer[12,32-35], whereas also anti-tumorigenic effects have been demonstrated for the corresponding CCR7 ligand CCL21[36]. However, we observed no correlation between CCR7 expression and HCC or CRLM, respectively. Similarly, the corresponding ligands CCL19 and CCL21 showed no significant difference in their gene expression between the tumor and tumor neighboring tissues in either cancer type. Therefore, we believe that an association of these chemokine/receptor pairs with the progression of CRLM or HCC is rather unlikely.

While investigating CXCL12/CXCR4 expression, we made the interesting observation that CXCR4 was significantly up-regulated in the CRLM compared with the tumor neighboring liver tissues, yet no significant difference in gene expression was detected for CXCR4 in the HCC tissues thus indicating a distinct difference in the CXCR4 expression pattern between HCC and CRLM. These results are in line with recent findings that report CXCR4 gene expression in primary CRC demonstrated significant associations with recurrence and survival suggesting CXCR4 as a prognostic factor for poor disease outcome[29]. Unlike CXCR4, the chemokine ligand CXCL12 displayed no significant difference in gene expression between the tumor and the tumor neighboring tissues in HCC or CRLM thus indicating that CXCL12 and CXCR4 are inversely expressed in CRLM. This type of expression pattern was also demonstrated for CRC cell lines[37]. CXCL12 is presently discussed controversially with respect to its role in promoting tumor growth and metastasis. Various studies suggest CXCL12 involvement in metastasis, angiogenic activity and modulation of tumor immunity[3,14,15,38-40], while others describe efficient antitumor responses promoted by the CXCL12/CXCR4 interaction, suggesting that secretion of CXCL12 in tumors may mediate T-cell-dependent antitumor responses[41-43].

CCL20 is also presently controversially discussed with respect to its role in tumorigenesis. With regard to the chemoattractant properties of CCL20 for dendritic cells (DC), Fushimi *et al* reported on the tumor suppressive properties of this chemokine showing that CCL20 transgenes attract DC to established murine tumors and suppress tumor growth[44]. However, other studies correlate CCL20 transfection into a mouse tumor cell line with decreased immunogenicity and enhanced tumor growth[45] and recent data correlate increased serum levels of CCL20 in HCC with cancer-related factors[46]. Other reports allocating tumor growth promoting qualities with CCL20, associate CCR6 expression with hepatic metastasis in a rodent model and recent results, based on chemotactic and actin polymerization assays, correlate CCR6 expression with intrahepatic metastasis of HCC[30,47]. In our study, CCL20/CCR6 was the only pair among the chemokine ligand/receptor pairs under investigation that displayed a prominent expression pattern in HCC and CRLM, showing significant up-regulation in the tumor tissues of patients of both cancer types in comparison to the tumor neighboring tissues, respectively. We assume that these high expression levels in the malignant liver tissues of different origins - in one case primary tumor, in the other case liver metastases - indicate a possible pathogenetic role of CCL20 and its receptor in the development of hepatic malignancies. Moreover, we detected significantly higher CCL20 expression in the CRLM tissues compared with the HCC tissues. One possible explanation for this marked CCL20 overexpression in the hepatic metastases could be related to the ferocious malignity of metastatic cells. In other words, the malignant status of a cancer cell might be correlated with CCL20 expression. It is well known that the survival rate for patients is far worse when they have developed metastases at the time of surgery as compared to patients with the same primary tumor who have not developed metastases. It seems that as the chemokine metabolism of a cancer cell becomes increasingly unbalanced the further the malignity of the cell proceeds and the more aggressive a cancer tissue turns. This assumption is supported by previous clinico-pathological findings of our group[19], which demonstrate a significant increase in CCL20 expression rates in HCC tissues from grade Ⅲ tumors in comparison to HCC tissues from grade Ⅱ tumors. Consequently, the marked CCL20 up-regulation in CRLM in comparison to the HCC tissues reported here could be interpretated as an indication of the extent of alteration in the tumor cells. In line with this theory, we would expect higher CCL20 expression rates in liver metastases in comparison to the moderately differentiated tumor cells of the HCC tissues, since metastatic cells virtually show the highest grading level of tumor differentiation, usually bearing no resemblance with normal liver cells.

In summary, the results presented in this study suggest an association of the CCL20/CCR6 pair and the development and progression of hepatic malignancies and we propose CCL20 as a predictive pathogenetic marker for tumor grading and the existence of liver metastases. Since we also presented evidence for a correlation between CXCR4 expression and CRLM, our results also support theories that suggest CXCR4 as a potential predictive factor for colorectal metastasis of the liver. Since the identification of chemokines as key targets in cancer and metastasis has emerged as a quickly progressing research topic, various chemokine receptor antagonist compounds are presently being developed. The identification of the CCL20/CCR6 and CXCL12/CXCR4 pairs as novel targets in CRLM and other metastatic processes may be of potential clinical value for the staging of primary tumors and the prevention of hepatic recurrences.

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*COLORECTAL CANCER*

# Survival benefit in patients after palliative resection *vs* non-resection colon cancer surgery

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## Abstract

**AIM:** To evaluate survival in patients undergoing palliative resection versus non-resection surgery for primary colorectal cancer in a retrospective analysis.

**METHODS:** Demographics, TNM status, operating details and survival were reviewed for 67 patients undergoing surgery for incurable colorectal cancer. Palliative resection of the primary tumor was performed in 46 cases in contrast to 21 patients with non-resection of the primary tumor and bypass surgery. Risk factors for postoperative mortality and poor survival were analyzed with univariate and multivariate analyses.

**RESULTS:** The two groups were comparable in terms of age, gender, preoperative presence of ileus and tumor stage. Multivariate analysis showed that median survival was significantly higher in patients with palliative resection surgery (544 *vs* 233 d). Differentiation of the tumor and tumor size were additional independent factors that were associated with a significantly poorer survival rate.

**CONCLUSION:** Palliative resection surgery for primary colorectal cancer is associated with a higher median survival rate. Also, the presence of liver metastasis and tumor size are associated with poor survival. Therefore, resection of the primary tumor should be considered in patients with non-curable colon cancer.



**Key words:** Palliative surgery; Colorectal cancer

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## INTRODUCTION

Colorectal cancer is the second leading cause of death by malignancy in western countries. About 30% of patients, detected by screening programs for colorectal cancer, present with distant metastases[1] and non-resectable distant metastases are the threshold for curative approaches in these patients. Regardless of the palliate situation, these patients often require surgical treatment due to acute complications such as bowel obstruction and perforation. In addition colon cancer can lead to chronic symptoms such as anaemia. In principal, two surgical approaches are optional. First, the primary colon cancer can be resected and the passage of the gut can be achieved by direct anastomosis or alternatively by a stoma. Second, the primary tumor can be left in place and the passage of the gut is achieved by bypassing the tumor or a stoma. Choices for surgical approach are based on several aspects. Frequently, patients with advanced colon cancer are poor risk candidates for surgery, considering that many of the patients are elderly with concomitant medical diseases and have been debilitated by the advanced malignancy. Obviously, leaving the primary tumor in place is often less demanding for the patient and surgical palliation can be as minimal as possible.

On the other hand, experimental evidence suggests that the primary tumor might control dormancy of distant metastasis. The phenomenon by which a primary tumor is able to inhibit growth of distantly spread foci of the same tumor has been demonstrated[2]. Thus removing the primary tumor in an advanced stage of colon cancer might promote metastatic progression and determine survival time of the patient.

We reviewed the results of non-curative surgery for patients with primary colorectal cancer and analyzed factors that might influence the operative mortality rate, postoperative morbidity and the survival of these patients.

## MATERIALS AND METHODS

Palliative surgery was defined as surgery with the presence of residual local disease in the operative field and the presence of non-resectable distant metastases. Between April 1993 and December 2003, 69 patients (45 men, 24 women; mean age 62 years, range 26-85) fulfilled study criteria and were subsequently entered prospectively into a database of colorectal malignancies in the Department of Surgery, University of Regensburg. Survival data, medical record, the locations of primary cancers and metastases,

**Table 1 Main presenting symptoms of patients with palliative surgery for colorectal cancer *n* (%)**

| | Resection | Non-resection | Total |
|---|---|---|---|
| Screnning Program | 16 (34) | 5 (23) | 21 (31) |
| Symptomatic | 28 (61) | 13 (61) | 41 (61) |
| Symptomatic with obstruction | 2 (4) | 3 (14) | 5 (7) |

**Table 2 Sites of the index primary tumor *n* (%)**

| | Resection | Non-resection | Total |
|---|---|---|---|
| Right colon | 13 (27) | 1 (5) | 14 (19) |
| Colon transversum | 1 (2) | 0 (0) | 1 (1) |
| Left colon | 2 (6) | 0 (0) | 2 (4) |
| Sigma | 16 (34) | 4 (19) | 20 (28) |
| Rectum | 14 (30) | 16 (76) | 30 (43) |

**Table 3 cTNM characteristics of patients with resection and with non-resection surgery *n* (%)**

| | Resection (*n* = 46) | Non-resection (*n* = 21) | Total (*n* = 67) |
|---|---|---|---|
| cTx | 0 (0) | 4 (19) | 4 (6) |
| cT2 | 4 (9) | 0 (0) | 4 (6) |
| cT3 | 14 (32) | 5 (24) | 19 (29) |
| cT4 | 28 (60) | 12 (57) | 40 (59) |
| cN0 | 7 (15) | 2 (10) | 9 (16) |
| cN1 | 12 (26) | 6 (29) | 18 (26) |
| cN2 | 19 (41) | 0 (0) | 19 (28) |
| cN3 | 8 (16) | 0 (0) | 8 (11) |
| cN+ | 0 (0) | 13 (62) | 13 (19) |
| cM0 | 8 (17) | 8 (39) | 16 (24) |
| cM1 | 38 (82) | 13 (61) | 51 (75) |

operative details, postoperative outcomes, and patients' demographics were reviewed. Morbidity was defined as any post-operative complication that led to a prolonged hospital stay, additional procedures or post-operative mortality. Operative mortality was defined as death that occurred within 30 d from surgery. Two patients left Regensburg shortly after the surgery. Their follow-up data were incomplete, and they were excluded from the survival analysis.

Forty-five (65%) patients were admitted to the hospital with symptoms. The leading cause of admission was rectal bleeding in 39 (57%) patients. Urgent presentation with intestinal obstruction or perforation occurred in 5 patients. In addition to symptomatic patients, 21 (30%) of the tumors were detected by screening programs. Concomitant medical diseases were present in 47%, particularly prevalent in elderly patients (Table 1).

Table 2 presents the sites of the primary cancer. Tumors distal to the splenic flexure were found in 54 (80%) patients. The TNM characteristics are listed in Table 3. No statistical difference between the two groups was found. The type of surgery is shown in Table 4. Resection of the cancer was performed in 46 of the 69 patients.

**Table 4 Types of surgery *n* (%)**

| | Resection | Non-resection |
|---|---|---|
| Rectum resection | 13 (28) | |
| Rectum exstirpation | 3 (7) | |
| Sigma resection | 10 (28) | |
| Hemicolectomy left | 2 (4) | |
| Hemicolectomy right | 11 (24) | |
| Sigma + Rectum resection | 5 (11) | |
| Other Resections | 2 (4) | |
| Ileostomy | | 21 (100) |

**Table 5 Characteristics of patients who underwent resection and non-resection surgery[1] *n* (%)**

| | Resection (*n* = 46) | Non-resection (*n* = 21) |
|---|---|---|
| Sex: M/F | 29/17 (63/37) | 14/7 (67/33) |
| Median age | 61.6 | 63.7 |
| Peritoneal seedings | 7 (15) | 5 (24) |
| Operative mortality | 2 (4) | 0 (0) |
| Morbidity | 3 (7) | 0 (0) |
| Presence of distant metastasis | 38 (82) | 13 (62) |
| Median day of hospital stay (range) (postoperative mortality excluded) | 16.6 (7-60) | 14.7 (7-41) |

[1]Mann-Whitney *U* test.

Categorical variables were analysed by the $\chi^2$ test or Fisher's exact test when appropriate. Continuous variables were presented in median values with range; they were analysed by the Mann-Whitney *U* test. Survival was calculated from the time of surgery. Analysis of survival excluded patients who died during the postoperative period. Survival data were analysed by the Kaplan-Meier method. Factors were compared by log-rank test. Multivariate analysis used the Cox proportional hazard model, and factors with *P* values less than 0.05 in univariate analysis were included in the multivariate analysis. Presentation of results includes hazard ratio (HR) and 95% confidence interval (CI). *P* values less than 0.05 were considered statistically significant.

## RESULTS

Patients who underwent resection of the primary tumor or a non-resection procedure were comparable in terms of age, peritoneal seedings and operative mortality, as shown in Table 5. Furthermore, there was no difference in the presence of co-morbidities. Surgery was elective in 65 patients, and the remaining 2 were operated on as urgent surgery. The operative mortality rate was 4 patients, 3% with resection and 0% with non-resection procedure. Mean hospital stay was 15, 9 d (range 7-60). The median range of hospital stay (d) was not different between the two groups.

Post-operative therapy (47 chemotherapy alone, 2 radiation therapy, 6 combined chemoradiation) was given to 55 patients and was most prevalent for those who were

**Table 6 Patients with post-operative therapies *n* (%)**

| | Resection (*n* = 46) | Non-resection (*n* = 21) |
|---|---|---|
| Chemo | 39 (85) | 8 (38) |
| Radiation | 0 (0) | 2 (10) |
| Chemo and radiation | 2 (4) | 4 (19) |

**Table 7 Factors affecting the survival of patients with non-curative resection**

| | Resection | | Non-resection | |
|---|---|---|---|---|
| | *n* | median survival (d) | *n* | median survival (d) |
| Sex | | | | |
| Male | 29 | 742.1 | 14 | 218.7 |
| Female | 17 | 589.0 | 7 | 261.1 |
| Peritoneal metastasis | | | | |
| Yes | 7 | 683.4 | 5 | 201.6 |
| No | 39 | 685.0 | 16 | 333.0 |
| Bilobar liver metastasis | | | | |
| Yes | 38 | 599.7 | 13 | 220.4 |
| No | 8 | 578.2 | 8 | 253.1 |
| Resection | | | | |
| Yes | 46 | 543.8 | 0 | |
| No | 0 | | 21 | 232.9 |
| Lymph nodea | | | | |
| Positive | 37 | 660.0 | 19 | 225.8 |
| Negative | 9 | 790.8 | 2 | 300.0 |

younger in age or had a lower incidence of co-morbid medical diseases (Table 6). Since type of chemotherapy might influence the post-operative survival rate, stratification of the survival rates was performed according to the different drugs used. 12 different chemotherapeutic agents or combination of agents were used and no significant impact on survival was observed.

The median survival of patients with resection and without resection was 544 *vs* 233 d, respectively ($P < 0.001$; Figure 1).The analysis of risk factors that might affect the survival is shown in Table 7. Multivariate analysis showed poor survival to be associated with non-resection surgery ($P < 0.001$) and differentiation of the tumor ($P < 0.001$; Figures 2 and 3).

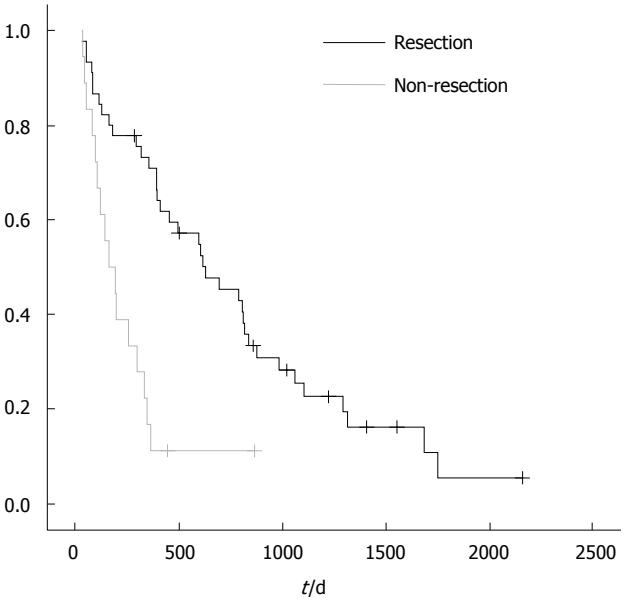


**Figure 1** Survival rates of patients with resection and non-resection.

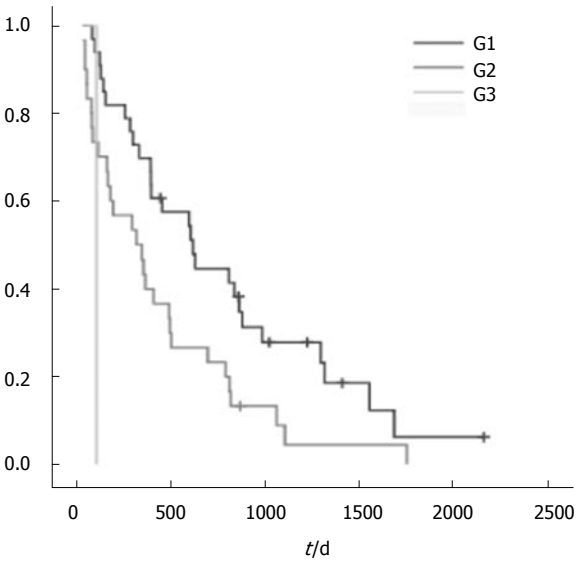


**Figure 2** Survival rates of patients dependent on differentiation of the tumor.

## DISCUSSION

Patients with incurable colorectal cancer are usually compromised by the presence of advanced malignancy and many of them also suffer from concomitant medical diseases, rendering surgical treatment an option with high risk. Therefore, the main objectives of the operative treatment are avoiding complications such as obstruction, perforation and profuse bleeding. In addition, surgery is worthwhile only if a reasonable length of survival can be achieved and the operative risk is acceptable. At present, only a few studies with detailed analysis of the results of palliative surgery for primary colorectal cancer are available making surgical treatment for patients with incurable colorectal cancer controversial[3-6]. We evaluated survival in patients undergoing palliative resection versus non-resection surgery for primary colorectal cancer in a retrospective analysis and found a significant longer survival rate for patients with resected primary tumors. Interestingly, patients with resection of the primary tumor did not benefit from chemotherapy to a greater extent compared to non-resected patients.

Bleeding and obstruction are reported to be the main presenting symptoms in patients with palliative surgery[3,7] in colorectal cancer. 67% of our patients had symptoms of bleeding or obstruction. The symptoms were usually discrete resulting in only 3% of emergency operations. As an alternative to urgent surgery, self-expanding metallic stents have been discussed as an effective non-operative means to relieve acute colonic obstruction[8-12]. However, stents may dislocate or cause bleeding in up to 30% of

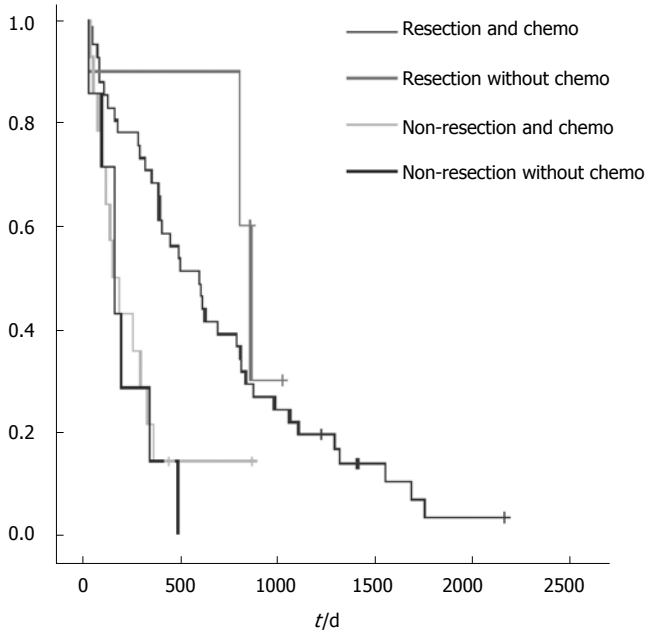


**Figure 3** Survival rates of patients with resection and non-resection and chemo therapy.

stent-treated patients. Furthermore after dilatation of the obstruction with a stent the tumor is obviously left in place. None of our patients was treated with a stent because the main symptom was bleeding, but further development of this technique might reduce side effects and it may be a better alternative to surgical treatment.

Our approach to palliative surgery was aggressive, aiming for resection of the primary tumor. Non-resection was carried out in patients who were in very poor condition or if the resection was hazardous. This point of view has also been offered by other authors[4] and is seen as better palliation. The resection rate in our cohort was 66%, which is comparable to other studies ranging from 69%[5] to 90%[3]. In contrast to other reports, age or location (rectum or rectosigmoid) of the cancer did not influence the likelihood for resection. There was a tendency to perform non-resection in patients with peritoneal seeding.

The operative mortality in this study was 2.8%. This is lower than in other studies reporting non-curative surgery[3,5]. Both patients died after anastomosis leakage and subsequent peritonitis. Anastomosis in palliative surgery for rectal cancer has been advocated by others[13] reporting one case of anastomotic leakage in 60 patients with palliative anterior resection. Our rate of anastomosis leakage is comparable and thus we would not recommend avoiding anastomosis in a palliative situation. Restoring bowel continuity is not a hazardous procedure and can improve quality of life. Non-resection was not an independent factor for high post-operative mortality (none of the non-resected patients died post-operatively). This stands in contrast to other studies, which reported high mortality rates up to 17% in patients without resection, while rates in those with resection were 9% and 5%. However, it should be emphasized that in those studies the patients who did not receive resection had advanced diseases, severe co-morbidities or unstable haemodynamic status during surgery. Both groups of our patients were comparable in these respects and lower operative trauma might be reflected in the lower mortality of non-resected patients.

Others found that extensive liver involvement and poorly differentiated tumors, the presence of ascites and the absence of other therapies, age over 75 years, and cardiovascular disease were associated with poor survival. We have found that poorly differentiated tumors are associated with poor survival in surgery for incurable colorectal cancer in our patients. Only 5 patients were older than 75 years and therefore no relation between age and outcome could be demonstrated in our study. Others propose that surgical treatment is only worthwhile if the patients do not have severe symptoms. Obviously the decision may need individual consideration, and the potential benefits and risks should be balanced carefully.

Thus non-resection, which is associated with similar morbidity and mortality rates and hospital stay lengths as resection, may be the only option to palliate complications such as obstruction or perforation. The aim must be to discharge the patient to enjoy a mediocre quality of life in the remaining days. This option, however, must be weighted against overall survival. In our cohort, the resected patients had a significant benefit in survival. Thus the enhanced operative mortality versus better survival should be considered in the individual patient, and good clinical judgment is needed to balance the benefits and risks of surgery.

We also found that the use of therapeutic chemotherapy and/or radiation therapy was not associated with better survival. Unfortunately, there were 12 different schemes of therapy given and thus no statistical difference was determined. There were only a few patients who did not receive palliative chemotherapy and thus no statistically significant level was reached. However, improved survival is likely to be achieved in current chemotherapeutic regimens. Traditional 5-fluorouracil based chemotherapy for metastatic colorectal cancer has a response rate of about 30%[14-18]. Better quality of life and improved survival have been demonstrated in patients who underwent chemotherapy than in those with the best supportive treatment[16]. Furthermore, irinotecan and oxaliplatin have been shown to produce a survival benefit and to improve quality of life in patients not responding to 5-fluorouracil based chemotherapy[19].

Taken together, palliative surgery for colorectal cancer is associated with an acceptable mortality rate, especially in patients without resection. Furthermore, anastomosis in a palliative situation is a safe procedure enhancing the quality of live for the patient. In addition, leaving the primary tumor in place is associated with poor survival. In the presence of these poor risk factors, good clinical judgment and careful consideration of balance between the risks and benefits are necessary before embarking on surgical palliation.

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*BASIC RESEARCH*

# Hemodynamic and antifibrotic effects of a selective liver nitric oxide donor V-PYRRO/NO in bile duct ligated rats

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## Abstract

**AIM:** To assess whether a liver specific nitric oxide (NO) donor (V-PYRRO/NO) would prevent the development of portal hypertension and liver fibrosis in rats with bile duct ligation (BDL).

**METHODS:** Treatment (placebo or V-PYRRO/NO 0.53 μmol/kg per hour) was administered i.v. to rats 2 d before BDL (D-2) and maintained until the day of hemodynamic measurement (D26). Intra-hepatic NO level was estimated by measuring liver cGMP level. Effects of V-PYRRO/NO on liver fibrosis and lipid peroxidation were also assessed.

**RESULTS:** Compared to placebo treatment, V-PYRRO/NO improved splanchnic hemodynamics in BDL rats: portal pressure was significantly reduced by 27% ($P < 0.0001$) and collateral circulation development was almost completely blocked (splenorenal shunt blood flow by 74%, $P = 0.007$). Moreover, V-PYRRO/NO significantly prevented liver fibrosis development in BDL rats (by 30% in hepatic hydroxyproline content and 31% in the area of fibrosis, $P < 0.0001$ respectively), this effect being probably due to a decrease in lipid peroxidation by 44% in the hepatic malondialdehyde level ($P = 0.007$). Interestingly, we observed a significant and expected increase in liver cGMP, without any systemic hemodynamic effects (mean arterial pressure, vascular systemic resistance and cardiac output) in both sham-operated and BDL rats treated with V-PYRRO/NO. This result is in accordance with studies on V-PYRRO/NO metabolism showing a specific release of NO in the liver.

**CONCLUSION:** Continuous administrations of V-PYRRO/NO in BDL rats improved liver fibrosis and splanchnic hemodynamics without any noxious systemic hemodynamic effects.



**Key words:** Nitric oxide; Portal hypertension; Liver fibrosis; Hemodynamics; Rats

Moal F, Veal N, Vuillemin E, Barrière E, Wang J, Fizanne L, Oberti F, Douay O, Gallois Y, Bonnefont-Rousselot D, Rousselet MC, Calès P. Hemodynamic and antifibrotic effects of a selective liver nitric oxide donor V-PYRRO/NO in bile duct ligated rats. *World J Gastroenterol* 2006; 12(41): 6639-6645

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## INTRODUCTION

Chronic liver diseases generally progress slowly from inflammation to fibrosis and in many cases to portal hypertension (PHT) and cirrhosis. PHT is responsible for the development of collateral venous circulation and esophageal varices which may bleed and are life-threatening[1,2]. PHT results from increased intrahepatic vascular resistance due to not only hepatic architectural changes inherent to fibrosis but also contraction of liver vascular smooth muscle cells, myofibroblasts and hepatic stellate cells (HSCs). Indeed, not only are HSCs involved in collagen deposition and development of liver fibrosis, but they also regulate intrahepatic blood flow by sinusoid contraction/constriction[3-5]. Therefore, liver fibrosis and PHT are usually two major therapeutic targets in chronic liver diseases that might be prevented by a single agent[6].

Nitric oxide (NO), a potent vasodilator was recently shown to modulate the intrahepatic vascular tone in normal rats[7,8]. Interestingly, NO has anti-fibrotic potentials which are related to its reactive oxygen species (ROS) scavenging capabilities. Cumulative evidences suggest that PHT in cirrhosis is partly due to a decreased liver NO production from the liver sinusoidal endothelial cells[9-11]. The deficiency of NO in the cirrhotic liver may also be involved in liver fibrosis and, inversely, intrahepatic NO supplementation may prevent liver fibrosis. Several NO donors (nitroglycerin, S-nitroso-N-acetyl penicillamine and sodium nitroprusside) were shown to exert a direct anti-

fibrogenic effect by inhibiting proliferation, motility, and contractility of HSCs induced by several ROS generating systems or by platelet-derived growth factor[12-14].

Contrasting with liver NO level, systemic NO level is increased in chronic liver diseases causing deleterious effects on systemic hemodynamics[11]. Thus, NO-based drug therapy in PHT should target the liver without systemic delivery of NO. Recently, it has been shown that transduction of liver with recombinant adenovirus carrying the neuronal NOS gene significantly reduced intrahepatic resistance and portal pressure in bile duct ligation and $CCl_4$ models of cirrhosis[15]. A promising pharmacological approach has also been reported with NCX-1000, a selective liver NO-releasing derivative of ursodeoxycholic acid (UDCA)[16]. NCX-1000, but not UDCA, was shown to reduce intrahepatic resistance in the $CCl_4$ model and to inhibit HSC contraction *in vitro*, suggesting that NCX 1000-derived NO was responsible for these effects. In contrast, both NCX 1000 and UDCA reduced liver collagen deposition in that study and it was unclear whether NCX 1000-derived-NO could have antifibrotic effects by itself. Beneficial effects of selective liver NO release need to be further assessed since UDCA derivative NCX 1000 yielded encouraging results for PHT and liver fibrosis treatment[16].

V-PYRRO/NO ($O^2$-vinyl 1-(pyrrolidin-1-yl) diazen-1-ium-1, 2-diolate) was reported to specifically deliver NO in the liver[17-19]. These (presumably cytochrome P450 and epoxide hydrolase) convert V-PYRRO/NO into PYRRO/NO by removal of the O-substituted vinyl ether group[17]. In contrast to V-PYRRO/NO, PYRRO/NO is an unstable anionic diazeniumdiolate with a very short half-life (3 s at pH 7.4, 37℃) and spontaneously decomposes into NO[17]. In other words, intra-hepatic production of NO from V-PYRRO/NO is due to the hepatic enzyme-dependent metabolism of V-PYRRO/NO. Using Alzet minipumps to deliver V-PYRRO/NO into rat systemic circulation for up to 24 h, Saavedra *et al* found no significant change in the mean arterial pressure, while liver protective effects were observed in TNFalpha / galactosamine hepatotoxic model[17]. This study suggested that NO derived from V-PYRRO/NO was present mainly in the liver rather than in the systemic circulation[17]. *In vitro* studies showed consistently that V-PYRRO/NO was specifically metabolized by hepatocytes and not by other cell types examined (endothelial cells plus Kupffer cells, pulmonary artery smooth muscle cells, pulmonary artery endothelial cells and the macrophage cell line Raw 264.7)[17].

The aim of our study was to determine the effects of an early and continuous administration of V-PYRRO/NO on liver fibrogenesis and on systemic/splanchnic hemodynamics in an appropriate model of liver injury. The bile duct ligation (BDL) model was chosen for several reasons. Like the $CCL_4$ model, BDL is one of the most common models of liver fibrosis and PHT. However, using BDL rather than $CCl_4$ to induce liver fibrosis and PHT should be preferable in our study because V-PYRRO/NO and $CCl_4$ both require cytochrome P450 to be metabolized. $CCL_4$ would therefore introduce a bias in our study. The main aim of the present study was to assess the effects of V-PYRRO/NO when administered continuously two days before BDL operation, for a total of four weeks. We found that V-PYRRO/NO was efficient in decreasing both hemodynamic disorders and liver fibrosis associated with BDL and that V-PYRRO/NO anti-fibrotic effect may be due to a decrease in lipid peroxidation.

## MATERIALS AND METHODS

### Animal model of cirrhosis

Male Sprague-Dawley rats (Faculty of Medicine, Angers, France) with an initial body weight of 210 to 350 g underwent BDL under ether anesthesia. The surgical procedure was performed on day 0, as previously described[20]. Under the same conditions, sham-operated rats with an initial body weight of 220 to 320 g had a laparotomy without ligation of bile duct and served as controls. According to published recommendations[21], all rats received weekly subcutaneous injections of vitamin K1 (50 μg) to decrease mortality from hemorrhagic diathesis. Protocols performed in this laboratory were approved by the French Agriculture Office in conformity with the European legislation for research involving animals.

### Therapeutic regimen

Our study aimed to assess the effects of early and continuous administration of V-PYRRO/NO in rats with BDL. For that purpose, treatment (V-PYRRO/NO or placebo) was administered two days before BDL or sham-surgical operation (D2) and maintained until the day of hemodynamic measurement four weeks later (D26). Continuous treatment was made using Alzet osmotic minipumps (model 2ML2, Alzet®, USA). Because of their two-week half-life characteristic, these pumps were replaced on D12 with new pumps filled with freshly diluted solutions of V-PYRRO/NO, prepared from stock solutions of V-PYRRO/NO (100 mg in 2 mL ethanol) diluted 1:10 in NaCl 0.9%. Final concentration of V-PYRRO/NO at 5 mg/mL was chosen in order to obtain a delivery rate of 0.53 μmol/kg per hour into the rat circulation using Alzet minipumps with a 2 mL volume capacity and a 5 μL/h delivery rate (giving a two-week half-life to these minipumps). The rat average weight was around 300 g. The minipumps were inserted subcutaneously to the back of the rats under anesthesia (ether) and connected to the left femoral vein of the animals with a polyethylene catheter (PE-60, Clay Adams, NJ, USA). The study included 4 groups of rats treated either with V-PYRRO/NO ($0.53\ \mu mol.kg^{-1}.h^{-1}$) or with placebo (ethanol diluted 1:10 in NaCl 0.9 %) from D2 to D26. The groups were as follows: sham with V-PYRRO/NO ($n$ = 13), sham with placebo ($n$ = 9), BDL with V-PYRRO/NO ($n$ = 21), and BDL with placebo ($n$ = 22).

### Rat conditioning

Hemodynamic measurement was performed on rats anesthetized with an intraperitoneal injection of 1mL/100 kg pentobarbital (Nesdonal® 0.5 g, Rhône-Poulenc, Paris, France). All rats were given free access to food and water until 14 to 16 h before the study. Food was withdrawn to avoid digestive influences on splanchnic hemodynamics[22].

During hemodynamic measurement, body temperature was maintained at 37℃ with a homeothermic blanket system (Homeothermic Blanket Control Unit, Harvard Apparatus Inc, Natick, USA). Hemodynamic measurement was performed 30 min after manipulation, i.e. when values had stabilized, which included: mean arterial pressure (MAP), heart rate (HR), portal pressure (PP), cardiac output (CO) or index (CI), systemic vascular resistance (SVR), and splenorenal shunt (SRS) blood flow. After hemodynamic measurement, the animals were killed by exsanguination under anesthesia. Body weight and liver mass were recorded at the time of death. Gains in body mass in each group were calculated as: (final body mass - initial body mass)/initial body mass.

### Hemodynamic and ascites measurement

MAP, HR, PP, SRS blood flow and CO were measured in anesthetized rats as described previously[23-25]. CO and SRS blood flow, an accurate index of collateral circulation blood flow, (as shown before[24]) were measured using the transit time ultrasound (TTU) devices. Amounts of ascites were estimated roughly and indicated with the following grades: 0: no ascites, 1: ascites absorbing less than half a compress, 2: ascites absorbing a compress, 3: ascites absorbing more than one compress.

### Liver fibrosis evaluation

Area of liver fibrosis. The area of liver fibrosis was measured by image analysis as described in our laboratory[26]. Briefly, three liver sections ( > 1 $cm^2$ each) were randomly taken from the right, median and left liver lobes of each rat. Liver sections were stained in 0.1% picrosirius red solution and histomorphometric analysis was performed on a Leica Quantimet Q570 image processor. Total liver area of fibrosis was expressed as the mean fibrosis percentage in the 3 liver sections. In each of them, 30 fields were evaluated.

Hepatic hydroxyproline content. As a liver fibrosis marker[27], hepatic hydroxyproline-content was measured using a modified version of the method described by Jamall *et al*[28] and Seifert *et al*[29]. Briefly, 3 liver fragments (250 mg) from each rat were homogenized in 6N HCl and then hydrolyzed at 110℃ for 18 h. After cooling, the hydrolysate was filtered through a 0.45-μm Millipore filter and aliquots of the hydrolysate were neutralized with NaOH 6N. Chloramine T was added to a final concentration of 2.5 mmol/L. After 5 min, 410 mmol/L paradimethyl-aminobenzaldehyde was added and the mixture was incubated for 30 min at 60℃. After cooling to room temperature, the samples were read at 560 nm with a control reagent which contained the complete system without added tissues.

The concentration of hydroxyproline in each sample was determined from a standard curve generated from known quantities of hydroxyproline. Mean hepatic hydroxyproline for each rat corresponds to three liver samples analyzed. The final result was expressed as μg of hydroxyproline/g liver protein. Liver protein content was measured in the fourth fragment of liver with a BCA Protein Kit (Pierce, Rockford, USA).

### Other biochemical measurements

Liver and kidney function tests. At the end of the hemodynamic measurement, blood samples (from the femoral artery) were immediately centrifuged at 4℃, and sera were kept at -80℃ until the biochemical assays were performed. Each rat underwent blood liver function tests including total bilirubin, alkaline phosphatases (ALP), alanine aminotransferase (ALT), aspartate aminotransferase (AST) activities and blood renal function tests including urea and creatinine.

Liver cGMP assay. Hepatic cGMP levels, reflecting hepatic NO level, were measured by enzyme immunoassay using a commercially available kit (Amersham-Pharmacia Biotech, Uppsala, Sweden).

Total liver NOS activity. NOS activity was measured by converting L[$^{14}$C] arginine into L[$^{14}$C] citrulline based on the modified method from Cahill *et al*[30]. Tissues were minced and homogenized by sonication (Vibracell, Bioblock, Illkirch, France) at 4℃ in a buffer containing 320 mmol/L sucrose, 50 mmol/L Tris-HCl, 1 mmol/L ethylendiaminetetra-acetic acid, 1 mmol/L DTT, 100 mg/L PMSE 10 mg/L leupeptin, 10 μg/ml trypsin-inhibitor and 2 mg/L aprotinin. After centrifugation (1000 × *g* for 5 min at 4℃), 50 μL of the supernatant (50-100 μg protein) was incubated in a total volume of 160 μL Tris-HCI (pH 7.4) containing 0.1 mmol/L ethylendiaminetetra-acetic acid, 3 μmol/L tetrahydrobiopterin, 1 mmol/L NADPH, 5 mmol/L valine, 5 mmol/L of ethylene glyco-bi-aminoethylether-N-tetra-acetic acid (EGTA), and 1 37 KBq/Ml [$^{14}$C] arginine. L[$^{14}$C] citrulline was separated by applying the samples to columns containing pre-equilibrated DOWEX AGW-X8 and eluting them with 1 mL of 1 mmol/L citrulline. The amount of radioactivity was measured by scintillation counting (Beckmann, LS 3801, Irvine, CA, USA). Enzyme activity was expressed as pmol of citrulline formed per mg of proteins per hour. Protein concentration was measured in the supernatant using a commercially available protein kit (BCA Protein Kit, Pierce, Rockford, USA).

Serum nitrate/nitrite levels. The systemic NO production was evaluated through the measurement of serum nitrate/nitrite levels using a commercially available colorimetric assay (Nitrate/Nitrite endpoint determination colorimetric assay kit, Cayman Chemical, Ann Arbor, USA).

Liver malonedialdehyde assay (MDA). Hepatic MDA level, a marker of lipid peroxidation, was determined by the thiobarbituric acid reaction and quantified by fluorometry (excitation wavelength: 515 nm, emission wavelength: 548 nm), using 1, 1, 3, 3-tetraethoxypropane as standard[31]. Hemodynamic, biochemical assays and morphometric studies of the liver were performed by two observers unaware of the treatment given.

### Statistical analysis

Quantitative variables were expressed as mean ± SD. Multiple quantitative variables were compared using the analysis of variance or by the Kruskall and Wallis test when variances were heterogeneous. Post hoc comparisons were performed using parametric tests for homogeneous (Tukey) or heterogeneous (Tamhame) variances or non-parametric test (Mann-Whitney). Box plots indicate median, interquartile range and extremes. An $\alpha$ risk < 5% was considered to be statistically significant. The qualitative variable 'mortality' has been studied using the $\chi^2$ test. The statistical software

**Table 1 General characteristics of rats**

| Characteristics | Sham | | BDL | | *P* |
|---|---|---|---|---|---|
| | Placebo *n* = 9 | V-PYRRO/NO *n* = 12 | Placebo *n* = 12 | V-PYRRO/NO *n* = 10 | |
| Initial body weight (g) | 299 ± 34 | 274 ± 29 | 288 ± 56 | 293 ± 67 | NS |
| Body weight gain (%) | 33 ± 21 | 22 ± 9 | 14 ± 12[a] | 21 ± 17 | 0.04 |
| Liver / body weight (%) | 2.3 ± 0.4 | 2.8 ± 0.3 | 5.9 ± 1.5[a] | 5.6 ± 2.0[c] | < 0.0001 |
| Ascites score | 0 | 0 | 0.3 ± 0.9 | 0 | NS |

[a]*P* < 0.05 *vs* sham, [c]*P* < 0.05 *vs* V-PYRRO/NO sham; NS: Not significant.

**Table 2 Liver and kidney serum function tests of rats**

| Parameter | Sham | | BDL | | *P* |
|---|---|---|---|---|---|
| | Placebo *n* = 9 | V-PYRRO/NO *n* = 12 | Placebo *n* = 12 | V-PYRRO/NO *n* = 10 | |
| Urea (mmol/L) | 10.2 ± 2.1 | 7.9 ± 1.7 | 9.2 ± 1.6 | 9.5 ± 2.7 | NS |
| Creatinine (μmol/L) | 77 ± 25 | 63 ± 24 | 66 ± 11 | 73 ± 18 | NS |
| AST (UI/L) | 172 ± 52 | 123 ± 60 | 1150 ± 1480[a] | 1164 ± 1349[c] | < 0.0001 |
| ALT (UI/L) | 48 ± 18 | 55 ± 36 | 88 ± 47 | 82 ± 20 | NS |
| Alkaline phosphatases (UI/L) | 160 ± 103 | 148 ± 80 | 380 ± 197[a] | 367 ± 141[c] | < 0.001 |
| Bilirubin (μmol/L) | 1.0 ± 0.0 | 1.3 ± 0.5 | 117.0 ± 22.0[a] | 101.0 ± 30.0[c] | < 0.0001 |

[a]*P* < 0.05 *vs* sham placebo, [c]*P* < 0.05 *vs* sham V-PYRRO/NO; NS: Not significant.

**Table 3 Hemodynamic data of rats**

| | Sham | | BDL | | *P* |
|---|---|---|---|---|---|
| | Placebo *n* = 9 | V-PYRRO/NO *n* = 12 | Placebo *n* = 12 | V-PYRRO/NO *n* = 10 | |
| MAP (mm Hg) | 119 ± 7 | 113 ± 11 | 104 ± 8[a] | 98 ± 13[b] | < 0.001 |
| Heart rate (beat/min) | 441 ± 29 | 428 ± 44 | 411 ± 29 | 422 ± 26 | NS |
| Cardiac index (mL/min·100 g) | 14 ± 5 | 15 ± 4 | 39 ± 11[a] | 33 ± 6[a,c] | < 0.0001 |
| SVR (dyn.s.cm$^{5}$·100 g$^{1}$·10$^{3}$) | 782 ± 355 | 636 ± 184 | 236 ± 89[a] | 248 ± 78[c] | < 0.0001 |
| Portal pressure (mmHg) | 9.2 ± 0.8 | 8.0 ± 1.2 | 16.3 ± 2.2[a] | 11.9 ± 2.7[a,c,e] | < 0.0001 |
| SRS BF (mL/min) | 0.22 ± 0.08 | 0.31 ± 0.12 | 1.33 ± 1.34[a] | 0.42 ± 0.22[e] | 0.007 |

[a]*P* < 0.05 *vs* Sham, [c]*P* < 0.05 *vs* sham V-PYRRO/NO, [e]*P* < 0.05 *vs* BDL placebo. MAP: Mean arterial pressure, SRS BF: Spleno-renal shunt blood flow; SVR: Systemic vascular resistance; NS: Not significant.

used was SPSS version 11.5.1 (SPSS Inc., Chicago, IL, USA).

## RESULTS

### General characteristics of rats

Initial body weight was not significantly different among the groups. As expected in placebo-treated rats, body mass gain was significantly lower in BDL rats than in sham rats. Moreover, liver mass/body weight ratio was significantly higher in BDL rats than in sham rats. V-PYRRO/NO treatment did not influence liver mass in any group but seemed to reduce the presence of ascites in BDL rats (no ascites was observed in the V-PYRRO/NO BDL rats, in contrast with placebo BDL rats) (Table 1).

### Liver and kidney serum function tests

BDL rats had a significant increase in AST and ALP activities and bilirubin level compared to their respective control groups. V-PYRRO/NO had no effect on liver or renal tests in sham or BDL rats (Table 2).

### Hemodynamics

As expected, BDL rats had a significant reduction in MAP and SVR, and a marked increase in CI and portal pressure as compared to their respective control groups. In the placebo groups, SRS blood flow was significantly increased in BDL rats *vs* sham rats. V-PYRRO/NO did not significantly change the systemic hemodynamics (MAP, HR, CI and SVR) in both BDL and sham rats. In contrast, V-PYRRO/NO significantly affected portal hemodynamics in BDL rats: a 27% decrease in PP and a 74% decrease in SRS blood flow (Table 3).

### Liver fibrosis and lipid peroxidation

Liver fibrosis was quantified in each group of rats based

**Table 4 Liver fibrosis of rats**

| | Sham | | BDL | | *P* |
|---|---|---|---|---|---|
| | Placebo *n* = 9 | V-PYRRO /NO *n* = 12 | Placebo *n* = 12 | V-PYRRO /NO *n* = 10 | |
| Area (%) | 2.5 ± 0.5 | 3.1 ± 0.8 | 12.1 ± 4.3[a] | 8.4 ± 3.6[c,e] | < 0.0001 |
| Liver hydroxyproline (μg/g protein) | 164 ± 26 | 171 ± 49 | 381 ± 88[a] | 268 ± 83[c,e] | < 0.0001 |

[a]$P < 0.05$ *vs* Sham, [c]$P < 0.05$ *vs* sham V-PYRRO/NO, [e]$P < 0.05$ *vs* BDL placebo.

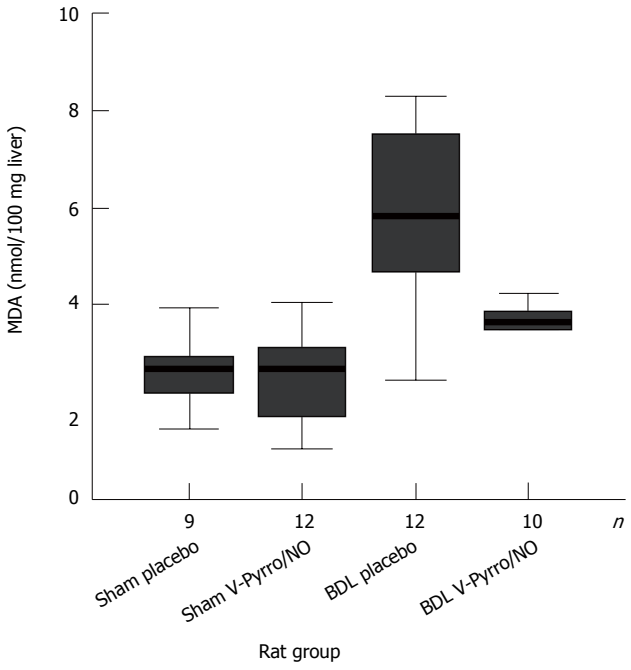


**Figure 1** Liver malonedialdehyde content (Kruskal-Wallis test: $P = 0.002$).

on the area of liver fibrosis and hepatic hydroxyproline content (Table 4). As expected, BDL operation induced a dramatic increase in both markers of liver fibrosis. V-PYRRO/NO significantly reduced the area of liver fibrosis and hepatic hydroxyproline content in BDL rats (by 31% and 30%, respectively). Since NO has ROS scavenging capabilities and lipid peroxidation products are known to stimulate fibrogenesis, we measured liver MDA levels in each group of rats to determine whether the anti-fibrotic effect of V-PYRRO/NO was related to the inhibition of lipid peroxidation. V-PYRRO/NO significantly decreased liver MDA level in BDL rats by 44%, $P = 0.007$ (Figure 1).

### *Measurements reflecting NO level*

V-PYRRO/NO significantly increased liver cGMP levels in BDL rats ($P = 0.04$) (Figure 2) but decreased liver total NOS activity ($P = 0.04$) (Figure 3). V-PYRRO/NO did not significantly change the serum nitrate/nitrite levels in BDL rats (82 ± 19 *vs* 72 ± 16 μmol/L, NS) (data not shown).

## DISCUSSION

V-PYRRO/NO had no noxious effects on body and liver

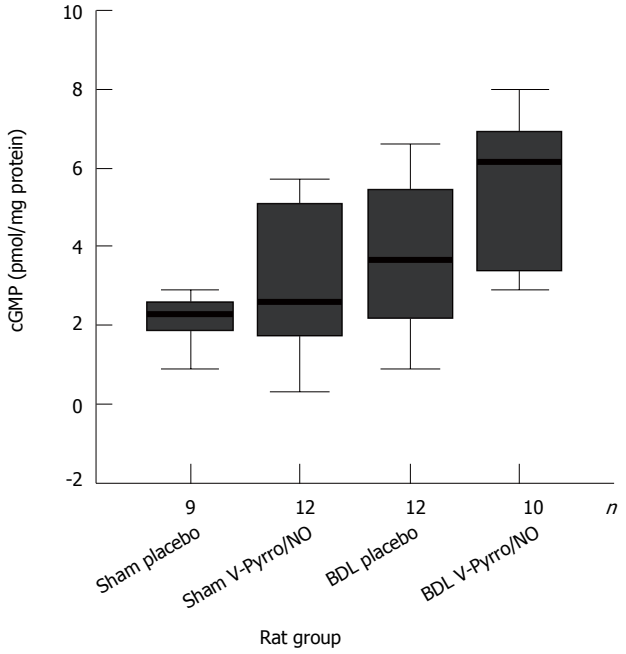


**Figure 2** Liver cGMP level (Kruskal-Wallis test: $P = 0.003$).

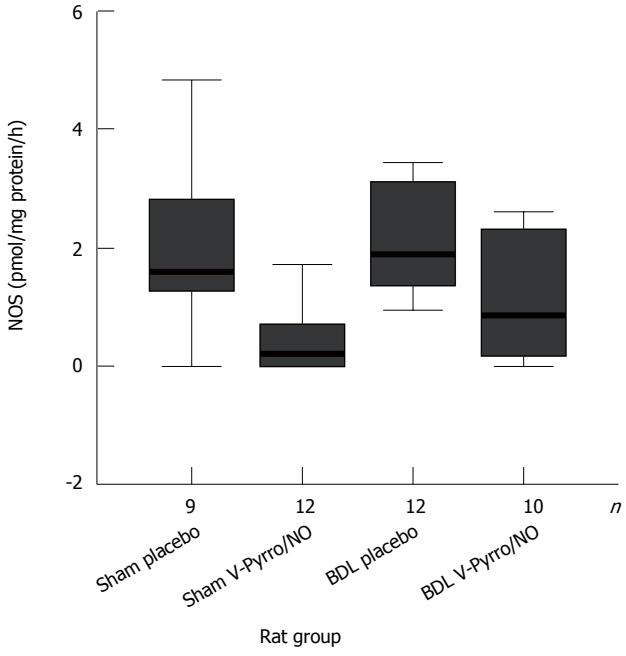


**Figure 3** Liver total NOS activity (Kruskal-Wallis test: $P = 0.001$).

weight gains, hepatic and renal functions, and did not significantly alter systemic hemodynamics (MAP, CI, or SVR) in both sham and BDL rats. Our data on V-PYRRO/NO systemic effects after continuous administration complete those of Saavedra *et al* showing that acute systemic administration of V-PYRRO/NO had no effect on MAP in the normal rats[17,19]. On the other hand, V-PYRRO/NO significantly decreased PP and SRS blood flow (an index of collateral circulation) in BDL rats. The decrease in PP can be attributed to reduced hepatic vascular resistance and/or diminished fibrogenesis. The decrease in SRS blood flow might be partly due to the reduction in PP or to other

mechanisms. Concerning the effect of liver-specific NO donor on PP, Fiorucci *et al*[32] reported a decrease in PP upon NCX-1000 administration for 5 d in four-week BDL rats whereas Loureiro-Silva *et al*[33] did not document such a decrease in their 10-12 week $CCl_4$ rats treated for 14 d with the same dosage of NCX-1000. The reason for NCX-1000 effect on PP is not obvious but may be model-related[34]. Our findings support this concept in the BDL model where V-PYRRO/NO did decrease PP.

Our study showed that V-PYRRO/NO limited the development of liver fibrosis in BDL rats (there was a significant decrease in the area of liver fibrosis and liver hydroxyproline content). Products of lipid peroxidation are known to stimulate fibrogenesis. To determine whether the anti-fibrotic effect of V-PYRRO/NO was related to the inhibition of lipid peroxidation, we measured MDA level (a marker of lipid peroxidation) in the livers of each group. Consistently with a study in the acetaminophen-induced hepatotoxicity murine model[35], we found that V-PYRRO/NO decreased hepatic MDA level in BDL rats. The decrease in liver lipid peroxidation in our V-PYRRO/NO rats may result from a reduction in hepatocyte death as suggested by several *in vitro* studies[17-19,35]. Thus, the mechanism underlying the anti-fibrotic effect of V-PYRRO/NO may be a NO-mediated protection against lipid peroxidation.

In the present study, V-PYRRO/NO treatment increased hepatic cGMP levels (reflecting hepatic NO generation) but decreased hepatic total NOS activity. This may be due to NO feedback inhibition on NOS activity, as suggested in a recent study[36]. As discussed in the introduction section, V-PYRRO/NO was designed to be a stable molecule until metabolized into PYRRO/NO by enzymes predominant in the liver. Hepatocytes contain high levels of enzymes capable of metabolizing V-PYRRO/NO and incubation with V-PYRRO/NO (24 h, 1 mmol/L) resulted in increased nitrite/nitrate levels in the supernatant of these cells in contrast to four other cell types examined[17]. Confirming other studies, we observed no systemic hemodynamic change and unaltered nitrate/nitrite levels in the serum of our BDL and sham rats after V-PYRRO/NO treatment (0.53 μmol/L per hour for 24 d), supporting the absence of systemic diffusion of NO from V-PYRRO/NO[17-19]. Moreover, in agreement with a study using acute administration of V-PYRRO/NO[17], we found that cGMP increased in the liver but not in a non-liver tissue (such as lungs, data not shown) of the rats chronically treated with V-PYRRO/NO. Our study also suggests that exogenous NO production in the liver is beneficial to the treatment of liver fibrosis and PHT. Additional experiments are however needed to rule out an effect of the bio-converted drug (i.e. the carrier compound without NO group).

In conclusion, the present *in vivo* study showed that early and continuous administration of V-PYRRO/NO via osmotic minipumps resulted in a decrease in liver lipid peroxidation and fibrosis, as well as splanchnic hemodynamic improvement (decrease in portal pressure and in collateral circulation development), without systemic hemodynamic effects. This result supports other studies showing that NO derived from V-PYRRO/NO was delivered preferentially to the liver rather than in the systemic circulation. Taken together, our study suggests that targeting NO delivery to the liver might be an interesting option for the treatment of liver fibrosis or early cirrhosis.

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*BASIC RESEARCH*

# Preventive effect of a pectic polysaccharide of the common cranberry *Vaccinium oxycoccos* L. on acetic acid-induced colitis in mice

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## Abstract

**AIM:** To study isolation and chemical characterization of pectin derived from the common cranberry *Vaccinium oxycoccos* L. (oxycoccusan OP) and the testing of its preventive effect on experimental colitis.

**METHODS:** Mice were administrated orally with OP two days prior to a rectal injection of 5% acetic acid and examined for colonic damage 24 h later. Colonic inflammation was characterized by macroscopical injury and enhanced levels of myeloperoxidase activity measured spectrophotometrically with o-phenylene diamine as the substrate. The mucus contents of the colon were determined by the Alcian blue dye binding method. Vascular permeability was estimated using 4% Evans blue passage after i.p. injection of 0.05 mol/L acetic acid.

**RESULTS:** In the mice treated with OP, colonic macroscopic scores (1.1 ± 0.4 *vs* 2.7, $P < 0.01$) and the total square area of damage (10 ± 2 *vs* 21 ± 7, $P < 0.01$) were significantly reduced when compared with the vehicle-treated colitis group. OP was shown to decrease the tissue myeloperoxidase activity in colons (42 ± 11 *vs* 112 ± 40, $P < 0.01$) and enhance the amount of mucus of colitis mice (0.9 ± 0.1 *vs* 0.4 ± 0.1, $P < 0.01$). The level of colonic malondialdehyde was noted to decrease in OP-pretreated mice (3.6 ± 0.7 *vs* 5.1 ± 0.8, $P < 0.01$). OP was found to decrease the inflammatory status of mice as was determined by reduction of vascular permeability (161 ± 34 *vs* 241 ± 21, $P < 0.01$). Adhesion of peritoneal neutrophils and macrophages was also shown to decrease after administration of OP (141 ± 50 *vs* 235 ± 37, $P < 0.05$).

**CONCLUSION:** Thus, a preventive effect of pectin from the common cranberry, namely oxycoccusan OP, on acetic acid-induced colitis in mice was detected. A reduction of neutrophil infiltration and antioxidant action may be implicated in the protective effect of oxycoccusan.



**Key words**: Common cranberry *Vaccinium oxycoccos* L; Pectin; Colitis; Anti-inflammatory; Mice

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## INTRODUCTION

Inflammatory bowel disease (IBD), including Crohn's disease and ulcerative colitis (UC), are characterized by chronic and spontaneously relapsing inflammation resulting in tissue destruction[1]. At present, there is no radical treatment for UC. Steroids and salicylic acid preparations are usually used to control and suppress the inflammation. However, adverse reactions and a high relapse rate are problems arising from these therapies[2]. Acute intestinal inflammation is associated with the infiltration of a large number of leukocytes into the bowel mucosa. A decrease in leukocyte number and function has been shown to prevent bowel inflammation[3]. Therefore, leukocytes are assumed to be a potential target of colitis drug research.

Pectic polysaccharides are well known to be a component of dietary fiber and to possess immunomodulating capacity with a predominant effect on neutrophils and macrophages. Feeding with pectins has been found to reduce a degree of experimental bowel injury induced by acetic acid[4] and by dextran sulfate

sodium[5]. Oral pretreatment with comaruman, a pectin from cinquefoil *Comarum palustre* L., has been shown to prevent the development of experimental colitis in mice[6].

It is well known that physiological effects are determined by the pectin structure[7]. Pectins used as food additives consist mostly of linear chains of galacturonic acid residues[8], whereas pectins of fruits, vegetables and herbs are characterized by an abundance of diversity of the macromolecular structure[7]. Therefore, the search and chemical characterization of new pectins with colon-protecting capacity is of great interest.

*Vaccinium* sp. (Ericaceae) is widespread throughout the cool temperate Northern Hemisphere, including the northern parts of Europe, Asia and North America. Cranberries are widely used in food as well as in ethnomedicine[9]. Regular drinking of cranberry juice has been shown to reduce the recurrence of urinary tract infections due to its action against coliform bacteria[10]. Cranberries were found to contain fructose[11] and a high molecular weight constituent of an unknown nature[12] that inhibits the adherence of bacteria to eukaryotic cells. The non-dialyzable material prepared from the cranberry juice concentrate has been shown to decrease the pro-inflammatory cytokine response of macrophages induced by LPS[13]. Cranberries are among the richest food sources of procyanidins, which are polyphenols possessing a potent anti-oxidant capacity[14]. Phenolic compounds of cranberries have been found to inhibit the growth of human gastrointestinal pathogens[15].

Cranberries have been far reported to contain a high amount of pectic polysaccharides[16,17]. However, the structure and anti-inflammatory activity of pectin derived from cranberries have not been studied until now.

The present work is devoted to testing the preventive effect of the pectin from the common cranberry *Vaccinium oxycoccos* L. on experimental colitis.

## MATERIALS AND METHODS

### Materials

Oxycoccusan OP was extracted from the ripe berries of the common cranberry *Vaccinium oxycoccos* L. (*Oxycoccus palustre* Pers.) with aqueous ammonium oxalate as described previously[18]. OP was shown to consist of primarily of galacturonic acid residues (82%) together with the minor residues of rhamnose (1.5%), arabinose (8%), glucose (5%) and galactose (3%) (Mw = 100-300 kDa). The apple pectin (MP Biomedicals, Inc.) was determined to consist of the galacturonic acid residues (70%) together with residues of glucose (29%) and traces of rhamnose, arabinose and galactose residues (Mw = 200-400 kDa). Prednisolone (Akrihin), *o*-phenylene diamine, horseradish peroxidase (Roanal), alcian blue, fetal calf serum, thiobarbituric acid (Sigma), and 96-well flat-bottom tissue culture plates (Linbro®, MP Biomedicals, Inc.) were used in this study.

### Animals

The structure of this study and the animal experimental procedures were approved by the Ethical Committee of the Komi Science Center of the Russian Academy of Sciences on Animal Care and Use.

Male A/HeJ mice weighing 20-25 g were used. They were housed in standard environmental conditions and fed with rodent diet and water ad libitum. The mice were fasted for a night before the induction of colitis but had free access to drinking tap water. Colitis was then induced and its severity was evaluated morphologically in three experiments. One of the biochemical parameters, including myeloperoxidase (MPO), malondialdehyde (MDA) or mucus content, was measured in the individual experiment after morphological observation. Each experimental group consisted of 7 animals.

### Application of OP and induction of experimental colitis

The mice were lightly anesthetized with ether. A plastic catheter (2 cm long, external diameter 1 mm) was inserted rectally into the colon so that the tip was 3 cm proximal to the anus. Acetic acid (5%, pH 2.5, 0.15 mL,) was instilled into the colon lumen through the catheter, and saline was instilled as a control[19].

Animals were treated orally with OP at the dose of 25-100 mg/kg (dissolved in water) by using a flexible rubber catheter two days before the induction of colitis. The control mice received the same amount (0.2 mL) of water. The positive control and reference groups received prednisolone (5 mg/kg) and commercial apple pectin (100 mg/kg), respectively.

### Assessment of colitis severity

Mice were killed by cervical dislocation one day after the administration of acetic acid. The entire colon was isolated and opened longitudinally, and rinsed with phosphate buffered saline (PBS). The macroscopic scoring of the colon damage was performed using the following criteria: 0, no macroscopic change; (1) mucosal erythema alone; (2) mild mucosal oedema, slight bleeding or small erosion; (3) moderate oedema, bleeding ulcers or erosion, oedema and tissue necrosis[20]. For each mouse, the ulcer area was determined by summing the sizes of lesions measured macroscopically by two blinded observers. The total area of damage was expressed as the relative percentage (%) of the total surface area of the colon.

### Determination of colonic myeloperoxidase (MPO) activity

After the macroscopic measurements, the excised colons (100-150 mg) were homogenized in PBS (pH 7.4) and centrifuged at 10 000 × *g* for 20 min at 4℃. MPO activity in the supernatants was then assayed by mixing the supernatant with citric phosphate buffer (pH 5.0) containing 0.4 mg/ml of *o*-phenylene diamine and 0.015% hydrogen peroxide. The change in absorbance (A) at 492 nm was measured spectrophotometrically and compared with a standard dilution of horseradish peroxidase[21]. MPO activity was expressed as units/mg of tissue.

### Measurement of the levels of lipid peroxidation

Malondialdehyde (MDA) formation was used to quantify lipid peroxidation in the intestinal wall and was measured as thiobarbituric acid-reactive material[22]. The intestinal samples were homogenized (100 mg/mL) in 1.2% KCl buffer. Two hundred microliters of the homogenates were then added to a reaction mixture consisting of 0.8%

aqueous thiobarbituric acid (0.75 mL), 8.1% sodium dodecyl sulfate (0.1 mL), 0.75 mL of 20% acetic acid (pH 3.5), and distilled water (0.3 mL). The mixture was heated at 90℃ for 45 min. After cooling to room temperature, 2.0 mL of a mixture of *N*-butanol and pyridine (15:1, v/v) was added to 2.0 mL of the samples. The mixture was shaken vigorously. After centrifugation at 4000 r/min for 10 min, the absorbance of the organic layer was measured at 540 nm. The MDA level is expressed as nanomoles per milligram of protein[23].

### Evaluation of the adherent colonic mucus

The mucus content of the colon was determined spectrophotometrically by the Alcian blue dye binding method[24]. The colon was excised and immersed for 2 h in 0.1% Alcian blue in 0.16 mol/L sucrose solution buffered with 0.05 mol/L aqueous sodium acetate. The unbound dye was then removed by two subsequent washings for 15 and 45 min in 0.25 mol/L sucrose solution and the mucus-bound dye was eluted by immersing the colon in a 0.5 mol/L $MgCl_2$ solution for 2 h. The solution obtained was centrifuged at 4000 r/min and *A* of supernatant was read at 650 nm. The amount of Alcian blue extracted per one gram of the wet colonic sample was then calculated from standard curves.

### Measurement of vascular permeability

Vascular permeability was measured in the healthy mice two days after a single oral administration of OP or indomethacin as a positive control. Mice were injected intravenously with 4% Evans blue (0.01 mL/g body weight). After injection of the dye, 0.05 mol/L acetic acid (0.01 mL/g body weight) was injected intraperitoneally. Fifteen minutes later, the mice were killed by an overdose of ether and the viscera were exposed after a 1 min period to allow blood to drain away from the abdominal wall. The animal was held by a flap of the abdominal wall and the viscera were irrigated with saline over a Petri dish. The washing was filtered through glass wool and transferred to a test tube. To each tube was added 0.1 mL of 0.05 mol/L NaOH to clear any turbidity due to protein, and *A* was read at 590 nm[25].

### Adhesion of peritoneal leukocytes

The peritoneal cells were harvested from mice by peritoneal lavage with phosphate buffered saline (5 mL). The cells were washed by centrifugation (1500 r/min, 10 min) and then suspended in Hank's medium supplemented with 10% fetal calf serum. The cell suspensions (0.1 mL) were incubated in a 96-well flat-bottom tissue culture plate in the presence of phorbol-12-myristate-13-acetate (PMA, 0.025 mg/L) as a stimulating agent at 37℃ for 15 min. The wells were washed to remove the nonadherent cells and the adherent cells were fixed in ethanol and stained with azure-eosin. The colored material was solubilized in MeOH and *A* of the solution obtained was measured at 650 nm[26].

### Statistical analysis

All data were expressed as the as mean ± SD. Nonparametric statistics were used to evaluate the results. Comparisons between groups were made with the Mann Whitney *U*-test (package "MedCalc" v.9.1.0.1, Mariakerke, Belgium). A value of $P < 0.05$ was considered significant.

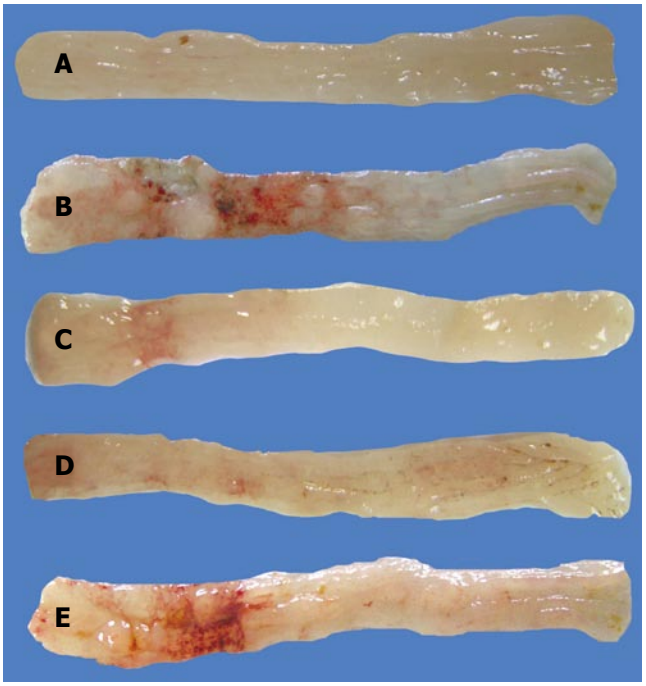


**Figure 1** Macroscopic examination of the colonic mucosa before (**A**) and 24 h after acetic acid injection (**B-E**). Mice were treated two days previously with (**B**) saline (damage score 4, square area of injury 40%); (**C**) prednisolone 5 mg/kg (damage score 2, square area of injury 1%); (**D**) OP, 100 mg/kg (damage score 2, square area of injury 3%); (**E**) apple pectin, 100 mg/kg (damage score 3, square area of injury 25%). The colon without the cecum was removed and opened along the mesenteric border.

## RESULTS

### Effect of OP on colitis severity

All of the mice were found to be sick after a rectal injection of acetic acid and to have diarrhea with blood in the stool and abdominal distention. In comparison with healthy mice (Figure 1A ), the colonic samples from mice with colitis were shown to have severe mucosal damage with edema, deep ulcerations and hemorrhages 24 h after the rectal injection of acetic acid (Figure 1B). A colonic inflammation was biochemically monitored by measuring the level of MPO activity in the colonic tissue taken from the site of inflammation. MPO activity was found to increase from 46 ± 8 to 112 ± 40 U/mg ($P < 0.01$, $n = 7$). Acetic acid infused rectally was shown to reduce the colon-bound mucus from 1.2 ± 0.4 to 0.4 ± 0.17 μg/mL ($P < 0.01$, $n = 7$). Damage of the colon reached the maximum level 24 h after the rectal injection of acetic acid. Signs of healing and regeneration of the mucosa were observed on the 5$^{th}$ d and the mucosa became almost normal on the 8$^{th}$ d after injection of acetic acid.

Oral administration of oxycoccusan OP two days before infusion of acetic acid into the colon was found to prevent the progression of colitis (Figure 1C). In the mice treated with OP, colonic macroscopic scores and the total square area of damage were significantly reduced when

**Table 1 Preventive effect on colon injury of oxycoccusan OP administered orally two days before infusion of acetic acid into the rectum of mice (means ± SD)**

| Pretreatment | Macroscopic damage score | Total square of injury, % |
|---|---|---|
| Vehicle-treated (Colitis) | 2.7 ± 0.8 | 21 ± 7 |
| Oxycoccusan OP | | |
| 25 mg/kg | 2.0 ± 0.4 | 17 ± 9 |
| 50 mg/kg | 2.0 ± 0.8 | 20 ± 9 |
| 100 mg/kg | 1.1 ± 0.4[b] | 10 ± 2[b] |
| Prednisolone 5 mg/kg | 1.3 ± 0.7[b] | 8 ± 3[b] |
| Apple pectin 100 mg/kg | 2.6 ± 0.7 | 23 ± 9 |

[b]$P < 0.01$ *vs* the vehicle-treated colitis group ('Colitis'). $n = 7$.

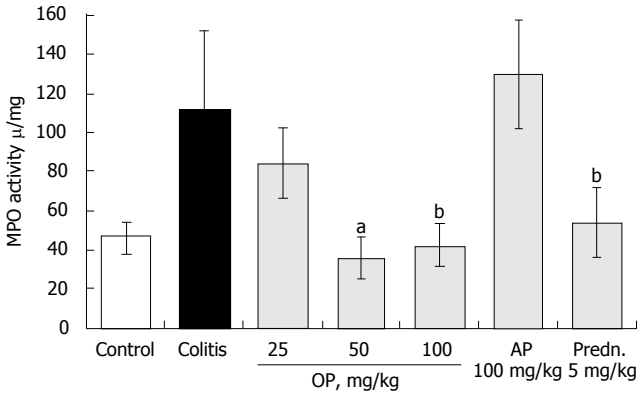


**Figure 2** Effect of oxycoccusan OP on myeloperoxidase (MPO) activity in colonic tissue of mice with acetic acid-induced colitis. Values are means ± SD ($n = 7$), [a]$P < 0.05$, [b]$P < 0.01$ *vs* the colitis group.

compared with the vehicle-treated colitis group (Table 1). The preventive effect of OP was dose-related and comparable with that of prednisolone in a dose of 5 mg/kg, administered p.o. (Figure 1D). The apple pectin used as a reference pectic substance failed to influence the colitis (Figure 1E).

The MPO activities were estimated in the colonic samples as a marker of granulocyte and monocyte influx into the tissue. A peroral pretreatment of mice with OP orally was shown to attenuate the tissue MPO activity in colons as compared with the colitis group (Figure 2).

In the next experiment, the macroscopic score (1.8 ± 0.4 *vs* 2.7 ± 0.6, $P < 0.05$) and square area of damage (7 ± 3 *vs* 28% ± 10%, $P < 0.01$) were also decreased in colitis mice receiving OP pretreatment. Corresponding to morphological alterations, MDA levels were increased in the colonic samples of colitis mice in comparison to healthy mice. MDA levels in the samples obtained from mice that received OP were found to decrease compared to those in the colitis group (Table 2).

In the third experiment, the production of mucus was measured in the colonic samples of healthy, colitis-affected (macroscopic score 2.8 ± 0.9; square area of damage 21% ± 7%) and OP-administered (macroscopic score 1.7 ± 0.5; damage square 8% ± 6%) mice. OP was found to stimulate the production of mucus by colons of colitis-affected mice. The evaluation of Alcian blue recovery from colon-bound mucus in the OP-treated animals with colitis showed that the levels of adherent mucus were comparable with those detected in native mice (Figure 3).

**Table 2 Preventive effects on colonic MDA levels of oxycoccusan OP administered orally two days before infusion of acetic acid into the rectum of mice (means ± SD)**

| Pretreatment | nmol/mg of protein |
|---|---|
| Healthy | 3.6 ± 0.8 |
| Vehicle-treated (Colitis) | 5.1 ± 0.8 |
| Oxycoccusan OP 100 mg/kg | 3.6 ± 0.7[b] |
| Apple pectin 100 mg/kg | 4.2 ± 1.4 |

[b]$P < 0.01$ *vs* vehicle-treated colitis group ('Colitis'). $n = 7$.

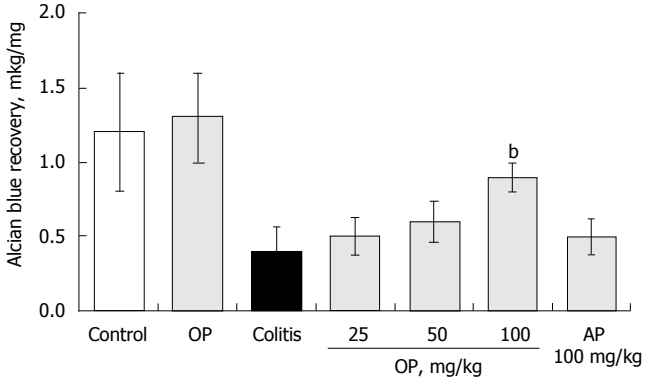


**Figure 3** Effect of oxycoccusan OP (100 mg/kg) on the levels of bound mucus in the colon of mice. Values are means ± SD ($n = 7$), [b]$P < 0.01$ *vs* the appropriate control.

### *Effect of OP on healthy mice*

Vascular permeability, the adhesion of peritoneal leukocytes, and colon-bound mucus were measured two days after oral administration of oxycoccusan to native mice as the initial step to elucidate the mechanism of the preventive effect of oxycoccusan. Oral administration of OP failed to influence the colon mucosa of healthy mice (Figure 3). OP was found to decrease the inflammatory status of mice as was detected by a reduction of vascular permeability (Figure 4). The adhesion of peritoneal neutrophils and macrophages was shown to slightly decrease after the administration of OP (Figure 5). The apple pectin, in comparison with oxycoccusan, failed to possess anti-inflammatory activity and failed to enhance the mucosal layer.

## DISCUSSION

Plant polysaccharides have been previously demonstrated to reduce colon damage in experimental colitis[4,5,27]. The described investigations were performed at an extended exposure (6-21 d) to polysaccharides starting after the induction of colitis. Various effects of polysaccharides are likely to interfuse during this long exposure. In the present study, pectin OP was found to prevent acetic acid-

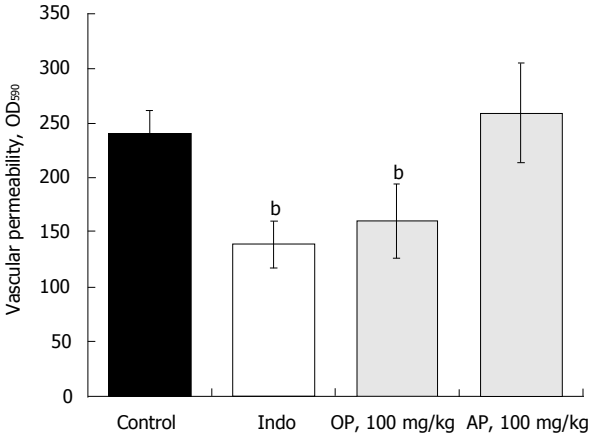


**Figure 4** Effect of oxycoccusan OP on the vascular permeability of the healthy mice. Values are means ± SD ($n$ = 7), $^{b}P < 0.01$ *vs* the control.

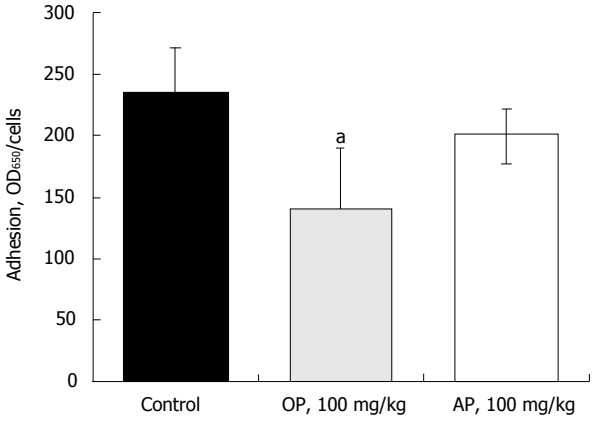


**Figure 5** Effect of oxycoccusan OP on the adhesion of peritoneal leukocytes of the healthy mice. Values are means ± SD ($n$ = 7), $^{a}P < 0.05$ *vs* the control.

induced colitis when given once beforehand at doses of 50-100 mg/kg. The doses of pectin used were chosen to approximate the estimated human intake as dietary fiber[28]. The protective effect of OP was comparable with that of prednisolone, which is a conventional medicine for the treatment of acute episodes of UC.

The exact mechanism of the preventive effect of OP on colitis remains unclear. The suppression of MPO activity by OP indicates that pectin inhibits the accumulation of neutrophils in the colonic mucosa. Indeed, MPO activity has been previously shown to be proportional to a number of infiltrating granulocytes, primarily neutrophils in the model of colonic inflammation[29]. Increasing amounts of transmigrating neutrophils induce significant epithelial disruptions resulting in epithelial discontinuities and erosions[30]. Therefore, it has been suggested that a delay in the influx of neutrophils into the intestinal wall is implicated in the preventive effect of cranberry pectin. A migration of neutrophils from the microvasculature is due to the influx of luminal proinflammatory bacterial products into the *lamina propria,* which results from disruption of the gastrointestinal barrier by oxygen radicals[31]. The role of reactive oxygen species has been stressed in recent years. Increased lipid peroxidation has been identified in the colons of patients with UC[32] as well as in experimental colitis induced by acetic acid[22]. Oxycoccusan was found to prevent an increase in MDA concentration. Plant polysaccharides, including pectins, are assumed to be capable of minimizing free radical-induced damage. A polysaccharide from *Angelica sinensis* has been found to possess a protective effect on immunological colon injury, which is probably due to the mechanism of antioxidation[33]. Ko *et al*[34] showed that colonic damage was significantly reduced by pretreatment of mice with an *Astragalus membranaceus* extract that contains polysaccharides and saponins. A polysaccharide from *Rheum tanguticum* has been shown to protect intestinal epithelial cells against oxidative stress[35]. A significant reduction in mucosal damage has been noted when pectin was administered before the perfusion of rat jejunum with peroxyl and hydroxyl radicals[36]. Therefore, it was proposed that the protective effect of OP against colitis was related to its antioxidant action. However, the presence of flavonoids in the OP preparations should be confirmed before elucidation of the mechanism of antioxidant action of polysaccharides *per se*.

Leukocyte adhesion represents one of the first steps in initiation of the inflammatory response and it is essential for the accumulation of active immune cells at sites of inflammation[37]. In light of this connection, the ability of OP to influence the adhesion of peritoneal leukocytes was determined in order to elucidate the involvement of leukocytes in the mechanism of the antiinflammatory effect. The data on leukocyte adhesion reduction may partially explain the preventive effect of OP on the accumulation of neutrophils in the intestinal wall.

A reduction of vascular permeability by OP indicates the suppression of the release of vasoactive mediators (histamine, serotonin) by competent cells. Sulfated glycosaminoglycan heparin has been shown to inhibit exocytosis in mast cells[38]. Oxycoccusan, as a pectin, possess structural similarity with heparin, which comprises a polyanionic macromolecule with a negative charge density. A possible effect of OP on the activity of basophils and mast cells is under investigation. It is possible that OP exerts its preventive effect on colitis by affecting plasma factors that regulate vascular permeability and cellular trafficking during the process of inflammation.

Thus, a preventive effect of pectin from the common cranberry, namely oxycoccusan OP, on acetic acid-induced colitis in mice was detected. Neutrophil infiltration reduction and antioxidant action may be implicated in the protective effect of oxycoccusan.

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*BASIC RESEARCH*

# Effects of recombinant human canstatin protein in the treatment of pancreatic cancer

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## Abstract

**AIM:** To examine the effect of canstatin, a newly discovered endogenous inhibitor of angiogenesis, in the treatment of pancreatic cancer *in vivo*.

**METHODS:** The canstatin cDNA fragment was synthesized and amplified from the total RNA extracted from human placenta tissues by RT-PCR. The resulting product was firstly cloned into pUCm-T vector, then into plasmid pET-22b (+) and transformed into *E. coli* BL21. Isopropyl-1-thio-b-Dgalactopyran-oside (IPTG) was used to induce the expression of canstatin protein and affinity chromatography was used to purify the protein. To determine the activity of purified recombinant human canstatin (rhCanstatin), orthotopic xenograft human pancreatic cancer models were established. Human pancreatic cancer cells (SW1990) were injected into the pancreas of BALB/c nude mice. Twenty-four nude mice with orthotopic xenograft tumor were randomly divided into 3 groups 10 d after the inoculation, and were treated with PBS 0.3 mL, or canstatin 5 mg/kg, or 10 mg/kg per day for 3 wk intraperitoneally. When the experiment was over, all tumors were resected and the effects of rhCanstatin on tumor growth, microvessel density (MVD) were analyzed.

**RESULTS:** After IPTG induction, SDS-PAGE showed a new monomeric 24 kDa protein band. This protein was purified through affinity chromatography and refolded through dialysis with a final concentration of 60 mg/L. In orthotopic pancreatic cancer models, the final tumor volume in groups treated with PBS, canstatin 5 mg/kg, 10 mg/kg were 355.21 ± 39.54 $mm^3$, 112.73 ± 10.47 $mm^3$, and 61.75 ± 6.99 $mm^3$ respectively. The immunohistochemical examination showed that the MVD in tumors treated with canstatin was significantly less than that in other group.

**CONCLUSION:** These findings demonstrate that the rhCanstatin effectively retards the growth of pancreatic cancer in a dose-dependent manner through inhibiting angiogenesis and may be a promising therapeutic agent for pancreatic cancer treatment in the clinic.



**Key words:** Canstatin; Angiogenesis; Pancreatic cancer; Anti-tumor agent

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## INTRODUCTION

The prognosis of patients with pancreatic cancer is poor, with or without treatment. The American National Cancer Institute (NCI) reported in its SEER Cancer Statistics Review that there are approximately 27 000 new cases of pancreatic cancer, resulting in around 26 000 deaths each year in the US. It is now the fourth most common cause of cancer deaths in USA and among the top ten common causes of cancer deaths in China. Being extremely aggressive, pancreatic cancer is often far advanced by the time symptoms occur, and a definitive diagnosis is established, with less than 20% resectable chance. The total 5-year survival rate is less than 5% from the time of diagnosis, although combination therapy has been accepted using radiation therapy or chemotherapy with or without surgery. Therefore, it is urgent to develop new treatment strategy for this malignant disease.

Angiogenesis, the formation of new capillary blood vessels, plays an essential role in normal and pathological processes, such as embryogenesis, wound healing and tumor growth[1-2]. Angiogenesis is also essential for the growth of solid tumors and their metastases[3-4]. Solid tumors cannot grow beyond a few millimeters in diameter

without generation of tumor vasculature. Furthermore, the denser the blood vessel formation, the higher the rate of tumor growth and the greater the metastatic potential[5]. Extensive research has led to the identification and isolation of several regulators of angiogenesis, some of which represent therapeutic targets.

Canstatin, a 24-kDa NC1 domain of the $\alpha_2$ chain of type Ⅳ collagen, is a newly discovered endogenous inhibitor of angiogenesis following the discovery of endostatin[6,7]. Previous studies have shown that canstatin efficiently suppresses the growth of human prostate carcinoma and renal cell carcinoma in models, even more potent than endostatin[8]. However, it is still unknown whether canstatin is effective in the treatment of pancreatic cancer. In this study, we cloned canstatin DNA sequence, expressed and purified recombinant human canstatin (rhCanstatin) and detected its anti-tumor effects on pancreatic cancer.

## MATERIALS AND METHODS

### Vectors, host bacteria and reagents

*E. coli* DH5α was preserved in our laboratory. *E. coli* BL21 and plasmid expression vector pET-22b (+) were purchased from the College of Life Sciences of Fudan University. pUCm-T vector was purchased from Shenergy Biocolor. *Bam*HI, *Hin*dⅢ, T4 DNA ligase and DNA molecular weight marker were supplied by New England Biolabs. Protein marker was purchased from Shanghai Sangon Co. Trizol was provided by Jingmei Biotech. One-step reverse transcription polymerase chain reaction (RT-PCR) kit was purchased from Takara. Isopropyl-1-thio-b-Dgalactopyranoside (IPTG), X-gal, plasmid mini kit, gel extraction mini kit and Coomassie Brilliant Blue R250 were purchased from Shanghai Watson. Nickelnitrilotriacetic acid-agarose column was purchased from Qiagen. The placental tissues were kindly provided by a woman who underwent caesarean operation in our hospital with informed consent. A pair of gene specific primers was designed according to canstatin DNA sequence presented in GenBank.

The forward primer (5'-CGGGATCCTGTCAGCAT CGCTACCTC-3') and reverse primer (59-CCCAAGCT TCAGGTTCTTCATGCACAC -3') were synthesized by Shanghai Sangon.

### Cell line, animals and antibody

A human pancreatic cancer cell line SW1990 was preserved in our laboratory. Cells were maintained in culture in DMEM supplemented with 10% fetal bovine serum (FBS) and antibiotics (100 units/mL penicillin and 100 μg/mL streptomycin). BALB/c male nude mice were purchased from Institute of Animal Center of Chinese Academy of Sciences in Shanghai. Rat anti-mouse CD34 monoclonal antibody was purchased from Hbt Biotechnology.

### RNA isolation

The fresh human placental tissues were frozen in liquid nitrogen immediately after dissection. The total RNA was isolated from the tissues using trizol according to the instruction. Pellets of the total RNA were dissolved in diethyl pyrocarbonate-treated water and finally quantitated by measurement of the optical density at 260 nm through spectrophotometer and stored at -80℃ before use.

### Amplification of target sequences by RT-PCR

The sequence encoding canstatin was amplified by RT-PCR from the total RNA. RT-PCR was performed in a 50 μL of reaction system containing 10 × reaction buffer (5 μL), 25 mmol/L $MgCl_2$ (10 μL), 10 mmol/L mixture of four dNTPs (5 μL), 40 MU/L ribonucleases inhibitor 1 μL, 5 MU/L AMV reverse transcriptase XL (1 μL), 5 MU/L AMV-Optimized Taq DNA polymerase (1 μL), 25 μmol/L forward primer (1 μL), 25 μmol/L reverse primer (1 μL), 1.8 g/L total RNA template (1 μL), ribonucleases free double distilled $H_2O$ (24 μL). RT-PCR conditions were: reverse transcription reaction at 50℃ for 40 min, initial denaturation at 94℃ for 2 min, 35 cycles, each cycle consisting of denaturation at 94℃ for 40 s, annealing at 52.6℃ for 40 s and extension at 72℃ for 1 min, and after the cycles a final extension at 72℃ for 5 min. The RT-PCR products were added to 6 × loading buffer, mixed and run on 1% agarose gel (0.5 μg/mL ethidium bromide). After electrophoresis at 100 volts for 40 min, the gel was examined under ultraviolet light and photographed. The PCR products were recovered from the gel following the instruction of gel extraction mini kit.

### Cloning of the PCR products

The ligation mixture included 10 × ligation buffer 1 μL, pUCm-T 1.5 μL, purified PCR products 6.5 μL and T4 DNA ligase 1 μL. After incubated overnight at 16℃, 3 μL of the ligation reactions was used to transform the competent *E. coli* DH5α by electroporation. The transformed cells were gently spread over the surface of the agar plate covered with IPTG and X-gal and containing appropriate ampicillin. The plate was inverted and incubated overnight at 37℃.

### Identification of bacterial colonies containing recombinant plasmids

Six white single bacterial colonies were picked from the agar plate and then inoculated into different LB medium containing ampicillin with the final concentration of 100 mg/L. The cultures were incubated overnight at 37℃ with vigorous shaking. Plasmid DNAs were isolated from each culture by the alkaline lysis method presented in the kit and then analyzed by gel electrophoresis after digestion with *Bam*HI or *Hin*dⅢ.

### DNA sequencing

The positive colony was sequenced on both strands with universal primers M13-/T7 by the Sanger Dideoxy-mediated chain-termination method. The nucleotide sequence was compared with that in GenBank database.

### Construction of prokaryotic expression vector

The recombinant plasmid DNAs were extracted by the method described earlier, digested with both *Bam*HI and *Hin*dⅢ, and finally ligated into predigested pET22b

vector by T4 DNA ligase. Plasmid constructs encoding canstatin were transformed into *E. coli* BL21 through electroporation. The transformed cells were gently spread over the surface of the agar plate containing ampicillin and incubated overnight at 37℃. Seven white single bacterial colonies were picked from the agar plate and grown in small-scale LB medium containing 100 mg/L ampicillin. Plasmid DNAs were isolated from each culture and restriction analysis was performed.

### Expression of rhCanstatin

An overnight bacterial culture was used to inoculate a 200-mL culture into LB medium. This culture was grown for approximately 3.5 h until the cells reached an A600 of 0.6. Then, protein expression was induced by addition of IPTG to a final concentration of 0.5 mmol/L. After a 3-h induction, cells were harvested by centrifugation at 12000 × *g*. The expression level of the protein was assessed by analysis on 12% SDS-PAGE gel followed by staining of Coommassie Brilliant Blue R250.

### Purification and refolding of rhCanstatin

The harvested cells were suspended in 1 mmol/L EDTA, 0.1 mol/L NaCl, 0.05 mol/L Tris-HCl, pH 8.0, sonicated briefly and centrifuged at 5000 g for 20 min at 4℃. The precipitated fraction was washed by 10 mL of 5 mmol/L imidazole, 0.5 mol/L NaCl, 0.02 mol/L Tris-HCl, pH 7.5 and again centrifuged at 5000 g for 20 min at 4℃. The supernatant fraction was discarded. The sediment was lysed in 8 mol/L urea, 0.5 mol/L NaCl, 0.02 mol/L Tris-HCl, pH 7.9 and passed over a 5-mL of nickelnitrilotriacetic acid-agarose column which had been equilibrated and washed with buffer 0 (8 mol/L urea, 0.5 mol/L NaCl, 0.02 mol/L Tris-HCl, pH 8.0) at a speed of 2 mL/min. The column was then stepwisely eluted by 15 mL each of 10, 25, 50, 125 and 250 mmol/L imidazole in buffer 0. The eluted fraction was collected separately and analyzed on SDS-PAGE gel. The eluted protein between 125 and 250 mmol/L imidazole was dialyzed three times against 2 liters of PBS at 4℃. Protein concentration was determined by the BCA assay.

### In vivo tumor studies

Human pancreatic adenocarcinoma cells (SW 1990) were harvested from culture, and $1 \times 10^7$ cells in sterile PBS were injected into the pancreas of 4-wk-old male BALB/c nude mice. The tumors grew for 10 d, after which animals were divided into groups of 8 mice each and treated with PBS 0.3 mL, or canstatin 5 mg/kg, or 10 mg/kg per day for 3 wk. All agents were injected intraperitoneally. When the experiment was over, all tumors were resected. Tumor length and width were measured using a Vernier caliper, and the tumor volume was calculated using the standard formula: length × width squared × 0.5.

### CD34 immunohistochemistry

At the end of treatment, mice were sacrificed, and the tumors were excised. The removed tumors were dissected with a scalpel into several pieces approximately 3-4 mm thick and then fixed in 4% paraformaldehyde for 24 h.

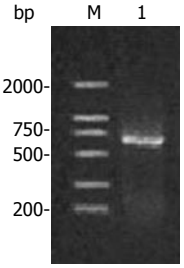


**Figure 1** Amplification products of human canstatin. Lane M: DNA marker; Lane 1: RT-PCR products.

Tissues were then transferred to PBS for 24 h before dehydration and paraffin embedding. After embedding in paraffin, 3-mm tissue sections were sliced and mounted. Sections were deparaffinized, rehydrated, and blocked with 10% rabbit serum. Next, slides were incubated at 4℃ overnight with a 1:50 dilution of rat anti-mouse CD34 monoclonal antibody, followed by two successive incubations at 37℃ for 30 min with 1:50 dilutions of biotin-conjugated secondary antibodies and HRP-conjugated streptavidin. Finally, diaminobenzidine was used as the chromogen. All slides were counterstained with Meyer's hematoxylin and observed under light microscope. Microvessel areas were quantified by manual counting of hotspots in sections, as described by Weidner *et al*[9].

### Statistical analysis

Results were expressed as mean ± SE. Unpaired *t* test was used to compare different groups of data as indicated. A 2-tailed *P* value of $< 0.05$ was considered significant.

## RESULTS

### Amplification of target sequences

The extracted total RNA was separated into three clear bands indicating 28S, 18S, and 5S after agarose gel electrophoresis. When the sample was diluted 50 times by diethyl pyrocarbonate-treated water, the values of $A_{260}$ and $A_{280}$ were 0.879 and 0.410 respectively with a $A_{260}$:$A_{280}$ ratio 2.095 through spectrophotometer. So the concentration of total RNA was 1.8 g/L according to the equation: total RNA concentration = 40 × $A_{260}$ × dilution power/1000. The target sequences were specifically amplified through RT-PCR, showing a clear band near the location of 684 bp DNA on agarose gel (Figure 1).

### Cloning in plasmid vectors and identification of bacterial colonies

After recovered from the gel, the resulting RT-PCR products were ligated into pUCm-T vectors, and the pUCm-T/canstatin constructs were then transformed into *E. coli* DH5α. Both blue and white colonies appeared on the agar plate after an overnight incubation. Six white colonies were selected. Restriction analysis of small-scale preparations of plasmid DNAs showed the plasmid DNAs in one white colony were separated into two bands near the locations of primary plasmid and objective gene fragment after digested by *Hin*dⅢ but only one band near

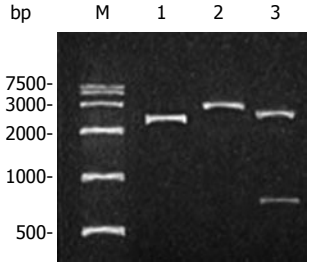


**Figure 2** Restriction analysis of plasmid pUCm-T/canstatin. Lane M: DNA marker; Lane 1: plasmid pUCm-T/ canstatin; Lane 2: pUCm-T/ canstatin digested by *Bam*H Ⅰ; Lane 3: pUCm-T/canstatin digested by *Hin*dⅢ.

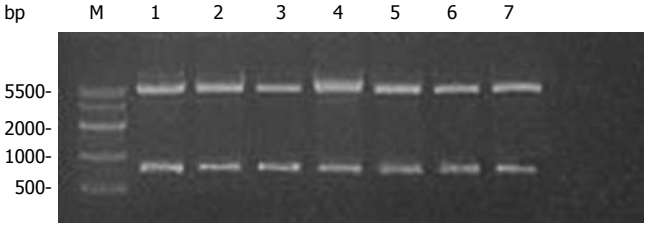


**Figure 3** Restriction analysis of plasmid pET-22b(+)/canstatin. Lane M: DNA marker; Lanes 1 to 7: plasmid DNAs of seven selected colonies digested by both *Bam*H Ⅰ and *Hin*dⅢ.

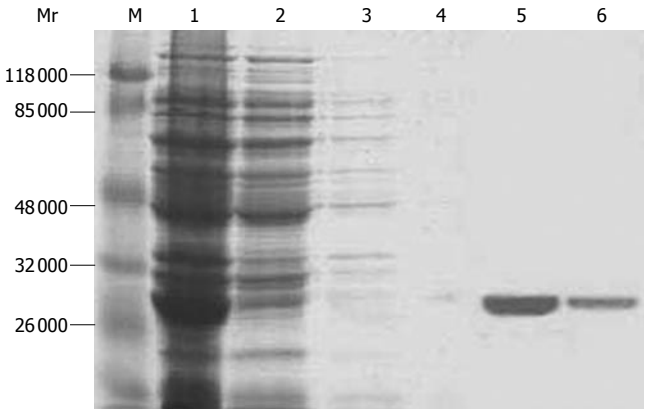


**Figure 4** SDS-PAGE gel electrophoresis of purified protein. Lane M: protein marker; lane 1: total bacterial protein; lane 2: 10 mmol/L imidazole elution; lane 3: 25 mmol/L imidazole elution; lane 4: 50 mmol/L imidazole elution; lane 5: 125 mmol/L imidazole elution; lane 6: 250 mmol/L imidazole elution.

the location of primary plasmid after digested by *Bam*HI (Figure 2). DNA sequencing demonstrated the sequence of this cloned gene was completely homologous to the canstatin gene sequence represented in GenBank.

### *Construction and identification of prokaryotic expression vector*

The canstatin cDNA was cut down from pUCm-T with *Bam*HI and *Hin*dⅢ and ligated into the vector pET-22b (+). The resultant plasmid pET-22b (+)/canstatin was then transformed into *E. coli* BL21. White colonies appeared on LB agar plate after an overnight incubation. Seven of them were picked and restriction analysis showed the plasmids DNA of all selected colonies were separated into two specific bands, one of which was the objective gene fragment (Figure 3).

### *Expression and purification of rhCanstatin*

After IPTG induction, SDS-PAGE analysis revealed a monomeric band at about 24 kDa. The relative quantitative values of expressed products over total bacterial proteins after 3 h of induction were 16.8 with the percentage of total protein 23.0% estimated by densitometry using Quantity One 4.1.1 protein analysis software (Figure 4). RhCanstatin was eluted from the column with two concentrations of imidazole (125 and 250 mmol/L). The concentration of dialyzed rhCanstatin was 60 mg/L.

### *In vivo tumor experiments*

Established orthotopic xenograft tumor models in mice were used to test the effectiveness of canstatin as an inhibitor of angiogenesis-dependent tumor growth. The final tumor volumes in groups treated with PBS, canstatin 5 mg/kg and canstatin 10 mg/kg were 355.21 ± 39.54 $mm^3$, 112.73 ± 10.47 $mm^3$, and 61.75 ± 6.99 $mm^3$ respectively (Figure 5A), and the tumor weights were 0.64 ± 0.08 g, 0.21 ± 0.02 g, and 0.12 ± 0.02 g respectively. The immunohistochemical examination showed that the MVD in tumors treated with canstatin 5 mg/kg (27.4 ± 6.1) or 10 mg/kg (20.2 ± 4.1) was significantly less than that in PBS group (40.2 ± 7.4) (Figure 5B). This decrease in tumor size was consistent with a decrease in CD34-positive vasculature (Figure 5C and D). In all of the *in vivo* studies, mice appeared healthy with no signs of wasting, and none of the mice died during treatment.

## DISCUSSION

Pancreatic cancer is a major cause of morbidity and mortality worldwide. It is an extremely life-threatening neoplasm due to its late diagnosis, rapid progression and resistance to chemo- and radiotherapy. Statistics data show it is the fourth leading cause of cancer death in the USA, with a median survival of less than 6 mo, and a 5-year survival rate of less than 5%[10,11]. Despite the great improvements in conventional therapy, including surgery, chemotherapy and radiotherapy, the mortality of this disease remains almost unchanged. There is, therefore, an urgent need for novel treatment strategies for this deadly disease.

Antiangiogenesis therapy is a new and attractive target for tumor therapy. By interrupting new vessel formation, tumor growth can be effectively arrested[12-16]. Compared to conventional therapy such as chemotherapy, anti-angiogenesis has several advantages theoretically[17,18]. Firstly, it has lower toxicity. Second, tumor vasculature is less mature and more sensitive to inhibiting agents than normal vessels. Third, due to the low mutagenesis rate of normal cell type, the endothelial cell is less likely to counteract therapeutic drugs through development of multi-drug resistance mechanisms. Fourth, drugs have easy access to their therapeutic target. In antiangiogenic therapy the target cells are those which constitute vessel walls, so intravenous antiangiogenic agents act on these cells directly. Fifth, antiangiogenic therapy has an inherent 'amplification' mechanism. Successful interference with only a few endothelial cells may lead to disruption of a whole vessel. Loss of a single vessel may deprive a significant number of tumor cells of essential nutrients, leading to cell death.

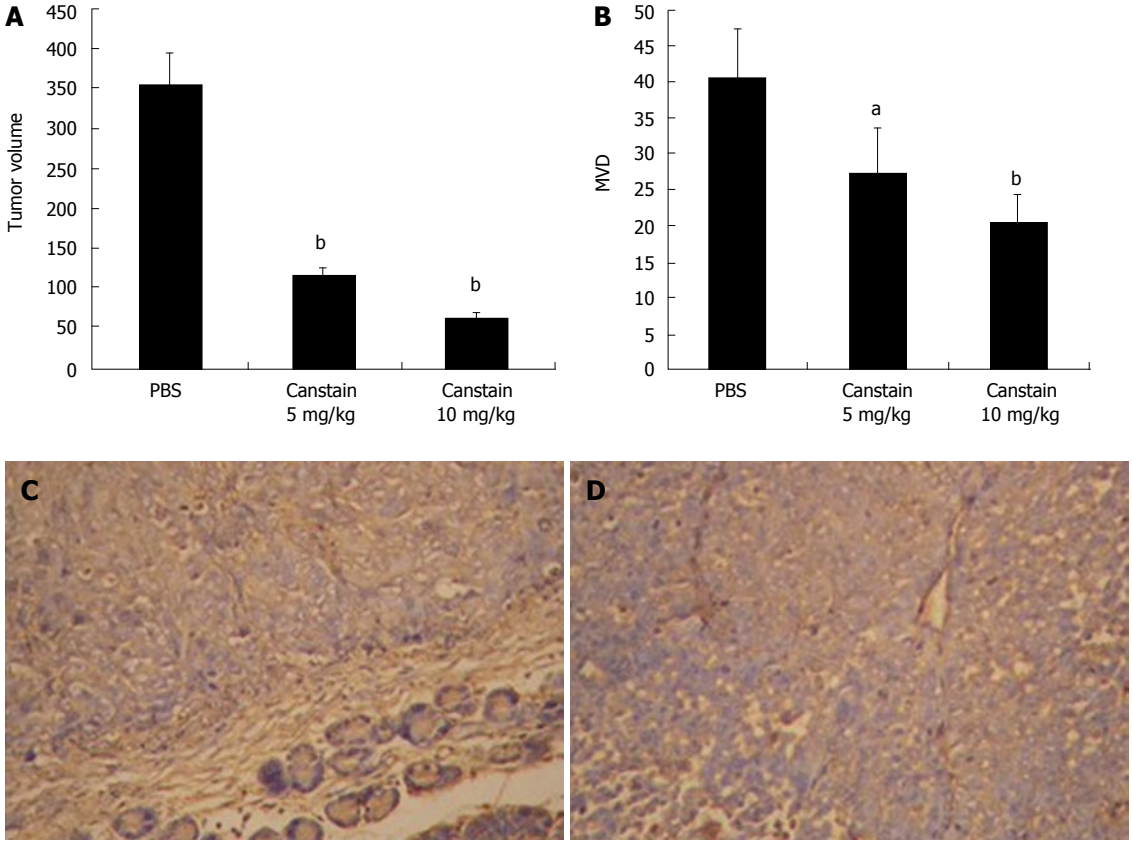


**Figure 5** *In vivo* antitumor effects in a xenograft model. The in vivo antitumor effect of the canstatin was analyzed in a SW1990 human pancreatic cancer cell orthotopic xenograft model. **A**: Tumor volumes in different groups: Canstatin 5 mg/kg or 10 mg/kg treatment showed a significantly stronger anti-tumor effect, compared with the PBS treated group ($^bP < 0.01$); **B**: MVD in xenograft models: significant inhibition of angiogenesis was observed in groups treated with canstatin 5 mg/kg or 10 mg/kg compared with that in the control group ($^aP < 0.05$, $^bP < 0.01$, respectively); **C**: CD34 immunohistochemistry in group treated with PBS (× 200): tumor sections showed extensive angiogenesis; **D**: CD34 immunohistochemistry in group treated with canstatin (× 200): tumor sections showed obviously decreased new vessels with a small focus of necrosis.

In light of these, this new therapy concept has aroused much attention and fueled the development of novel anti-angiogenic agents.

Despite some initial setbacks and negative clinical trial results, major progress has been made over the past few years in targeting angiogenesis for human cancer therapy. In Feb 2004, the US Food and Drug Administration (FDA) approved bevacizumab, a humanized anti-VEGF (vascular endothelial growth factor) monoclonal antibody, for the treatment of metastatic colorectal cancer, following a phase Ⅲ study showing a survival benefit[19]. In Dec 2004, the FDA approved pegaptinib, an aptamer that blocks the 165 amino-acid isoform of VEGF-A, for the treatment of the wet (neovascular) form of age-related macular degeneration (AMD)[20]. Angiogenesis inhibitors for the treatment of cancer have now been approved by the FDA in the US and in 28 other countries, including the European Union. When Avastin was approved in the US, Mark McClellan, the Director of the FDA, stated that: "Angiogenesis inhibitors can now be considered as the fourth modality of cancer therapy."

Canstatin, derived from the C-terminal globular non-collagenous (NC1) domain of the $\alpha_2$ chain of type Ⅳ collagen, is a new endogenous inhibitor of angiogenesis, which has been shown to successfully suppress the growth of implanted human prostate carcinoma and renal cell carcinoma in mice, even more potent than endostatin[21,22].

In the present study, placental tissues with abundant vessels were chosen to extract total RNA. And we successfully constructed a prokaryotic expression system to express rhCanstatin protein. *E. coli* was used as host bacteria because of its ability of rapid growth, well-characterized genetic background, high expression level, and cheapness, which is considered as the most attractive and ideal system for heterologous protein expression. The pET-22b (+) vector, as the expression vector, carries an N-terminal pelB signal sequence plus C-terminal His·Tag® sequence[23,24]. In our study, the resulting cDNA fragment was digested with *Bam*HI and *Hin*dⅢ and ligated into predigested pET-22b (+).This placed canstatin downstream of and in-frame with the pelB leader sequence, allowing for periplasmic localization and expression of soluble protein. The 3' end of the sequence was ligated in-frame with the polyhistidine tag sequence which also facilitated the purification of this target protein. After IPTG induction, SDS-PAGE analysis revealed a 24 kDa monomeric band, demonstrating the successful expression of target protein. Through affinity chromatography and dialysis, the production of rhCanstatin was purified and refolded with a final concentration of 60 mg/L and chick chorioallantoic membrane (CAM) assay demonstrated that rhCanstatin successfully inhibited newly formed blood

vessels in a dose-dependent manner.

Moreover, *in vivo* experiments showed that rhCanstatin successfully inhibited the growth of human pancreatic tumors by 3.15 fold or 5.75 fold at the dose of 5 mg/kg or 10 mg/kg respectively compared with placebo-treated mice. And this decrease in tumor size was consistent with a decrease in CD34-positive vasculature. Our findings demonstrate that rhCanstatin suppressed the growth of pancreatic cancer through inhibition of angiogenesis in a dose-dependent manner and suggest it may be a new potent anti-tumor agent for these patients. These findings add meaningful information to our understanding of the biological activity of rhCanstatin.

In conclusion, our findings verify the viewpoint that angiogenesis is an important target for cancer therapy. Animal tumor experiments demonstrate that the soluble rhCanstatin with antiangiogenic activity is a potential agent for pancreatic cancer therapy. It is meaningful to explore the applications of rhCanstatin in clinical use and study how to achieve the most effective combinations of antiangiogenic agents with chemotherapy or other biological agents in future.

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*BASIC RESEARCH*

# Effects of IκBα and its mutants on NF-κB and p53 signaling pathways

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## Abstract

**AIM:** To study the effects of IκBα and its mutants (IκBαM, IκBα243N, IκBαM244C) on NF-κB, p53 and their downstream target genes. The relationship of NF-κB, p53, and IκBα was further discussed.

**METHODS:** pECFP-IκBα, pECFP-IκBαM (amino acids 1-317, Ser32, 36A), pECFP-IκBα243N (amino acids 1-243), pECFP-IκBα244C (amino acides 244-317), pEYFP-p65 and pp53-DsRed were constructed and transfected to ASTC-α-1 cells. Cells were transfected with pECFP-C1 as a control. 30 h after the transfection, location patterns of NF-κB, p53 and IκBα (IκBαM, IκBα243N, IκBα244C) were observed by a laser scanning microscope (LSM510/ConfoCor2, Zeiss). RNA extraction and reverse transcription were performed in cells transfected or co-transfected with different plasmids. Effects of IκBα and its mutants on the transpiration level of NF-κB, NF-κB downstream target gene TNF-α, p53 and p53 downstream target gene Bax were observed by real time QT-PCR. In all experiments β-actin was reference. Results are expressed as the target/reference ratio of the sample divided by the target/reference ratio of the control. Different transfected cells were incubated with CCK-8 for 2 h in the incubator. Then the absorbance at 450 nm was measured by using a microplate reader.

**RESULTS:** Cells that were transfected with p53-DsRed revealed a predominant nuclear localization. YFP-p65 mainly existed in the cytoplasm. Cells were transfected with CFP-IκBα, CFP-IκBαM, and CFP-IκBα243N respectively and revealed a predominant cytosolic localization. However, cells transfected of CFP-IκBα244C revealed a predominant nuclear localization. The mRNA levels of p65, TNF-α, p53 and Bax in CFP-IκBα transfected cells did not change significantly, while in YFP-p65/CFP-IκBα co-transfected cells, IκBα decreased the transcription of p65 downstream gene TNF-α (2.24 ± 0.503) compared with the YFP-p65/CFP-C1 co-transfected cells (5.08 ± 0.891) ($P < 0.05$). Phosphorylation defective IκBα (IκBαM) decreased the transcription levels of all the four genes compared with the control ($P < 0.05$). The N terminus of IκBα (IκBα243N) increased the transcription of NF-κB (1.84 ± 0.176) and TNF-α (1.51 ± 0.203) a little bit. However, the C terminus of IκBα (IκBα244C) increased the transcription of NF-κB, TNF-α, p53 and Bax significantly (8.29 ± 1.662, 14.16 ± 2.121, 10.2 ± 0.621, 3.72 ± 0.346) ($P < 0.05$). The CCK-8 experiment also showed that IκBα244C and p53 synergistically mediate apoptosis.

**CONCLUSIONS:** IκBα and its mutants (IκBαM, IκBα243N, IκBαM244C) have different effects on NF-κB and p53 signaling pathways, according to their different structures. IκBαM bounds with NF-κB and p53 in cytoplasm steadily, and inhibits both of the two signaling pathways. p53 and IκBα244C may be co-factor in inducing apoptosis. The C terminal of IκBα enhanced cell death, which suggests that it may be a pro-apoptotic protein existed in cells.



**Key words:** Nuclear factor-κB; Inhibitor of NF-κB alpha; p53; Real-time QT-PCR

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## INTRODUCTION

Nuclear factor κB (NF-κB) is a family of pleiotropic transcription factors[1]. It regulates the transcription of a large number of genes that play key roles in embryonic development, lymphoid differentiation, apoptosis, and immune and inflammatory responses[2-5]. They are characterized by the presence of so called Rel homology domain, RHD, with a length of about 300 amino acids. Their active DNA-binding forms are homodimeric or heterodimeric complexes consisting of combinations of

these protein family members. The most abundant forms of NF-κB are p65/p50 hetero-dimers and p65/p65 homo-dimers[6-7]. In most cells, NF-κB complexes are normally localized to the cytosol as inactive complexes with inhibitory IκBα protein[8]. Activation of NF-κB in response to stimuli involves activation of IκB kinase (IKK), phosphorylation and degradation of IκBα at two serine residues (Ser32 and Ser36), followed by rapid ubiquitin-dependent degradation by the 26S proteosome and release of activated NF-κB[9-11]. Activated NF-κB then translocates to the nucleus, where it binds to its target DNA sequence and activates the transcription of a vast number and wide range of genes[12-15].

RelA, the p65 subunite of NF-κB is constitutively activated in certain neoplastic cells, such as pancreatic cancer cells and acute leukemia cells[16-19]. Approaches to suppress NF-κB activation in malignant cells have been considered as a potential treatment for neoplasia. Studies show that inhibition of NF-κB activation by expression of a dominant-negative mutant form IκBα (Ser 32, 36A) (IκBαM) completely inhibited liver metastasis of a pancreatic cancer cell line, and reduced angiogenesis in an ovarian cancer cell line[20-22].

Recent studies have shown that IκBα is found to inhibit p53 tumor suppressor protein by binding p53 to form a cytoplasmic p53 · IκBα complex, thus it prevents p53 nuclear translocation[23]. On the basis of this data, we hypothesized that when IκBα or its mutants were used to mediate activities of NF-κB in cells, they might affect the p53 signaling pathway simultaneously. In this study, pECFP-IκBα and its three mutants, pECFP-IκBαM (amino acides 1-317, S32, 36A), pECFP-IκBα243N (N terminus of IκBα, amino acides 1-243) and pECFP-IκBα244C (C terminus of IκBα, amino acides 244-317), were constructed. The location patterns of NF-κB, p53 and IκBα (IκBαM, IκBα243N, IκBα244C) were observed by laser confocal scanning microscopy. The effects of IκBα (IκBαM, IκBα243N, IκBα244C) on p53 and NF-κB, as well as the downstream genes of these two signaling pathways, were studied with real time QT-PCR. The relationship of NF-κB, p53, and IκBα activities is further discussed.

## MATERIALS AND METHODS

### Materials

Mammalian cell expression vector pEYFP-p65 was provided by Professor Johannes A. Schmid[24]. A human full-length IκBα cDNA was found in the universal GenBank database (gene number: AY033600) and was obtained from Funeng company (vector: RB01-IκBα). pDsRed-Mit vector was provided by Dr. Fuminori Tsuruta[25]. Wild-type p53 cDNA was provided by Dr. Ye KH (Jinan University, Guangzhou). Dulbecco's modified Eagle medium (DMEM) was purchased from GIBCO (Grand Island, NY). The RNA isolation kit and LightCycler FastStart DNA Master SYBR Green I kit were obtained from Roche. M-MLV Reverse Transcriptase was provided by BBI. Lipofectamine™ Reagent was purchased from Invitrogen. Cell Counting Kit-8 (CCK-8) was purchased from Dojindo Laboratories (Kumamoto, Japan).

### Construction of CFP-IκBα, YFP-p65 and p53-DsRed variants

Four expression constructs were constructed with the pECFP-C1 vector (cloning site, *Eco*RI and *Bam*HI; Clontech): (1) IκBα, the entire coding region (amino acides 1-317)(primers: FW3/RV1); (2) IκBαM, dominant negative IκBα construct made by altering Ser-32 to Ala-32 and Ser-36 to Ala-36, (amino acides 1-317, Ser32A,Ser36A) using primers FW1/RV1, FW2/RV1 and FW3/RV1 in turn; (3) IκBα243N, the N-terminal ankyrin region (amino acides 1-243 ) (primers: FW3/RV2); (4) IκBα244C, the C-terminal domain (amino acids 244-317) (primers: FW4/RV1). Wild-type p53 cDNA were cloned into the *Nhe*I and *Bam*HI sides of pDsRed-Mit vector (primers: p53F/p53R). The synthetic primers used for making these constructs by PCR are FW1: 5'-gag cgg cta ctg gac gac cgc cac gac gcc ggc ctg gac gcc atg aaa gac gag gag ta-3', FW2: 5'-g gag tgg gcc atg gag ggc ccc cgc gac ggg ctg aag aag gag cgg cta ctg gac gac c-3', FW3: 5'-c cgg aat tca ttc cag gcg gcc gag cgc ccc cag gag tgg gcc atg gag ggc c-3', FW4: 5'-gg aat tct aac aga gtt acc tac cag ggc ta-3', RV1: 5'-cgc gga tcc tca taa cgt cag acg ctg gcc tcc aaa cac aca gtc -3',RV2: 5'-cg gga tcc tta tca atg gtg atg gtg atg gtg gac atc agc ccc aca ctt-3', p53F: 5'-c tag cta gcg gaa gct tcc acc atg gag gag ccg cag tca gat-3', p53R: 5'- c ggg atc ccg gtc tga gtc agg ccc ttc tgt-3'. All constructs were verified by restriction and sequence analysis.

### Cell culture and transfections

Cell line, ASTC-α-1, was cultured in DMEM medium, supplemented with HEPES and 100 mL/L new born calf serum, and maintained at 37℃ at an atmosphere of 5% $CO_2$. Transient transfections were performed using the Lipofectamine™ Reagent (Invitrogen). Cells were transfected with CFP-C1 as a control. Microscopy of cells, RNA extraction and RT were performed 30 h after transfection.

### Laser scanning microscopy

YFP-p65, p53-DsRed and CFP-IκBα were visualized by using a laser scanning microscope (LSM510/ConfoCor2, Zeiss, Jena, Germany) with a 37℃ stage incubator. The distribution of YFP-p65 was observed by 514nm laser (HFT458/514, LP530). Cells transfected with p53-DsRed were observed with a 543nm laser and fluorescent images were collected with a 560 nm long-pass filter (HFT700/543, NFT545, LP560nm). CFP-IκBα, CFP-IκBαM, CFP-IκBα243N and CFP-IκBα243C were observed with an Argon-ion laser with 458 nm output and a band pass barrier filter (HFT458 nm, NFT545 nm, BP470-500 nm).

### RNA extraction and RT

Total RNA was isolated by using a high purity RNA isolation kit (Roche) according to the manufacturer's instructions. Total RNA (1 μg) was reverse transcribed with 20 U of M-MLV Reverse Transcriptase, using Oligo(dT)$_{18}$ primers (BBI) according to the manufacturer's instructions.

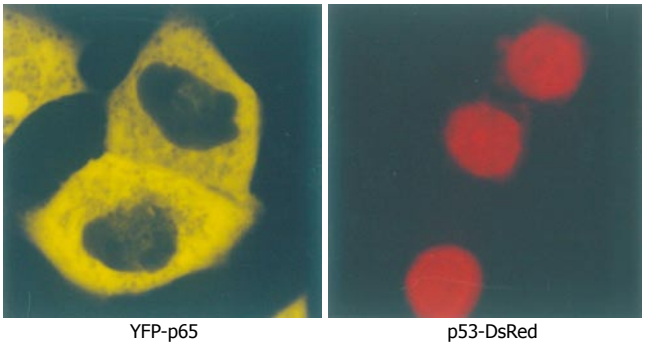


**Figure 1** Microscopy of YFP-p65 and p53-DsRed in living cells.

### *LightCycler real-time QT-PCR*

cDNA amplification by QT-PCR was carried out with the LightCycler FastStart DNA Master SYBR Green I kit (Roche). For QT-PCR, a mastermix of the following reaction components was prepared: 0.8 μL $MgCl_2$ stock solution, (25 mmol/L), 2 μL LightCycler FastStart DNA Master SYBR Green I, 2 μL (0.3 μmol/L) each of the primers, 11.2 μL water. LightCycler mastermix was filled in the glass capillaries and 2 μL of total cDNA temple was added. PCR primers were target gene 1 (p65: forward primer, 5'- GGCTATAACTCGCCTA GTGA -3'; reverse primer, 5'- CGAAG GAGCTGATCTGACTCA -3'), gene 2 (NF-κB downstream gene, TNF-α[26]: forward primer, 5' - CAGAGG GAAGAGTTCCCCAG -3'; reverse primer, 5'- CCTTGGTCTGGTAGGA GACG -3'), gene 3 (p53: forward primer, 5'-AGGTTGGCTCTGACTGTA-3'; reverse primer, 5'- GCAGCTCGTGGTGAGGCTC -3'), and gene 4 (p53 downstream gene, Bax[27]: forward primer, 5'- CTGACA TGTTTTC TGACGGC -3'; reverse primer, 5'-TCAGCCCATCTTCTTCCAGA-3'). In all experiments, β-actin was the reference (forward primer, 5'-GAAAT CGTGCGTGACATTAA-3'; reverse primer, 5'- GGA CTCGTCATACTCCTG-3').

The following LightCycler experimental run protocol was used: denaturation program (95℃ for 10 min), amplification and quantification program repeated 40 times (95℃ for 10 s, 55℃ for 5 s, 72℃ for 10 s), melting curve program (65-95℃ with a heating rate of 0.1℃ per second and a continuous fluorescence measurement) and finally a cooling step to 40℃. For the mathematical model it is necessary to determine the crossing points (CP) for each transcript. CP is defined as the point at which the fluorescence rises appreciably above the background fluorescence. The 'Fit Point Method' must be performed, at which CP will be measured at a constant fluorescence level[28]. Results are expressed as the target/reference ratio of the sample divided by the target/reference ratio of the control.

### *CCK-8 experiment*

Different transfected group cells were cultured in 96-well microplates for 48 h. CCK-8 was added to the cells and incubated for 2 h. OD450, the absorbance value at 450 nm, was read with a microplate reader (DG5032, Hua dong, Nanjing, China). The value is directly proportional to the number of viable cells in a culture medium and the cell proliferation.

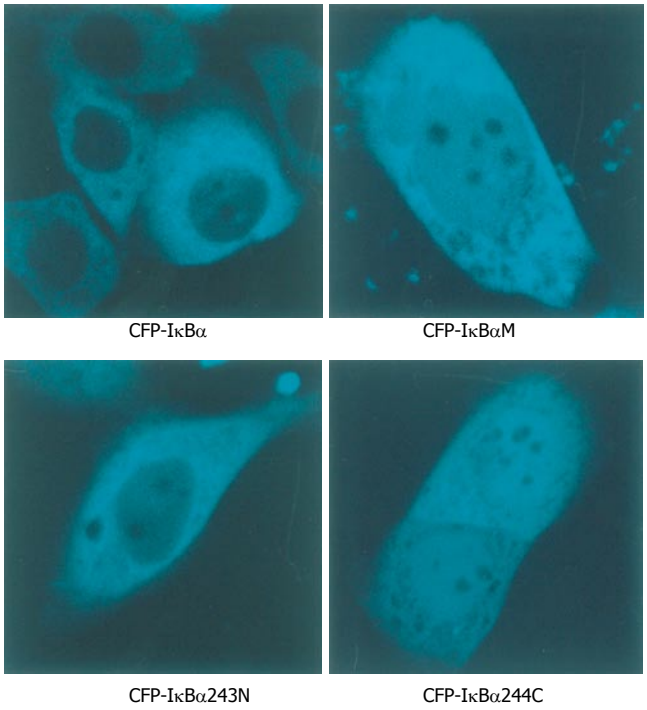


**Figure 2** Localization patterns of CFP-IκBα, CFP-IκBαM, CFP-IκBα243N and CFP-IκBα244C in living cells.

### *Statistical analysis*

Statistical results were obtained using the statistical software SPSS. The significant difference tests were based on analysis of variance with a single factor and two sample *t*-tests were performed.

## RESULTS

### *Localization patterns of p53-DsRed, YFP-p65, CFP-IκBα and its mutants in living cells*

Cells transfected with p53-DsRed revealed a predominant nuclear localization. YFP-p65 mainly existed in the cytoplasm (Figure 1). Cells were transfected with CFP-IκBα, CFP-IκBαM and CFP-IκBα243N respectively, and revealed a predominant cytosolic localization, while cells transfected with CFP-IκBα244C revealed a predominant nuclear localization of CFP-IκBα244C (Figure 2).

### *Standard curve for real time QT-PCR*

The concentration of the standards covers the expected concentration range of all samples. Dilution folds of the cDNA template for the standard curve run were 10ul to 3.20E-3 μL (Figures 3 and 4). The standard curves were analyzed with Real Quant Software to create a coefficient file. The coefficient file was used later in the relative quantification analysis.

### *Effects of IκBα and its mutants on the NF-κB signaling pathway*

Results are expressed as the target/reference ratio of

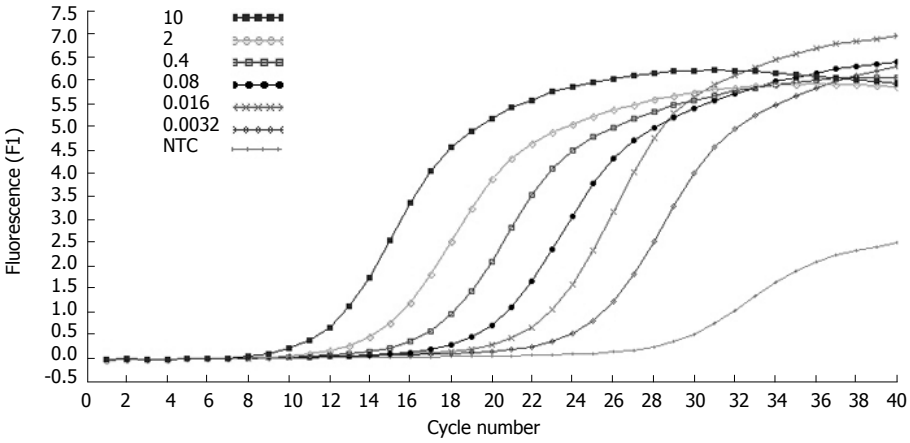


**Figure 3** Amplification plots of five fold serial dilutions of β-actin cDNA. The fluorescence values versus cycle number are displayed.

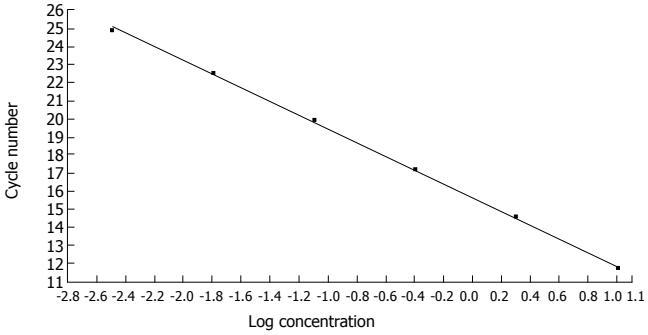


**Figure 4** Standard curve constructed with the β-actin cDNA standards from 1.00E + 1 to 3.20E + 3 by plotting the logarithmic concentration of the standard versus the crossing points (cycle number).

the samples divided by the target/reference ratio of the control ($n$ = 3). In all experiments β-actin cDNA was the reference. Results for the analysis of different transfected cells by QT-PCR showed that the level of p65 cDNA/β-actin cDNA (0.945 ± 0.152) and TNF-α cDNA /β-actin cDNA (1.05 ± 0.106) in CFP-IκBα transfected cells did not change significantly compared with the control(1.000 ± 0.000) (Figure 5), while in YFP-p65/CFP-IκBα co-transfected cells, IκBα decreased the transcription of p65 downstream gene TNF-α (2.24 ± 0.503) compared with the YFP-p65/CFP-C1 co-transfected cells(5.08 ± 0.891) ($^cP$ < 0.05) (Figure 6). In CFP-IκBαM transfected cells, the transcription level of the two genes (0.548 ± 0.086, 0.53 ± 0.056) decreased compared with the control ($^aP$ < 0.05). The level of p65 cDNA /β-actin cDNA and TNF-α cDNA /β-actin cDNA in CFP-IκBα243N transfected cells increased a bit (1.84 ± 0.176, 1.51 ± 0.203) ($^aP$ < 0.05). The most prominent was CFP-IκBα244C. It increased the transcription level of all the genes significantly (8.29 ± 1.662, 14.16 ± 2.121) compared with the control ($^aP$ < 0.05) (Figure 5).

### *Effects of IκBα and its mutants on the p53 signaling pathway*

Results for the analysis of different transfected cells by QT-PCR indicated that the effect of IκBα and its mutants on p53 and its downstream gene, Bax, were different (Figure 7). The level of p53 cDNA /β-actin cDNA and Bax cDNA /β-actin cDNA in CFP-IκBα (0.9 ± 0.126, 1.04 ± 0.109) and CFP-IκBα243N (0.806 ± 0.129, 0.79 ± 0.108) transfected cells did not change very much. In CFP-IκBαM transfected cells, the transcription level of the genes decreased (0.43 ± 0.061, 0.53 ± 0.063) compared with the control ($^aP$ < 0.05), however, CFP-IκBα244C increased the transcription level of p53 and Bax significantly (10.2 ± 0.621, 3.72 ± 0.346) ($^aP$ < 0.05) (Figure 7), which suggested that IκBα244C may play an important role in inducing apoptosis[14].

### *IκBα 244C and p53 synergistically mediates apoptosis*

To study the effect of IκBα244C on cell death, a CCK-8 experiment was performed. As Figure 8 shows, transient expression of p53-DsRed (1.206 ± 0.099 ) or CFP-IκBα244C (1.259 ± 0.072) resulted in enhancement of cell death compared with the control (1.531 ± 0.168) ($n$ = 6, $^aP$ < 0.05). The synergistic effect in mediating apoptosis by p53-DsRed/CFP-IκBα244C (0.805 ± 0.047) ($^dP$ < 0.01) was obtained.

## DISCUSSION

NF-κB and p53 are important transcription factors present in the majority of cells[27-30]. Constitutively activated NF-κB has been associated with increased cell proliferation and survival in cancer cells. Inhibitor of NF-κB alpha, IκBα, participates in both NF-κB and p53 signaling pathways[23,31-33] (Figure 9). The functional NF-κB and p53 activities may modulate each other, which in turn could affect the subsequent responses.

Previous studies demonstrated that IκBα interacts with NF-κB and p53 with different interaction sides[2,8,23]. IκBα and its mutants might have different effects on the transcription of NF-κB, p53 and their downstream genes, according to their different structures. Our studies showed that IκBα did not influence the transcription level of NF-κB, p53 and their downstream target genes in static cells compared with controls, which maybe due to the integrity of IκBα and the self-regulation capability of the cells. IκBα243N (amino acides 1-243), with lack of the PEST domain that regulates basal level protein turnover and is required for inhibition of DNA binding of NF-κB, increased the transcription of NF-κB and TNF-α slightly. Because IκBα243N cannot interact with p53, it has no effect on the transcription of p53 and Bax.

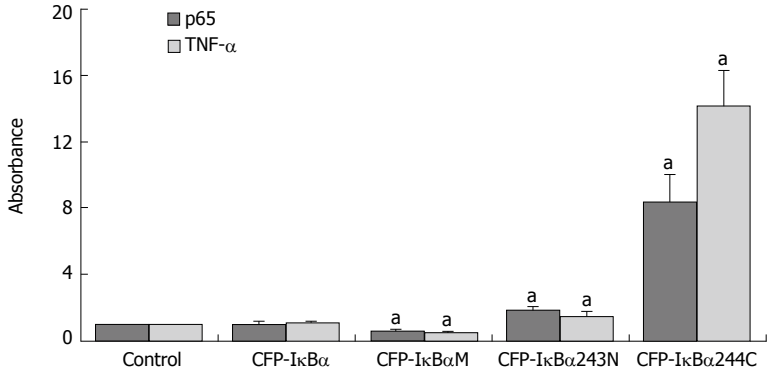


**Figure 5** Effects of IκBα and its mutants on NF-κB and NF-κB downstream gene TNF-α. Abscissa showed different transfected Cells. Y-coordinate expressed the target/reference ratio of the samples divided by the tar = get/reference ratio of the control. In all experiments β-actin cDNA was reference. ([a]*P* < 0.05 *vs* control).

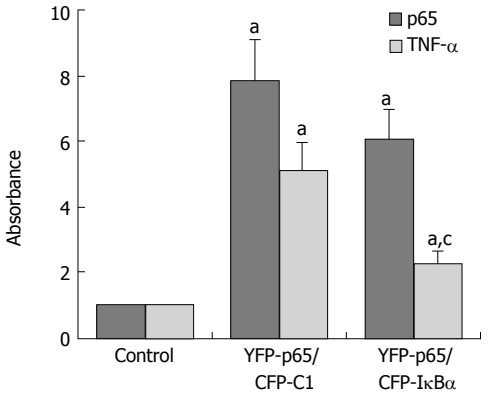


**Figure 6** Effects of IκBα on over expressed NF-κB. ([a]*P* < 0.05 vs control; [c]*P* < 0.05 *vs* YFP-p65/CFP-C1 group).

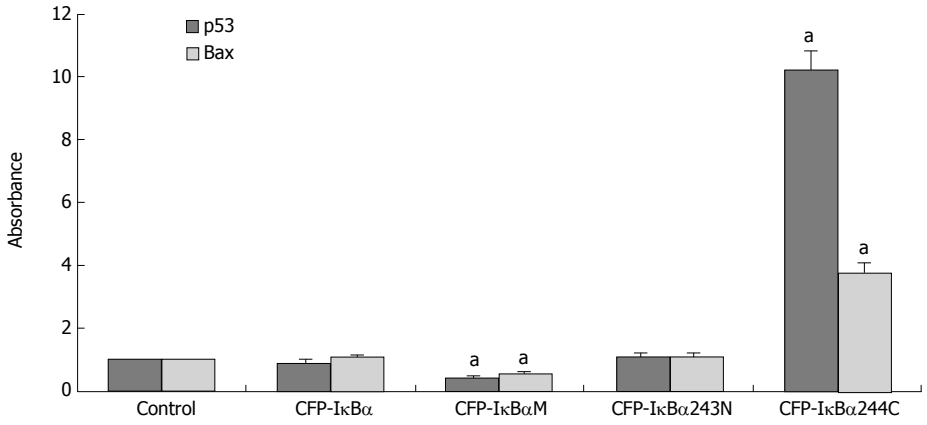


**Figure 7** Effects of IκBα and its mutants on p53 and p53 downstream gene Bax. ([a]*P* < 0.05 *vs* control).

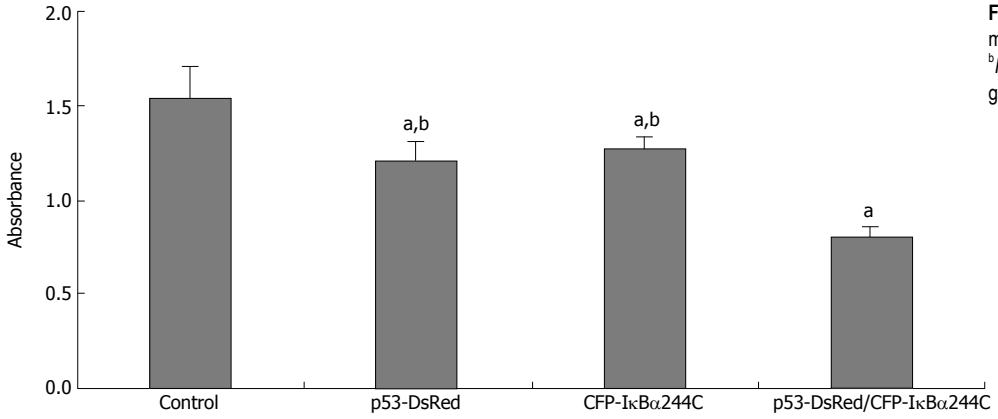


**Figure 8** IκBα244C and p53 synergistically mediates apoptosis. ([a]*P* < 0.05 *vs* control; [b]*P* < 0.01 *vs* p53-DsRed/CFP-IκBα244C group).

Dominant negative IκBα (IκBαM, Ser32, 36A) and the C terminus of IκBα (IκBα244C, amino acides 244-317) are notable because of their significantly different effects. IκBαM has mutations in Ser32 and Ser36. It can not phosphorylate at Ser32 and Ser36 and degradate, so IκBαM bound with NF-κB and p53 in the cytoplasm steadily and inhibited the transcription of their downstream genes, which is consistent with the report that IκBαM has been found to represses p53-dependent apoptosis in acute lymphoblastic leukemia cells[34]. In particular, transfection of IκBαM in human colon carcinoma and breast cancer cell lines did not increase sensitivity to daunomycin or Taxol[35,36]. IκBαM may repress p53 expression in two ways: (1) A portion of IκBαM directly interacts with p53 in cytoplasm and inhibits p53 translocate to the nucleus; (2) IκBαM binds to NF-κB in the cytoplasm and NF-κB·IκBαM complex is formed, which in turn inhibits the NF-κB activity and the NF-κB dependent p53 activity, for the NF-κB signaling cascade is a potential modulator of p53 activity, and NF-κB is a co-factor of p53 in mediating cell death[37-39].

IκBα244C does not have the ARD (ankyrin repeat domain) and NES in N terminus. It could not prevent NF-κB from translocating to the nucleus, and IκBα244C itself

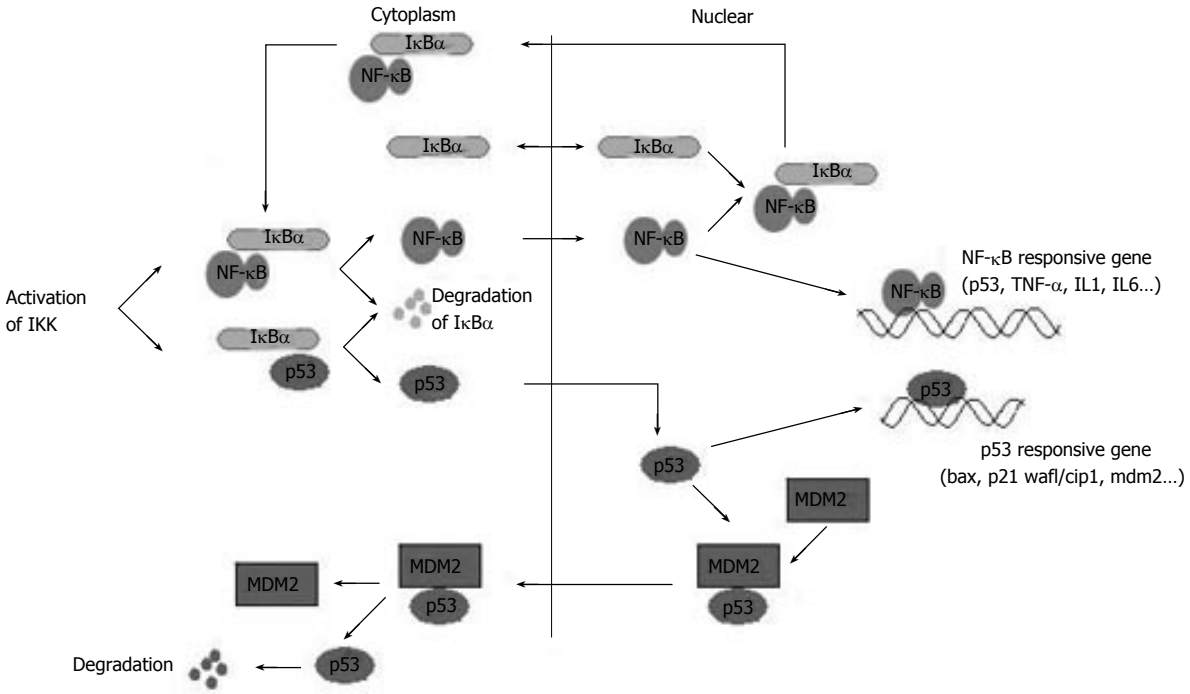


**Figure 9** IκBα participates in NF-κB and p53 signaling pathways.

mainly existed in the nucleus. IκBα244C enhanced the transcription level of p53, NF-κB and their downstream genes. The CCK-8 experiment showed that co-expression of p53 with IκBα244C resulted in enhancement of p53-mediated cell death. p53 and IκBα244C are possibly co-factors in inducing apoptosis, and the C terminus of IκBα may serve as a pro-apoptotic protein in living cells.

NF-κB has been considered a target for cancer treatment[17,40]. The function of IκBα as an inhibitor in regulating NF-κB activation has been well studied. Findings from the present study suggest that mutants of IκBα have different effects on NF-κB and p53 signaling pathways, and may result in different therapy results. The inhibition effect of IκBαM indicates drugs that induce apoptosis by a p53-dependent mechanism may be inhibited by the use of IκBαM constructs through inhibition of p53 function by these agents. The C terminal of IκBα enhanced cell death, which suggests that it may be a pro-apoptotic protein existing in cells, but the mechanism remains to be determined and there may exist NF-κB and p53 independent pathways.

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*CLINICAL RESEARCH*

# Hematopoietic cell transplantation for Crohn's disease; is it time?

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## Abstract

**AIM:** To review all studies in the literature that have assessed Hematopoietic cell transplantation (HCT) and Crohn's disease (CD) with the ultimate aims of determining if this is a viable treatment option for those with CD. A secondary aim was to review the above literature and determine if the studies shed further light on the mechanisms involved in the pathogenesis of CD.

**METHODS:** An extensive Medline search was performed on all articles from 1970 to 2005 using the keywords; bone marrow transplant, stem cell, hematopoietic cell, Crohn's disease and inflammatory bowel disease.

**RESULTS:** We identified one case in which a patient developed CD following an allogeneic HCT from a sibling suffering with CD. Evidence for transfer of the genetic predisposition to develop CD was also identified with report of a patient that developed severe CD following an allogeneic HCT. Following HCT it was found that the donor (that had no signs or symptoms of CD) and the recipient had several haplotype mismatches in HLA class Ⅲ genes in the IBD3 locus including a polymorphism of NOD2/CARD15 that has been associated with CD. Thirty three published cases of patients with CD who underwent either autologous or allogeneic HCT were identified. At the time of publication 29 of these 33 patients were considered to be in remission. The median follow-up time was seven years, and twenty months for allogeneic and autologous HCT respectively. For patients who underwent HCT primarily for treatment of their CD there have been no mortalities related to transplant complications.

**CONCLUSION:** Overall these preliminary data suggest that both allogeneic and autologous HCT may be effective in inducing remission in refractory CD. This supports the hypothesis that the hemolymphatic cells play a key role in CD and that resetting of the immune system may be a critical approach in the management or cure of CD.



**Key words:** Crohn's disease; Inflammatory bowel disease; Bone marrow transplant; Stem cells; Hematopoietic cell transplantation

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## INTRODUCTION

Crohn's disease (CD) is a chronic inflammatory disease of the gastrointestinal tract which commonly affects young adults. It follows a relapsing and remitting course and there is no known cure. However, approximately 10% to 15% have chronic unremitting active disease, with only 10% remaining in remission over many years[1]. A recent systematic review of population based cohorts estimated the prevalence of CD to be 26.0 to 198.5 cases per 100 000 persons and the incidence to be 3.1 to 14.6 cases per 100 000 person-years in North America[1]. In Europe the overall incidence per 100 000 person-years was 5.6[2]. Health related quality of life and general quality of life is lower in Crohn's disease patients compared to the normal population[3] and quality of life is inversely related to active disease, hospitalization and surgery[4-7]. Achieving disease remission improves overall health related quality of life, employment rate, mental and physical functioning[8,9]. In three trials patients with CD have increased mortality compared to the normal population. Standardized mortality ratios were 1.4, 1.51 and 1.3 respectively[10-12]. Loftus *et al*[13] also showed that 20 year survival was 79% for those with CD versus 86% in the normal population. A recent study revealed that up to 32% of all deaths in patients with CD are directly related to complications of their disease[14].

Economic consequences for CD relates both to the loss of productivity, as CD most commonly affects adults in their working years, and the costs of hospitalization, medical and surgical treatment. Longobardi *et al*[15]

estimated the cost of non participation in the labour force in 1998 to be 104.2 million dollars in Canada for patients with inflammatory bowel disease. A study of Crohn's disease patients in the United States found that surgery accounts for the majority of hospitalization and 40% of the total cost of hospitalization[16]. Crohn's disease patients who suffer an acute exacerbation requiring hospitalization in the United Kingdom have a 20 fold increase in healthcare costs compared to patients with stable disease[17].

Present therapy is aimed at relieving inflammation and treating signs and symptoms. Therapy consists of non-specific anti-inflammatory agents such as 5-ASA, glucocorticoids, immunomodulators, and anti-tumor necrosis factor therapy. The goals of therapy should include the induction and maintenance of remission and an attempt to heal mucosa with the ultimate goal being restitution of normal intestinal function. Despite the growing use of immunomodulators and new biologic agents the need for intestinal resection has remained stable[18]. Within five years of diagnosis of CD up to 43% of patients require surgical intervention[19]. The impact of infliximab, a monoclonal antibody to tumor necrosis factor has been substantial in treating patients with CD who were previously refractory to standard treatment. In our center response rates to infliximab are 75%-78%. However, the use of infliximab is often limited by its cost. In Canada the median cost of one year of infliximab maintenance is $27 000. For those patients who do not respond to infliximab currently there is no standard medical therapy. The goal of this review is to discuss one current area of research in hematopoietic cell transplantation and Crohn's disease.

## HEMATOPOIETIC CELL TRANSPLANTATION (HCT) IN OTHER AUTOIMMUNE DISEASES

HCT includes conditioning (high dose chemotherapy, total body irradiation and/or anti-lymphocyte antibodies) followed by the infusion of the hematopoietic cells. The hematopoietic cells are either directly harvested from the marrow or mobilized from marrow to blood (for e.g. by filgrastim) and harvested by apheresis. In case of autologous transplantation for autoimmune diseases, the graft is typically depleted of T cells (e.g. by immunomagnetic selection of CD34 cells) to reduce the likelihood of reinfusing presumed disease-causing T cells. In spite of this, a small number of T cells may be reinfused and some T cells may survive the conditioning. Allogeneic HCT should theoretically be associated with a higher likelihood of cure of an autoimmune disease than autologous HCT, as typically there is a graft-*vs*-host reaction that eliminates the presumed disease-causing T cells that survived conditioning. On the other hand, due to graft-vs.-host disease (GVHD), allogeneic transplantation is typically associated with higher transplant-related morbidity and mortality. For this reason, far more autologous than allogeneic transplants have been performed for various autoimmune diseases, in an attempt to "reset the immune system".

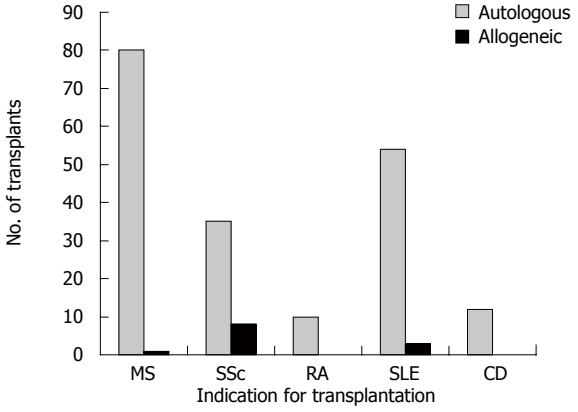


**Figure 1** Number of hematopoietic cell transplants for multiple sclerosis (MS), scleroderma/systemic sclerosis (SSc), rheumatoid arthritis (RA), systemic lupus erythematosus (SLE) and Crohn's disease (CD) registered to the Center for International Blood and Marrow Transplant Research (CIBMTR) by 30 teams in North America, by September, 2005.

HCT has been performed in more than 700 patients with autoimmune diseases[20]. The most frequent indications have been systemic sclerosis (SSc), multiple sclerosis (MS), rheumatoid arthritis (RA), and systemic lupus erythematosus (SLE) (Figure 1). The results are reviewed below. For MS, the European Blood and Marrow Transplant (EBMT) registry reported data on autologous transplantation in 85 patients with severe progressive disease given cyclophosphamide and/or growth factors for stem cell mobilization, then combination chemotherapy ± total body irradiation (TBI) and anti-lymphocyte globulin. Transplant related mortality at three years was 6% with progression free survival of 74% and overall survival 90%[21]. Marked improvement in gadolinium-enhancing MRI lesions was seen. Unfortunately, filgrastim-induced neurological deterioration was seen in 22 patients; this was typically transient but six patients had persistent deficits. Recently updated analysis of 183 patients reported to the EBMT registry reveals 5.5% treatment-related mortality[20].

Several single-center Phase Ⅰ/Ⅱ studies with 15 or more patients have been completed in Europe and show a progression-free survival at one to three years ranging from 70% to 80% with transplant-related mortality ranging from 0%-3%[22]. A US multicenter Phase Ⅰ/Ⅱ study of 26 MS patients who received autologous stem cell transplant after conditioning with cyclophosphamide, TBI and anti-thymocyte globulin (ATG) conditioning and granulocyte colony stimulating factor (G-CSF) mobilization demonstrated a three year projected survival of 91% and disease progression of 27% at 24 mo median follow-up[23]. One patient had a neurological flare during filgrastim therapy and one deteriorated during transplant; prophylactic steroid use may have prevented this in further patients. A Phase Ⅲ randomized trial is underway in Europe (ASTIMS trial) comparing autologous HCT using high dose chemotherapy (carmustine, etoposide, araC and melphalan) and ATG *vs* low dose chemotherapy (mitoxantrone) alone.

Systemic sclerosis (SSc) is a debilitating multiorgan disease with a mortality rate of 40% to 50% at five years

in high risk patients with high skin scores and involvement of the heart, lung or kidney[24-26]. Per the EBMT/EULAR (European League Against Rheumatism) registry, of the first 57 patients transplanted for SSc, 50 have been followed up for more than 6 mo (median 22.9 mo) and had an overall functional response rate of 92% (14 complete responses, 35 partial responses) and no response in 8%[27]. Skin score improvement > 25% was durable at two years in 79% of patients and lung function remained stable overall. Of responding patients, 35% showed evidence of relapse at nine months with projected 48% disease progression at five years and 72% survival at one year. Treatment-related mortality was 8.7%. In a US study of 30 SSc patients of autologous HCT using conditioning with cyclophosphamide, TBI and anti-thymocyte globulin (ATG) resulted in 75% of patients surviving at median follow-up of 36 mo with 12 of 12 assessable patients showing significant improvement in skin scores or the Health Assessment Questionnaire (HAQ) disability index at 12 mo[28]. Two of the first eight patients died of pulmonary toxicity, prevented in subsequent patients by lung shielding during TBI, two other patients died of treatment-related causes and two of progressive disease. In 19 assessable patients major organ function (lung, cardiac, renal) was stable. A French study showed response in 8 of 11 patients given autologous transplant with 36% of patients deceased at 18 mo follow-up (three of disease, one of treatment-related mortality)[26]. Four other patients had active SSc requiring treatment 6-12 mo post transplant. A phase Ⅲ trial is now accruing patients in Europe (ASTIS trial), comparing monthly pulse IV cyclophosphamide 750 mg/m$^2$ for 12 mo *vs* autologous HCT using mobilization with cyclophosphamide/filgrastim and conditioning with cyclophosphamide/ATG. No deaths have been seen in the first 42 patients. Another Phase Ⅲ trial has been recently opened in the US (SCOT trial), comparing monthly pulse Ⅳ cyclophospamide (also 750 mg/m$^2$ for 12 mo) versus autologous HCT using cyclophosphamide/filgrastim mobilization and conditioning with cyclophosphamide, ATG and total body irradiation.

For RA treated with autologous HCT, EBMT registry data revealed no treatment-related mortality in 76 patients who had failed a median of five disease-modifying anti-rheumatic drugs (DMARDS) at a mean follow-up of 16 (3-55) mo[29]. Two thirds of patients obtained American College of Rheumatology score improvements of 50% or better (ACR50), improved function on the HAQ, and tender joints improved for at least 18 mo despite the need for 73% of patients to restart DMARDs within 1 year. In a small study of 14 Dutch patients, 8 of 12 patients responded although 57% required reintroduction of DMARDS at a median of 105 d; three of these heavily treated and refractory patients then responded to DMARDS[30]. Thirty three Australian patients revealed no treatment related mortality with an ACR20 in 70% of patients and ACR20 in 39%, but disease recurred in 29 patients, at a mean of 180 d[31]. Further studies may clarify the role of HCT in rheumatoid arthritis in the context of newer biologic therapies.

Systemic lupus erythematosus has diverse clinical manifestations and an overall survival at ten years of 90%, with a subset of patients refractory to standard immunosuppressive therapy. In a study of 17 patients with refractory SLE given cyclophosphamide for stem cell collection, followed by cyclophosphamide, ATG, and methylprednisolone, 11% died after mobilization of treatment-related toxicity[32]. Follow up at 2 to 66 mo, revealed no deaths in the other 15 patients, with significant reductions in autoantibody titers, prednisone requirements, proteinuria and improved serum complement levels. Two of seven patients followed more than 30 mo relapsed and required further treatment. In the EBMT database, 27 of 51 patients improved, another 14 improved with subsequent relapse, and 7 patients (11%) died[33].

Across all disease groups, mobilization regimens have been associated with toxicity - in the EMBT registry database there was a mortality rate of 1.5%, largely from cyclophosphamide cardiotoxicity and filgrastim- induced disease flares[34].

Treatment-related mortality in the first 470 patients of the EBMT registry who received an autotransplant was 7 to 8% with marked differences between disease groups; although there was a rate of 12.5% in scleroderma patients, only 1 of 70 patients with rheumatoid arthritis died[34]. Death rates are likely related to severity of underlying illness/organ dysfunction and in SSc is associated with pulmonary artery pressure > 50 mm Hg and total body irradiation without lung shielding[35]. Patient selection is key; there have been no deaths in the last 2 years in MS and SSc trials.

## BONE MARROW DERIVED CELLS PLAY A CRITICAL ROLE IN IBD

Genetic studies suggest that in patients with inflammatory bowel disease (IBD) there may be several defects in the hemolymphatic ("inflammatory", hematopoietic cell-derived) cells[36]. Mutations in the NOD2/CARD15 gene increase the risk of developing Crohn's disease. NOD2/CARD15 is expressed in intestinal epithelial cells and monocytes/macrophages[37,38]. Recognition of intracellular bacteria in intestinal epithelial cells is impaired in 2030insC mutant NOD2[39]. Interestingly presence of NOD2 gene polymorphism, has also been associated with risk of gut GVHD[40,41]. Holler *et al*[41] found that the presence of single nucleotide polymorphisms of the NOD2/CARD15 gene in both the recipient and donor increased the incidence of GVHD to 56% compared to 18% in cases with no mutations from either recipient or donor.

Interestingly, a 37 years old patient developed CD after receiving an allogeneic marrow transplant from a sibling that had CD[42]. Furthermore, genetic studies showed that another patient that developed severe CD following an allogeneic bone marrow transplant may have developed the disease due to the transfer of known susceptibility genes[43]. In this case, CD developed following receiving marrow from a donor (that did not exhibit CD) but had several haplotype mismatches in HLA class Ⅲ genes in the IBD3 locus including a polymorphism of NOD2/CARD15 that has been associated with CD[43].

The role of bone marrow-derived cells in the intestine following transplant is presently unclear. Both in animal

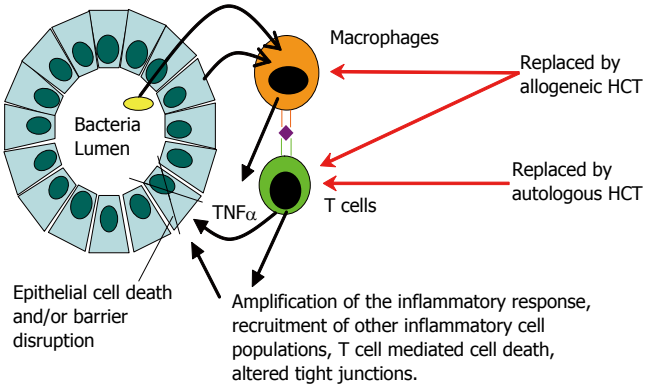


**Figure 2** Pathogenesis of CD and proposed mechanism of action of HCT. See text for details.

models as well as in patients it appears that bone marrow derived cells are involved in the healing process following intestinal injury and may contribute to various components of the mucosa including myofibroblasts, endothelium as well as possibly the epithelium[44,45].

Given the above evidence for hematolymphatic cell playing an important role in IBD pathogenesis, a hypothesis has been put forth that HCT may induce remission of IBD. See Figure 2 for the proposed (speculative) mechanism of action. In this scenario macrophages (orange) present peptides (purple diamond) derived from patient cells or intestinal microorganisms (yellow) to T cells (green). The T cells become stimulated and secrete cytokines like tumor necrosis factor alpha (TNF-α) which can directly damage enterocytes and/or amplify the inflammatory response. The macrophages present the peptide by major histocompatibility complex (MHC) class Ⅱ (e.g., HLA-DR) and the T cells recognize the MHC-peptide complex through T cell receptors. Only macrophages and T cells are depicted here for simplicity, though other hematolymphatic cells (cells derived from hematopoietic stem cells) may participate in the pathogenesis, including neutrophils, eosinophils, dendritic cells and mast cells. Likewise, only enterocytes are depicted here despite other intestinal non-hematolymphatic cells like fibroblasts may participate in the pathogenesis. Genetic predisposition to CD may involve non-hematolymphatic cells (e.g., altered genes needed for the healing of epithelial cells from injury may be missing or altered) or hematolymphatic cells (e.g., altered genes needed for T cells to undergo apoptosis). The latter mechanism may prevail given the ability of HCT to induce durable remission of CD. The genetic predisposition to CD involving the hematolymphatic cells may be due to inherited DNA sequences (e.g., coding for HLA-DR type capable of presenting the pertinent peptide) or DNA sequences generated during ontogeny (e.g., coding for T cell receptor capable of recognizing the peptide). Allogeneic HCT may cure CD by replacing all recipient hematolymphatic cells with donor hematolymphatic cells harboring DNA sequences that do not predispose to CD. Autologous HCT may cure CD by replacing the hematolymphatic cells which (or the ancestors of which) generated the predisposing DNA sequence in ontogeny (e.g., the T cell clone capable of recognizing the peptide) with healthy autologous hematolymphatic cells (e.g., T cell clones generated de novo in the thymus after the HCT that cannot recognize the peptide). The cells that are thought to be replaced by the allogeneic or autologous HCT are pointed out by the red arrows. The pathogenesis of CD is based in part on the following references; [1-4].

## ALLOGENEIC HEMATOPOIETIC CELL TRANSPLANTATION (HCT) IN CROHN'S DISEASE

To date there are no reported cases of allogeneic bone marrow or HCT performed for the primary treatment of CD. There are 14 patients reported in the literature that had CD and underwent allogeneic HCT for hematological disorders (Table 1). Ditschkowski *et al*[46] reported a case series in which 10 out of 11 patients remained free of inflammatory bowel disease following allogeneic HCT for hematological malignancy with a median follow-up time of 34 mo. Seven out of the eleven patients had Crohn's disease. All patients received cyclophosphamide as part of different conditioning regimens. All patients except for two received total body irradiation. The one patient with recurrent IBD symptoms after transplant failed to demonstrate any endoscopic or radiological recurrence of IBD. There was one transplant-related mortality reported at ten months from opportunistic infection. The usefulness of this study for the determination of whether allogeneic HCT may cure CD is limited by the fact that at the last follow up only two patients were off of immunosuppressive drugs.

Lopez-Cubero *et al*[47] reported a case series of six patients with Crohn's disease who underwent allogeneic marrow transplantation for leukemia. Five of these patients had active CD at the time of transplant. All patients received cyclophosphamide and total body irradiation as conditioning therapy. Two patients had sclerosing cholangitis. One patient died of sepsis at 3 mo post-transplant. The remaining five patients were free of CD related symptoms more than one year post transplant. Four of these five patients had sustained remission of CD at 54 to 183 mo post-transplant. One patient had relapse of CD at d 545 post transplant. Interestingly, the relapsed patient had mixed donor-host hematopoietic chimerism, whereas the four patients who achieved sustained remission were assumed to be complete chimeras as they had GVHD in the first two years after HCT. Immunosuppressive drugs were discontinued at least six months prior to the last follow up in the four patients with sustained CD remission.

An additional case has been reported involving a 35 year old male with CD who was transplanted for AML and at eight years post transplant was free of symptoms and signs of CD[48]. In summary of the 14 published cases of allogeneic bone marrow or stem cell transplant in patients with CD, 11 patients have achieved remission (Table 2). The median follow up time was seven years. Of seven patients followed for CD after they have discontinued immunosuppressive drugs, six patients are in remission of

**Table 1 Allogeneic transplant cases**

| | Demographics & indication for HCT | Disease activity before HCT | Complications of IBD before HCT | IBD duration (yr) | Donor type | Conditioning therapy | T cell deplet. |
|---|---|---|---|---|---|---|---|
| Ditschkowski *et al*[46] Total 11 patients CD-7 UC-4 | Median age 41<br>Male: 5<br>Female: 6<br>CML: 9<br>AML: 1<br>MDS: 1 | Inactive: 5<br>Low Act: 6 | Fistulae: 3<br>Obstruction: 2<br>EIM: 2<br>Surgery: 4 | Median: 10 | HLA- identical sibling - 8<br>HLA- identical unrelated donor- 2<br>Mismatched unrelated donor- 1 | TBI + Cy: 6<br>TBI + Cy + ATG: 1<br>TBI + Cy + ATG + thiotepa: 1<br>TBI + Cy + thiotepa:1<br>Busulfan + Cy: 2 | Yes: 3<br>No: 8 |
| Lopez-Cubero *et al*[47] | 27 Male CML | Inactive | EIM | 11 | HLA- match brother | TBI + Cy | No |
| | 27 Male CML | Active | Obstruction Fistula | 5 | HLA- match sister | TBI + Cy | No |
| | 41 Male CML | Active | Surgery Fistula EIM | 9 | HLA- match sister | TBI + Cy | No |
| | 41 Male CML | Active | Sclerosing cholangitis | 8 | HLA- match unrelated donor | TBI + Cy | No |
| | 38 Male CML | Active | Surgery EIM, ↑ ALP | 3 | One antigen mismatched brother | TBI + Cy | No |
| | 46 Male AML | Active | Sclerosing cholangitis | 29 | HLA- match sister | TBI + Cy | No |
| Talbot *et al*[48] | 35 Male AML | Active | Surgery | 7 | HLA- match brother | TBI + Cy | No |

ALP: Alkaline phosphatase; AML: Acute myeloid leukemia; ATG: Antithymocyte globulin; CD: Crohn's disease; CML: Chronic myeloid leukemia; Cy: Cyclophosphamide; EIM: Extraintestinal manifestations; HCT: Hematopoietic cell transplantation; MDS: Myelodysplastic syndrome; TBI: Total body irradiation; UC: Ulcerative colitis; Act: Activity; deplet: depletion.

**Table 2 Outcomes of allogeneic transplant cases**

| | GVHD | Duration of immunosuppressant post HCT (mo) | Length of follow up (mo) | Remission of CD | Ongoing CD medication or immunosuppressant |
|---|---|---|---|---|---|
| Ditschkowski *et al*[46] | Acute: 8<br>Chronic: 0 | 2 patients off immunosuppressants | 3-117<br>Median 34 | Yes: 6<br>No: 1 | Not stated |
| Lopez-Cubero *et al*[47] | Acute and Chronic | 10 | 183 | Yes | No |
| | Acute and Chronic | 70 | 119 | Yes | No |
| | Acute | 6 | 70 Died of suicide | No - recurrent d 545 | Prednisone |
| | Acute and Chronic | 18 | 101 | Yes | No |
| | Acute and Chronic | 48 | 54 Died of myocardial infarction | Yes | No |
| | Acute | Not detected | Died of septic shock d 97 | Not detected | Not detected |
| | Acute | 2 | 96 | Yes | No |

GVHD: Graft versus host disease.

CD. There have been two transplant-related deaths among the 14 cases.

## AUTOLOGOUS HEMATOPOIETIC CELL TRANSPLANTATION IN CROHN'S DISEASE

To date there have been 19 patients who have been reported to have both Crohn's disease and autologous HCT. Patients who underwent transplantation for the primary treatment of Crohn's disease had previously failed standard medical treatment including infliximab. The first case was published in 1993. A 41 year old female with non-Hodgkin's lymphoma and CD underwent an autologous bone marrow transplant. The follow-up period was short. However the patient was symptom free at six months[49]. Three additional cases who underwent transplant for malignancies have also remained in clinical remission[50-52].

Recently, Oyama *et el*[53] and colleagues reported a study in which 12 patients with refractory CD underwent autologous hematopoietic cell transplantation. All of their patients had failed traditional medical treatment including infliximab with a minimal CDAI $\geqslant$ 250. Majority of these patients had a history of surgery. Mobilization was with cyclophosphamide and filgrastim, and conditioning with cyclophosphamide and equine ATG. The peripheral blood stem cells were T-cell depleted. There was sustained

**Table 3 Autologous transplant cases**

| | Demographics & indication for HCT | Disease activity before HCT | Complications of CD | Duration of CD (yr) | Mobilization | Conditioning | T cell deplet | Length of follow-up (mo) | Remiss. of CD | Ongoing meds |
|---|---|---|---|---|---|---|---|---|---|---|
| Oyama[53] | 21 Female CD | Active | Fistulae, Surgery | 10 | Cy + GCSF | Cy + ATG | Yes | 37 | Yes | No |
| | 16 Male CD | Active | Fistulae, Obstruction, GR | 7 | Cy + GCSF | Cy + ATG | Yes | 36 | Yes | No |
| | 38 Female CD | Active | Fissures, Obstruction, Surgery | 20 | Cy + GCSF | Cy + ATG | Yes | 27 | Yes | No |
| | 27 Female CD | Active | Fistulae, EIM, Surgery | 12 | Cy + GCSF | Cy + ATG | Yes | 25 | No | Mtx, pred. |
| | 35 Male CD | Active | Fistulae, Obstruction, Surgery | 12 | Cy + GCSF | Cy + ATG | Yes | 24 | Yes | No |
| | 27 Female CD | Active | Fistula, Surgery | 6 | Cy + GCSF | Cy + ATG | Yes | 20 | Yes | No |
| | 25 Male CD | Active | Perianal disease, Surgery | 13 | Cy + GCSF | Cy + ATG | Yes | 17 | Yes | No |
| | 15 Male CD | Active | EIM, GR, Surgery | 8 | Cy + GCSF | Cy + ATG | Yes | 16 | Yes | No |
| | 31 Female CD | Active | Perianal disease, EIM, Surgery | 10 | Cy + GCSF | Cy + ATG | Yes | 14 | Yes | No |
| | 37 Male CD | Active | Perianal disease | 6 | Cy + GCSF | Cy + ATG | Yes | 14 | Yes | No |
| | 16 Male CD | Active | Fistula, EIM | 1.5 | Cy + GCSF | Cy + ATG | Yes | 7 | Yes | No |
| | 27 Male CD | Active | Perforation, EIM, Surgery | 14 | Cy + GCSF | Cy + ATG | Yes | 7 | Yes | No |
| Scime[58] | 55 Male CD | Active | EIM | 2 | Cy + GCSF | Cy + ATG | Yes | 5 | Yes | No |
| Kreisel[59] | 36 Male CD | Active | Fistulae, Abscess, Surgery | 14 | Cy + GCSF | Cy | Yes | 10 | Yes | Mtx, pred. |
| Soderholm[52] | 57 Female AML | NS | Obstruction, Fistula, Surgery | NS | NS | Cy + TBI | No | 60 | Yes | No |
| Musso[60] | 30 Male Hodgkin's Lymph. | Active | Surgery | 10 | Cy + GCSF | Idarubicin Melphalan | NS | 39 | Yes | No |
| Kashyap[51] | 20 Male NHL | NS | Perianal disease, Obstruction, Surgery | 8 | DHAP | Cy + TBI + VP-16 | No | 84 | Yes | No |
| Castro[50] | Age NS, Female Breast ca | Active | NS | 11 | NS | NS | NS | 36 | Yes | Asacol |
| Drakos[49] | 41 female NHL | Inactive | Fistula, Surgery | 22 | NS | BCNU + VP-16 + Cy + Ara-C + melphalan | NS | 6 | Yes | No |

AML: Acute myeloid leukemia; ATG: Antithymocyte globulin; BCNU: Carmustine; CD: Crohn's disease; Cy: Cyclophosphamide; D: Duration of Crohn's disease in years; DHP: Dexamethasone, high dose Ara-C, platinum; EIM: Extraintestinal manifestation; GR: Growth retardation; NHL: Non Hodgkin's lymphoma; NS: Not stated; TBI: Total body irradiation. VP-16: Etoposide; Mtx: Methotrexate; pred: prednisone; Remiss: Remission; meds: medications; deplet: depletion.

remission (CDAI $\leqslant$ 150) in 11 of 12 patients after a median follow-up of 18.5 mo. One patient experienced CD relapse at 15 mo post-transplant. All patients tolerated the BMT well and there were no transplant-related deaths. One patient died of accidental cause at 37 mo post HCT. Follow-up was at 6 and 12 mo post HCT and then yearly, and included at each visit small bowel radiograph and colonoscopy.

Collectively, 18 out of 19 patients have achieved clinical remission over a median follow up time of 20 mo (Table 3). Only two of these patients are on any ongoing medications again suggesting that HCT may result in CD remission without any need for ongoing medications. Fourteen of these patients underwent autologous HCT for primary treatment of their CD. The majority of these 18 patients had active disease prior to HCT with a history of CD complications including surgery and failure of infliximab therapy in patients where the primary indication for HCT was CD. There has been no mortality related to transplant complications.

## HCT VERSUS IMMUNOSUPPRESSION/ GCSF ALONE

Cyclophosphamide and GCSF were used as the mobilization regimen in the study by Oyama *et al*[54]. Arguably these two agents may explain part of the success of the HCT as cyclophosphamide has been shown to be effective in achieving remission in Crohn's disease and GCSF may shift the Th1/Th2 balance toward Th2 (whereas Th1 cells predominate in CD)[55]. In pediatric patients with IBD, IBD improved during treatment of glycogen storage disease with GCSF[56]. More recently a randomized placebo control trial showed an improvement in CDAI score and rates of remission with GCSF in CD[57].

However, three cases suggest that sustained clinical remission with HCT is likely not due just to cyclophosphamide and GCSF. Scime *et al*[58] reported a case of a 55 year old male who underwent HCT for primary treatment of CD. He showed no improvement in colonic lesions one month after mobilization therapy with cyclophosphamide and GCSF. He subsequently achieved both significant endoscopic and histological improvement after autologous HCT. Five months after HCT the patient remains in clinical remission. Kreisel *et al*[59] describe a 36 year old male who also underwent HCT for the primary treatment of CD. He continued to have persistent histological changes and recurrent CD nine months after mobilization therapy with cyclophosphamide and GCSF (before HCT). Ten mo after HCT the CD was improved,

in clinical remission with minimal histological changes. A third case involved a 30 year old male who underwent an autologous transplant for Hodgkin's lymphoma. He had partial improvement of his CD after mobilization therapy, however, required persistent steroid therapy. At 38 mo after HCT he remains in clinical remission[60].

In Europe a randomized Phase Ⅲ trial of autologous stem cell transplantation in Crohn's disease (ASTIC) has been established that should answer the question of whether the autologous transplantation adds significantly to the effect of immunosuppression/GCSF alone. Patients are randomized into those receiving mobilization chemotherapy with GCSF only versus mobilization chemotherapy with GCSF followed by autologous transplantation[61].

## SUMMARY OF HCT CASES IN CD

In total there are now 33 cases published of patients with CD who underwent either autologous or allogeneic bone marrow or hematopoietic stem cell transplantation. Twenty nine out of these thirty three patients are considered in remission. There were two cases of transplant related mortality. Importantly of the fourteen cases transplanted primarily for treatment of CD there have been no mortalities related to the transplant. Overall these preliminary data suggest that both allogeneic and autologous HCT may be effective in inducing remission in refractory CD. This supports the hypothesis that the hematolymphatic (inflammatory, bone marrow-derived) cells play a key role in CD and that resetting of the immune system may be a critical approach in the management or cure of CD.

The course of many autoimmune diseases including CD is by nature relapsing and remitting, underscoring the need for control populations with comparable severity of disease to evaluate outcomes in HCT. There is a need for objectively validated disease severity scoring, and for establishing prognostic indicators to identify candidates at high risk for poor disease outcome who might benefit from aggressive treatment. Of the cases where transplant was the primary treatment for CD, there have been no transplant related mortalities. However HCT for CD has to be approached with caution as transplantation for other autoimmune diseases has been associated with a transplant-related mortality of 0 to 25% (Passweg, personal communication on the ASTIS trial)[21,22,26-28, 33, 34].

Potential candidates for phase Ⅱ/Ⅲ trials would include patients who have failed standard medical treatment including infliximab (or can not tolerate it), who are unlikely to benefit from further surgery, and those with severe fistulizing disease not responding to conventional treatment.

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*RAPID COMMUNICATION*

# Late-onset acute rejection after living donor liver transplantation

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## Abstract

**AIM:** To investigate the incidence and risk factors of late-onset acute rejection (LAR) and to clarify the effectiveness of our immunosuppressive regime consisting of life-long administration of tacrolimus and steroids.

**METHODS:** Adult living donor liver transplantation recipients ($n$ = 204) who survived more than 6 mo after living donor liver transplantation were enrolled. Immunosuppression was achieved using tacrolimus and methylprednisolone. When adverse effects of tacrolimus were detected, the patient was switched to cyclosporine. Six months after transplantation, tacrolimus or cyclosporine was carefully maintained at a therapeutic level. The methylprednisolone dosage was maintained at 0.05 mg/kg per day by oral administration. Acute rejections that occurred more than 6 mo after the operation were defined as late-onset. The median follow-up period was 34 mo.

**RESULTS:** LAR was observed in 15 cases (7%) and no chronic rejection was observed. The incidence of hyperlipidemia, chronic renal failure, new-onset post-transplantation diabetes, and deep fungal infection were 13%, 2%, 24%, and 17%, respectively. Conversion from tacrolimus to cyclosporine was required in 38 patients (19%). Multivariate analysis revealed that a cyclosporine-based regimen was significantly associated with LAR.

**CONCLUSION:** Both LAR and drug-induced adverse events happen at a low incidence, supporting the safety and efficacy of the present immunosuppression regimen for living donor liver transplantation.



**Key words:** Living donor liver transplantation; Steroid; Acute rejection

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## INTRODUCTION

Standard regimens for immunosuppressive therapy after liver transplantation include calcineurin inhibitors and steroids, which result in a reduced incidence of acute rejection and improved recipient survival[1]. The long-term complications of chronic immunosuppression, such as diabetes mellitus, renal toxicity, hyperlipidemia, and opportunistic infections, however, are a great concern with regard to improved survival.

Because the majority of acute rejections occur within the first few months after liver transplantation, most centers try to taper off steroids and minimize maintenance trough levels of calcineurin inhibitors within the first 6 mo after liver transplantation[2,3]. Recently, more rapid steroid withdrawal programs (within 2 wk) were introduced[4,5]. The shorter steroid regimen increases the possibility of late-onset acute rejection (LAR), which might result in graft loss and serious morbidity[6].

The appropriate doses of immunosuppressive drugs over the long-term should be balanced against the incidence of LAR and drug complications. Our immunosuppressive regimen after adult living donor liver transplantation (LDLT) consists of life-long administration of steroids and tacrolimus strictly controlled with therapeutic drug monitoring. In the present study, we retrospectively investigated the incidence and risk factors of LAR to clarify the safety and efficacy of our immunosuppressive regime.

## MATERIALS AND METHODS

### Patients

A total of 247 LDLTs were performed in adult patients at the University of Tokyo Hospital between January 1996 and March 2005. Among the 224 recipients, 18 patients were excluded because of death within the 6 mo. Moreover, another two cases were excluded, one who received the graft from his identical twin and another who received auxiliary partial orthotopic liver transplantation. The remaining 204 patients (102 men and 102 women; age range: 18-67 years) were enrolled in the study. The median postoperative follow-up period was 34 mo (range, 6-114). The most common indication for LDLT was viral cirrhosis ($n$ = 77) followed by primary biliary cirrhosis ($n$ = 46).

Pre-operative aspartate transaminase, total bilirubin levels, and serum creatinine were 19-308 IU/L, 4-400 mg/L, and 2-44 mg/L, respectively. The median score for model for end-stage liver disease was 14 (range, 4-34).

### Immunosuppression

Our surgical technique for recipient and donor surgery is described elsewhere[7]. All the patients received the same immunosuppressive regimen (Table 1) consisting of tacrolimus (FK, Prograf, Astellas Pharma Inc., Tokyo, Japan) and methylprednisolone. If adverse effects occurred with FK, the patient was switched to cyclosporine (CsA). The indications for conversion are described elsewhere[8]. More than 6 mo after LDLT, FK and CsA were maintained at 5 to 10 μg/L and 100 to 150 μg/L, respectively, with therapeutic drug monitoring at least once a month. A trough level of less than 5 μg/L FK or less than 100 μg/L CsA was regarded as sub-therapeutic. Methylprednisolone was maintained at 0.05 mg/kg per day by oral administration for lifetime of the recipients after the initial 6 mo.

### Postoperative care

Diagnosis of acute rejection was based on internationally accepted histologic criteria[9]. Acute rejection occurring more than 6 mo after LDLT was regarded as LAR. Biopsy-proven acute cellular rejection scored more than 3 in Banff classification was treated with high-dose methylprednisolone (20 mg/kg per day) followed by recycling. Patients with steroid-resistant cellular rejection were treated with mycophenolate mofetil and anti-T-cell monoclonal antibody (OKT3, Ortho-Biotech Corporation, Raritan, NJ).

The cytomegalovirus status of the patient was monitored by pp65 antigenemia assay once a week for 3 mo postoperatively. When there were more than 5 antigen-positive cells/50 000 white blood cells, antiviral therapy was started until the antigenemia assay was negative. Details of antibacterial and antifungal prophylaxis are described elsewhere[10].

Hyperlipidemia was diagnosed when the serum cholesterol level was over 2300 mg/L in two successive examinations. Chronic renal failure was defined as serum creatinine of more than 25 mg/L on at least two successive determinations more than 6 mo after LDLT. Patients with normal glucose tolerance preoperatively, who required pharmacologic assistance to control blood sugar more than 6 mo after the transplantation, were diagnosed with new-onset post-transplantation diabetes.

**Table 1 Target trough levels of calcineurin inhibitors and steroid dosage**

| Postoperative time (d) | Tacrolimus (μg/L) | Cyclosporine (μg/L) | Methylprednisolone (mg/kg daily) |
|---|---|---|---|
| 1-7 | 15-20 | 300-350 | 20-0.75 |
| 8-14 | 14-16 | 250-300 | 0.5-0.3 |
| 15-90 | 10-15 | 200-250 | 0.3-0.12 |
| 91-180 | 8-10 | 150-200 | 0.08-0.12 |
| 180- | 5-10 | 100-150 | 0.05 |

### Statistical analysis

The incidence of acute rejection and post-transplantation complications more than 6 mo after LDLT was examined. Preoperative factors included age, gender, disease, serum total bilirubin levels, model for end-stage liver disease score, donor/recipient blood type, gender match, donor age, and the result of donor/recipient human leukocyte antigens (HLA) and the lymphocytotoxic crossmatch. Intraoperative factors included anhepatic phase duration, blood loss, and graft weight/standard liver volumes[11]. Postoperative factors were initial immunosuppressive regimen (FK or CsA), cytomegalovirus infection, and episodes of early acute rejection.

Categorical data were compared using chi-square or Fisher's exact tests. Continuous data were compared using the t-test or Mann Whitney U test. Multiple regression analysis was performed using the proportional hazards models to identify factors that were independently associated with LAR. The trough levels of the calcineurin inhibitors 6 mo after LDLT were recorded and compared between patients with LAR and those without. LAR-free survival was stratified by immunosuppressive regimen using the log-rank test. A $P$ value of less than 0.05 was considered statistically significant. Values of measured variables were expressed as median and range.

## RESULTS

### Clinical outcome

None of the patients experienced immediate graft non-function or chronic rejection. Postoperative vascular, hemorrhagic, and biliary complications that required re-operation within 6 mo, occurred in 75 patients (37%). Conversion from FK to CsA was required in 38 patients (19%) during the initial 6 mo after surgery. The most common reason for conversion was neurotoxicity ($n$ = 15), followed by hematopoietic disorder ($n$ = 7), diabetes mellitus ($n$ = 6), gastrointestinal intolerance ($n$ = 4), hepatotoxicity ($n$ = 3) and cardio-pulmonary disorder ($n$ = 3). The median time to conversion was 16 d (range: 6-165). The cumulative 1- and 3-year patient survival rates were 90% and 87%, respectively.

**Table 2 Incidence of LAR for selected baseline factors**

| Factors | Variables (*n*) | % LAR | *P* |
|---|---|---|---|
| Preoperative | | | |
| Age (yr) | < 40 (53) *vs* ≥ 40 (151) | 12 *vs* 6 | 0.03 |
| Gender | Men (102) *vs* women (102) | 8 *vs* 8 | 0.79 |
| Disease: Viral | Yes (77) *vs* No (127) | 9 *vs* 6 | 0.41 |
| Total bilirubin (mg/L) | < 50 (100) *vs* ≥ 50 (104) | 8 *vs* 7 | 0.72 |
| MELD score | < 10 (123) *vs* ≥ 10 (81) | 7 *vs* 9 | 0.57 |
| Blood type match | Identical (163) *vs* compatible (41) | 8 *vs* 5 | 0.5 |
| Gender match | Yes (91) *vs* No (113) | 9 *vs* 6 | 0.48 |
| Donor age (yr) | < 40 (84) *vs* ≥ 40 (120) | 10 *vs* 6 | 0.32 |
| HLA-A mismatch (*n*) | 0 (75) *vs* 1 or 2 (128) | 9 *vs* 6 | 0.64 |
| HLA-B mismatch (*n*) | 0 (38) *vs* 1 or 2 (166) | 11 *vs* 7 | 0.41 |
| HLA-DR mismatch (*n*) | 0 (43) *vs* 1 or 2 (161) | 5 *vs* 8 | 0.44 |
| T-LCX | Negative (195) *vs* positive (9) | 8 *vs* 0 | 0.39 |
| B-LCX | Negative (116) *vs* positive (88) | 5 *vs* 7 | 0.71 |
| Operative | | | |
| Anhepatic time (min) | < 150 (116) *vs* ≥ 150 (88) | 8 *vs* 7 | 0.8 |
| Blood loss (mL/kg) | < 100 (88) *vs* ≥ 100 (116) | 7 *vs* 8 | 0.8 |
| Graft weight/SLV (%) | < 50 (69) *vs* ≥ 50 (135) | 7 *vs* 7 | 0.97 |
| Postoperative | | | |
| Immunosuppressive regimen | FK (166) *vs* CsA (38) | 32 *vs* 2 | < 0.0001 |
| Proceeding CMV infection | Yes (82) *vs* No (116) | 7 *vs* 8 | 0.91 |
| Early acute rejection | Yes (61) *vs* No (143) | 11 *vs* 6 | 0.14 |

LAR: Late onset acute rejection; MELD: Model for end stage liver disease; HLA-n: Human leukocyte antigen n allele; LCX: Lymphocytotoxic crossmatch; SLV: Standard liver volume; CMV: Cytomegalovirus.

### *Postoperative complications*

Serum cholesterol and serum creatinine levels were 1130 mg/L (range, 560-2110) and 10 mg/L (range, 2-44) preoperatively, and 1850 mg/L (range, 900-3760) and 8.6 mg/L (range, 3-31) at 6 mo after transplantation, respectively. The incidence of hyperlipidemia, chronic renal failure, and new-onset post-transplantation diabetes was 13% ($n$ = 27), 2% ($n$ = 3), and 24% ($n$ = 48), respectively. To date, none of the patients has developed symptomatic cardiovascular diseases or end-stage renal disease that required hemodialysis or kidney transplantation.

Positive cytomegalovirus antigenemia occurred in 40% (82/204), all within 6 mo of surgery. The incidence of deep fungal infection that occurred more than 6 mo after surgery was 17% (35/204), which included candidiasis ($n$ = 20), aspergillosis ($n$ = 5), Pneumocystis carinii pneumonia ($n$ = 3), and cryptococcosis ($n$ = 2). The median time to the diagnosis was 192 d (range: 181-326). These deep infections were all successfully treated, except for one patient who died due to cryptococcosis.

### *Late-onset acute rejection*

LAR was observed in 15 cases (7%, Table 2). The median time to LAR was 302 d (range: 182-1490). All LAR cases were on a maintenance dose of steroid (methylprednisolone 0.05 mg/kg per day), except for one patient who had undergone steroid withdrawal 10 mo before LAR because of aseptic necrosis of the femoral head. Univariate analysis revealed that CsA-based immunosuppressive regimen at the onset of LAR and lower recipient age were significantly associated with LAR (Table 2). Multivariate analysis revealed that only the CsA-based regimen was an independent predictor (Hazard ratio, 0.033; range, 0.007 to 0.142; $P < 0.0001$).

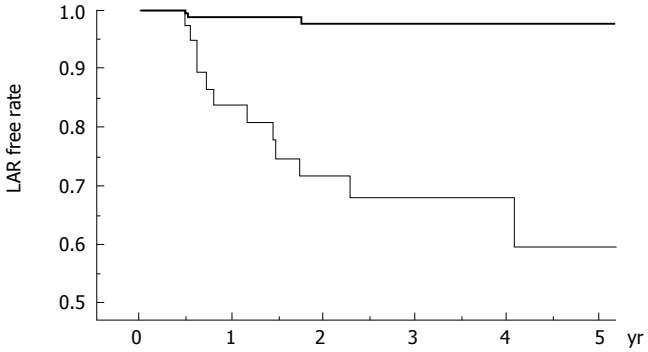


**Figure 1** Kaplan-Meier plot of LAR-free survival based on immunosuppression. Thick line: patients with tacrolimus ($n$ = 166); thin line: those with cyclosporine ($n$ = 38). $P < 0.0001$ comparison between patients with tacrolimus and cyclosporine.

**Table 3 Actual trough levels of calcineurin inhibitors six months after transplantation**

| Group | All | LAR | No LAR | *P* |
|---|---|---|---|---|
| | Mean (range) | Mean (range) | Mean (range) | |
| Tacrolimus (μg/L) | 7.0 (4.5-11.3) | 7.4 (6.5-9.2) | 7.0 (4.5-11.3) | 0.47 |
| Cyclosporine (μg/L) | 133 (70-210) | 135 (79-210) | 128 (70-190) | 0.35 |

LAR: Late onset acute rejection.

LAR-free survival stratified by immunosuppression regimen (FK-based or CsA-based) is shown in Figure 1. In those who developed LAR, only one patient in the CsA group had a sub-therapeutic level at least once during the preceding 8 wk. The trough levels more than 6 mo after transplantation were properly maintained within the target range in both FK-based and CsA-based recipients and were not related to LAR (Table 3). All LAR patients were successfully treated with steroid recycle therapy only, except for two who required additional mycophenolate mofetil and anti-T-cell monoclonal antibody administration. None of them developed chronic rejection.

## DISCUSSION

In the present study, there was a 7% incidence of LAR, which is an acceptable rate compared with previous studies (7%-23%)[12-16]. The incidence of early acute rejection in this population was 30% (61/204), and HLA-DR mismatching and positive T-lymphocytotoxic crossmatch had been proved to be independent significant predictors of early acute rejection[17]. Neither HLA compatibility nor lymphocytotoxic crossmatch was associated with LAR. Episodes of early acute rejection were not related to LAR, as reported previously[12,15]. The present study indicated that a CsA-based immunosuppressive regimen and lower

recipient age were significant risk factors for LAR, which is consistent with previous studies[18].

Whether FK or CsA is the primary immunosuppressant depends on the transplant center, although FK is currently prescribed for nearly 90% of new liver transplantation recipients[1]. In recent randomized trials in adults, there was no difference in the incidence of acute rejection over 1 to 30 mo[19]. It indicates that CsA and FK are equivalent immunosuppressants when maintained at high therapeutic levels. Previous reports emphasized that sub-therapeutic levels of calcineurin inhibitors are related to LAR[12-15], which was not supported by the present data (Table 3). Based on the properly maintained trough level of FK and CsA in our series, the patient drug-compliance bias is negligible and the higher LAR incidence in the CsA group might represent incomplete immunosuppression in the present CsA-based regimen especially for young recipients.

Steroid withdrawal after liver transplantation is still controversial. There are reports of successful steroid withdrawal in randomized studies[2,5]. In contrast, Pageaux *et al*[4] reported a higher incidence of rejection in a steroid withdrawal group. Yoshida *et al*[14] reported that low-dose steroids increased the incidence of LAR. The aim of steroid withdrawal is to reduce long-term complications, such as diabetes mellitus, hyperlipidemia, and opportunistic infections. When compared to steroid free protocols our results with steroid maintenance showed comparable incidence in opportunistic infection, normal serum creatinine and cholesterol level (6-12 mg/L and 1290-2320 mg/L, respectively) although incidence of postoperative diabetes mellitus (24%) seemed higher. Our protocol of life-long steroid maintenance might be justified by the lower incidence of LAR without increased risk of opportunistic infections and metabolic complications.

In conclusion, an FK-based regimen with life-long steroid maintenance (0.05 mg/kg per day) is safe and effective after liver transplantation. In young (< 40 years of age) recipients who require conversion from FK to CsA, careful observation for LAR is necessary.

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*RAPID COMMUNICATION*

# Arterial steroid injection therapy can inhibit the progression of severe acute hepatic failure toward fulminant liver failure

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## Abstract

**AIM:** To utilize transcatheter arterial steroid injection therapy (TASIT) *via* the hepatic artery to reduce hepatic macrophage activity in patients with severe acute hepatic failure.

**METHODS:** Thirty-four patients with severe acute hepatic failure were admitted to our hospital between June 2002 to June 2006 providing for the possibility of liver transplantation (LT). Seventeen patients were treated using traditional liver supportive procedures, and the other 17 patients additionally underwent TASIT with 1000 mg methylprednisolone per day for 3 continuous days.

**RESULTS:** Of the 17 patients who received TASIT, 13 were cured without any complications, 2 died, and 2 underwent LT. Of the 17 patients who did not receive TASIT, 4 were self-limiting, 7 died, and 6 underwent LT. Univariate logistic analysis revealed that ascites, serum albumin, prothrombin time, platelet count, and TASIT were significant variables for predicating the prognosis. Multivariate logistic regression analysis using stepwise variable selection showed that prothrombin time, platelet count, and TASIT were independent predictive factors.

**CONCLUSION:** TASIT might effectively prevent the progression of severe acute hepatic failure to a fatal stage of fulminant liver failure.

## INTRODUCTION

Although it is accepted that severe acute hepatic failure is self-limiting in most patients, it progresses to fulminant liver failure with a high mortality rate in some patients[1]. Liver transplantation (LT) is the only effective treatment for patients with fulminant liver failure[2]. It seems that early treatment for severe acute hepatic failure could effectively reduce the mortality, but it is challenged with several reasons. First, it is difficult to determine the likelihood of progression from severe acute hepatic failure to fulminant liver failure. Although studies have proposed several predictive parameters, such as coagulopathy, jaundice, etiology, and aging[3-7], a reliable and widely accepted procedure for distinguishing self-limiting cases from fatal cases has not been established. Second, even if it might be possible to predict the prognosis of severe acute liver failure, no effective procedures exist for preventing the progression from severe acute hepatic failure to fulminant liver failure. Plasma exchange and hemodiafiltration have been used as artificial liver support systems, but they are only partially effective[8-11].

The development of methods for inhibiting the progression of severe acute hepatic failure to fulminant liver failure has been hampered by a lack of understanding of the mechanisms of fulminant liver failure. The reason why the condition is fatal in some patients but remits spontaneously in others even among cases sharing a common etiology remains unclear. Several recent studies

indicate that activated macrophages in the liver might play a key role in the development of fulminant liver failure[12-15]. If this hypothesis is correct, then suppressing intrahepatic macrophage activity might be a useful treatment.

It is well known that macrophage activity is suppressed by corticosteroids[16,17], which have been used in the past as a treatment for severe acute hepatic failure, but their effectiveness has not been definitively established[18-20]. We speculated that the disappointing results might be due to an insufficient concentration of corticosteroids in the hepatic circulation, and therefore injected corticosteroids *via* the hepatic artery as a treatment for patients with severe acute hepatic failure. Selective arterial injection has been used for the treatment of hepatocellular carcinoma[21,22]. Infusion of 5-flourouracil or cisplatin *via* the hepatic artery is effective while peripheral intravenous injection *via* the hepatic artery is not effective against hepatocellular carcinoma. In this study, patients with severe acute hepatic failure were enrolled and prognostic factors, including arterial steroid injection therapy, were analyzed.

## MATERIALS AND METHODS

### Patients

The patients enrolled between June 2002 to June 2006 in this study were admitted to our hospital with a diagnosis of severe acute hepatic failure and fulfilled at least one of the following criteria: progressive and sustained prothrombin time (PT) (PT-international normalized ratio (INR) > 1.5 for more than 3 d), presence of ascites, and presence of hepatic encephalopathy. When obvious liver atrophy and hepatic coma were observed on admission, the patient was immediately prepared for liver transplantation and excluded from the study. Transcatheter arterial steroid injection therapy (TASIT) was administered with the consent of the patient or in cases of severe encephalopathy greater than grade Ⅱ, with the written informed consent of the patient's family. When consent was not obtained, traditional supporting treatments were continued. Therefore, this study was not a randomized trial. Plasma exchange was repeated to keep PT-INR lower than 1.7, and hemodiafiltration was performed if necessary. Regardless of the method of treatment, LT was implemented in cases whose liver insufficiency progressed when an appropriate donor could be found.

### Arterial steroid injection protocol

A 5-frame catheter was inserted from the right femoral artery to the common hepatic artery. Before the steroid injection, angiography was performed to determine whether an anomaly of the hepatic artery existed. When no replaced hepatic artery was found, the tip of the catheter was set at the proper hepatic artery. When the liver blood was supplied from two hepatic arteries, the branch that showed the widest feeding area was selected for positioning of the catheter. After insertion of the catheter, 1000 mg methylprednisolone was infused for 2 h per day. The arterial steroid injections were continued for 3 d, and the catheter was removed just after injection on the third day. When a bleeding tendency was observed during this protocol, once-daily plasma exchange was added to the regimen.

The details of the informed consent statement and the arterial steroid injection protocol were approved by the Ethics Committee of Kyushu University.

### Statistic analysis

Differences in clinical backgrounds, symptoms, and laboratory data between patients who did or did not receive TASIT were analyzed using $\chi^2$ test and Student *t*-test. Factors potentially associated with the prognosis of severe acute hepatic failure were analyzed by univariate analysis followed by multivariate logistic regression analysis using stepwise variable selection. Factors with a *P* value > 0.10 were removed from the multivariate analysis.

## RESULTS

Between June 2002 to June 2006, 34 patients (21 males and 13 females) were enrolled in this study. Seventeen patients gave their consent to receive TASIT and underwent the treatment. The characteristics of the patients on admission are shown in Table 1. The overall outcome was as follows: 17 patients were conservatively cured, 9 died, and 8 underwent LT. Since it is difficult to find donors for orthotopic transplantation in Japan, living donor LT was performed if necessary. All patients who underwent LT survived the procedure. Pathological examination of liver samples resected during LT showed that the injured liver was markedly atrophic and wholly necrotized in each case, indicating that the cases selected for LT were correctly identified as candidates for LT.

Patient backgrounds on admission did not differ significantly except for the encephalopathy-grading, serum albumin and LDH concentration between patients who did or did not undergo TASIT. In patients who did not receive TASIT, 4 were conservatively cured, 7 died, and 6 underwent LT. In patients who received TASIT, 13 were conservatively cured, 2 died, and 2 underwent LT. All deaths among patients who did not receive TASIT were due to progression to liver failure, while in the TASIT group, one patient died of liver failure and the other died of pneumonia seven days after initial recovery from hepatic insufficiency.

Rapid improvement of liver function was observed in the conservatively cured 13 survivors who received TASIT during the 3-d protocol. Encephalopathy was observed in 9 of the conservatively cured survivors on admission, and completely disappeared on the second day of the TASIT protocol. As shown in Figure 1, serum ALT concentrations decreased remarkably and coagulopathy rapidly improved. Serum ALT concentrations rebounded slightly for a few days in most of the survivors just after the end of the TASIT protocol, and subsequently decreased and normalized within 3 wk after TASIT in all but 1 patient. Even in 1 exceptional case showing prolonged serum ALT elevation after TASIT, the ALT level was much lower compared to that on admission and PT stayed within the normal range. The hepatic function of the patient normalized five weeks after TASIT, but the

**Table 1 Characteristics and outcomes of patients ( mean ± SD)**

| | Arterial steroid injection therapy | | | |
|---|---|---|---|---|
| | Yes | No | Total | *P* |
| *n* | 17 | 17 | 34 | |
| Sex (male/female) | 11/6 | 10/7 | 21/13 | 0.7242 |
| Age (yr) | 45.1 ± 16.9 | 44.8 ± 19.0 | 45.0 ± 17.7 | 0.9623 |
| Ascites (none/present) | 12/5 | 7/10 | 19/15 | 0.0842 |
| Encephalopathy (0/1/2/3) | 5/4/3/5 | 11/0/5/1 | 16/4/8/6 | 0.0242 |
| T.bilirubin (mg/dL) | 9.04 ± 9.21 | 14.48 ± 9.62 | 11.76 ± 9.67 | 0.1014 |
| D.bilirubin (mg/dL) | 6.15 ± 6.73 | 9.08 ± 6.71 | 7.61 ± 6.79 | 0.2131 |
| D/T bilirubin ratio | 0.66 ± 0.06 | 0.61 ± 0.12 | 0.64 ± 0.10 | 0.1785 |
| Albumin (g/dL) | 3.55 ± 0.46 | 3.25 ± 0.32 | 3.40 ± 0.42 | 0.0362 |
| ChE (mg/dL) | 111.7 ± 40.5 | 95.4 ± 31.3 | 103.8 ± 36.7 | 0.2047 |
| AST (U/L) | 5817.5 ± 4223.6 | 3203.71 ± 2829.2 | 4510.62 ± 3780.2 | 0.0530 |
| ALT (U/L) | 4056.7 ± 2427.3 | 3643.71 ± 2677.1 | 3850.21 ± 2525.0 | 0.6407 |
| AST/ALT ratio | 1.58 ± 1.16 | 0.99 ± 0.68 | 1.28 ± 0.98 | 0.0833 |
| ALP (U/L) | 539.5 ± 258.3 | 517.8 ± 178.0 | 528.6 ± 218.1 | 0.7775 |
| γ-GTP (U/L) | 336.4 ± 351.2 | 182.0 ± 182.0 | 261.5 ± 288.7 | 0.1228 |
| LDH (U/L) | 4349.3 ± 4479.0 | 1343.3 ± 2553.1 | 2846.3 ± 3900.6 | 0.0238 |
| PT-INR | 2.50 ± 1.60 | 2.93 ± 1.92 | 2.71 ± 1.76 | 0.4890 |
| Platelet ($\times 10^4/\mu L$) | 13.9 ± 5.5 | 11.8 ± 4.2 | 12.8 ± 4.9 | 0.2121 |
| AFP (ng/mL) | 16.7 ± 27.2 | 29.6 ± 43.8 | 23.2 ± 36.5 | 0.3125 |
| $NH_3$ (μg/dL) | 90.5 ± 87.0 | 63.6 ± 32.2 | 77.1 ± 66.1 | 0.2451 |
| BUN (mg/dL) | 14.1 ± 10.9 | 14.8 ± 18.6 | 14.4 ± 15.0 | 0.8850 |
| Creatinine (mg/dl) | 1.20 ± 1.30 | 1.16 ± 1.24 | 1.18 ± 1.25 | 0.9210 |
| Outcome (Survival/death/LT) | 13/2/2 | 4/7/6 | 17/9/8 | 0.0085 |

*P*-values were calculated to compare the patients treated with and without TASIT.

etiology remained unknown.

To determine prognostic factors, patients were assigned to 2 groups based on their clinical outcomes: one group consisted of conservatively cured survivors who did not receive LT and the other included fatal cases and LT recipients who were judged to have a poor prognosis without LT, based on pathological findings of the injured liver. Univariate logistic analysis was performed for age, sex, etiology of hepatitis, administration of TASIT, laboratory data, and symptoms on admission (Table 2). Among these parameters, presence of ascites, concentration of serum albumin (> 3.3 g/dL), PT-INR (> 2.0), platelet count (> 12 × $10^4/\mu L$), and TASIT were significant factors ($P < 0.05$). Stepwise multiple regression analysis was performed using variables with *P* values ⩽ 0.1, which revealed that PT-INR, platelet count, and TASIT were independent prognostic factors (Table 3). The *P* value of the prediction equation was < 0.0001.

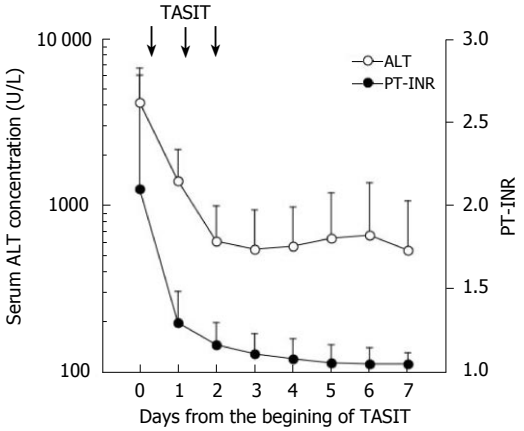


**Figure 1** Improvement of serum ALT concentrations (open circle) and PT-INR (closed circle) in the conservatively cured survivors after TASIT (mean ± SD).

## DISCUSSION

It is difficult to predict whether an individual patient with acute hepatitis develops fulminant liver failure because the condition often resolves spontaneously without any aftereffects. Severe acute hepatic failure has been generally defined as a clinical continuum between acute hepatitis without serious coagulopathy (PT > 50%) and acute liver disease complicated by clinical encephalopathy[1], which is considered a transient stage giving warning of fulminant liver failure. However, according to this definition of severe acute hepatic failure, two-thirds of patients spontaneously recover without developing encephalopathy[3]. Since such a high rate of conservative recovery is inappropriate for evaluating the effects of treatments designed to prevent progression of severe acute hepatic failure to fulminant liver failure, we established a more stringent criterion as a pre-stage of fulminant liver failure, namely acute severe hepatitis that has any of the following conditions: progressive and sustained PT

**Table 2 Univariate logistic analysis for survival probability**

| Variables | Estimate | *P* | Lower 95% | Upper 95% | Odds ratio |
|---|---|---|---|---|---|
| Age (50 *vs* ⩽ 50 yr) | -0.6061 | 0.0912 | -0.0802 | 1.3415 | 3.3608 |
| Sex (male *vs* female) | 0.1247 | 0.7244 | -0.5708 | 0.8322 | 1.2833 |
| Ascites (none *vs* present) | 1.2079 | 0.0036 | 0.4465 | 2.1030 | 11.1997 |
| Encephalopathy (none *vs* present) | 0 | 1.0000 | -0.6799 | 0.6799 | 1 |
| T.Bil (< 8 mg/dL *vs* ⩾ 8) | 0.4745 | 0.4930 | -0.8767 | 1.8627 | 1.6071 |
| D/T. Bil ratio (> 0.65 *vs* ⩽ 0.65) | 0.4884 | 0.4870 | -0.8825 | 1.9022 | 1.6296 |
| Albumin (> 3.3 *vs* ⩽ 3.3 g/dL) | 1.4816 | 0.0440 | 0.0865 | 3.0029 | 4.4000 |
| ChE (> 100 *vs* ⩽ 100 mg/dL) | 0.8574 | 0.2306 | -0.5244 | 2.3092 | 2.3571 |
| AST (> 3500 *vs* ⩽ 3500 U/L) | 0.4745 | 0.4930 | -0.8767 | 1.8627 | 1.6071 |
| ALT (> 4000 *vs* ⩽ 4000 U/L) | 0.9628 | 0.1735 | -0.3993 | 2.4025 | 2.6190 |
| AST/ALT ratio (> 1 *vs* ⩽ 1) | 0.2356 | 0.7317 | -1.1151 | 1.6042 | 1.2656 |
| LDH (> 600 *vs* ⩽ 600 U/L) | 1.2122 | 0.0912 | -0.1603 | 2.6831 | 3.3608 |
| PT-INR (> 2 *vs* ⩽ 2) | 1.4816 | 0.0440 | 0.0865 | 3.0029 | 20.1446 |
| Platelet (>12 × $10^4$/μL *vs* ⩽ 12 × $10^4$) | 1.7509 | 0.0200 | 0.3335 | 3.3197 | 5.7600 |
| AFP (< 5 *vs* ⩾ 5 ng/mL) | 0.2356 | 0.7317 | -1.1151 | 1.4616 | 1.2656 |
| NH3 (< 55 *vs* ⩾ 55 μg/mL) | 0.2356 | 0.7317 | -1.1151 | 1.6042 | 1.2656 |
| BUN (< 10 *vs* ⩾ 10 mg/dL) | 0.2356 | 0.7317 | -1.1151 | 1.6042 | 1.2656 |
| Etiology | | | | | |
| HBV (*vs* HAV) | 0.3269 | 0.5761 | -0.8196 | 1.5405 | 1.9230 |
| Others (*vs* HAV) | 0.1446 | 0.7567 | -0.7763 | 1.0744 | 1.3354 |
| TASIT (yes *vs* no) | 1.1787 | 0.0036 | 0.4311 | 2.0387 | 10.5624 |

(PT-INR > 1.5 more than 3 d), presence of ascites, and presence of hepatic encephalopathy. We speculated that PT at a point could not predict progression of severe acute hepatic failure to fulminant liver failure since a transient severe decrease of PT-INR is often observed even in self-limited acute hepatitis. Therefore, coagulopathy should be present for a specific period of time to enroll the patients close to fulminant liver failure. In this study, only 4 of 17 patients (23.5%) who were not treated with TASIT were conservatively cured. The remaining 13 patients died or underwent LT, which led to a markedly poorer prognosis than that reported in previous investigations in patients with severe acute hepatic failure[3,6]. The fatal outcomes indicate that our method for assessment of severe acute hepatic failure can identify patients who have a high risk of developing fulminant liver failure.

Multivariate logistic analysis revealed that TASIT could effectively prevent the progression of severe acute hepatic failure to fulminant liver failure. In light of past reports showing that corticosteroids have no effect on severe acute hepatic failure[18-20], we suggest that the effectiveness of TASIT may be due to the high concentrations of corticosteroids achieved in the hepatic circulation. However, we have not yet elucidated the mechanisms by which high concentrations of corticosteroids affect hepatic macrophages or damage hepatocytes. Furthermore, the role of macrophages in severe acute hepatic failure needs to be further clarified.

We believe that our protocol is safe and effective, but the ideal dose and duration of corticosteroid treatment should be verified. Although excessive use of corticosteroids generally induces infections that would hinder LT, 2 patients in this study who underwent LT after TASIT recovered without any complications, suggesting that our protocol does not seem to influence the outcome of LT. However, lower corticosteroid doses might be sufficient to prevent the progression of the disease. Randomized, controlled studies are needed to confirm this possibility.

**Table 3 Multivariate logistic regression analysis using stepwise variable selection**

| Variables | Estimate | *P* | Lower 95% | Upper 95% | Odds ratio |
|---|---|---|---|---|---|
| TASIT (done) | 1.6535 | 0.0076 | 0.6220 | 3.2312 | 27.3009 |
| Platelet (< 12 × $10^4$/μL) | 2.6871 | 0.0261 | 0.6450 | 5.7805 | 14.6895 |
| PT-INR (< 1.0) | 1.8711 | 0.0687 | -0.0119 | 4.1505 | 6.4953 |

Among the patients who received TASIT, 4 were not conservatively cured, 2 underwent LT and 2 died without LT. Since one of the deaths resulted from pneumonia after recovery from hepatic failure, TASIT was found to be ineffective for severe acute hepatic failure in 3 patients. A common finding in the 3 patients was rapid atrophy of the liver, showing an obvious decrease in liver volume within 24 h of admission, indicating that TASIT is not effective for patients who are at the later stage of fulminant liver failure. Therefore, TASIT should be performed when possible warning signs of the progression of severe acute hepatic failure to fulminant liver failure are observed. However, early administration of TASIT would involve patients who might otherwise recover following traditional supportive measures. Thus it is possible that some of the patients treated with TASIT in this study may have been cured without the treatment. Nonetheless, considering the results of multivariate logistic analyses and the high cost of LT, we believe that TASIT is advantageous over the traditional liver supportive procedures.

In this study, we demonstrated a new treatment strategy for preventing the progression of severe acute liver failure to fulminant liver failure. Our results indicate that TASIT is a safe and effective treatment for severe acute hepatic failure. However, its effect is insufficient in patients showing marked and rapid progression of liver atrophy. Therefore, the option of LT should be considered simultaneously. Randomized, controlled trials are needed to further evaluate this treatment strategy.

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*RAPID COMMUNICATION*

# Immunoproteome analysis of soluble and membrane proteins of *Shigella flexneri* 2457T

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## Abstract

**AIM:** To profile the immunogenic proteins of *Shigella flexneri* (*S. flexneri*) expressed during human infection using a proteomic approach.

**METHODS:** Soluble and membrane protein extractions of *S. flexneri* 2457T were separated by two-dimensional gel electrophoresis (2-DE). Proteins were transferred to PVDF membrane and immunoblotted with sera from shigellosis patients. Reactive protein spots were matched to Coomassie stained gels run in parallel, cut out and trypsin digested. Matrix-assisted laser desorption/ionization time of flight-mass spectrometry (MALDI-TOF-MS) was used to determine the peptide mass fingerprints, which were searched in the MASCOT database to identify the protein.

**RESULTS:** A total of 8 immunoreactive proteins were successfully identified from the Coomassie stained gels in three repeats. Six of these proteins have not previously been reported as immunogenic in *S. flexneri*. These proteins could be potential candidates for vaccine or attenuation studies.

**CONCLUSION:** Soluble and membrane proteins of *S. flexneri* 2457T have been screened by 2-DE and immunoblotting with sera from shigellosis patients. Eight proteins are identified as immunogenic.



**Key words:** *Shigella flexneri*; Immunogenetics; Vaccine antigen; Immunoblotting

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## INTRODUCTION

Shigellosis is a public health concern in developing countries, particularly for young children who make up 69% of all cases. The majority of shigellosis cases in the Asian, African and Central American regions are caused by *Shigella flexneri* (*S. flexneri*)[1]. Bacteria are transmitted *via* the fecal oral route and require as little as 100 organisms to cause the disease. *S. flexneri* penetrates the epithelial layer of the large intestine, invading and spreading throughout the intestinal epithelial cells. A number of proteins are known to be important for these invasion steps, particularly the products of the *mxi-spa* and *ipa* loci located on the virulence plasmid[2,3].

The immune response against *S. flexneri* appears to be predominantly a humoral response and it still remains unclear whether the host's cellular immunity plays any protective role[4]. The humoral immunity appears to be protective against reinfection, generally against the homologous serotype[5-7]. This is because both the mucosal and systemic immune responses seem to be primarily directed against the lipopolysaccharide (LPS), the structure of which differs between the serotypes. Besides the LPS, *S. flexneri* proteins, which do not show serotype-specificity, have been identified as immunogenic during *S. flexneri* infection of higher primates. The invasion plasmid antigens (Ipa) are antigenic in monkey and human studies, and capable of inducing a protective immune response in mice and guinea pigs when delivered as a multiprotein complex[8-11]. Additionally, some major outer membrane proteins of 30-35 kDa are recognized by the mucosal immune response of *Shigella* patients and are protective in guinea pigs, mice and rabbits[8,12,13].

The escalation in antibiotic resistance in *S. flexneri* isolates is adding increased strain to the limited health services of developing countries. Consequently, the World Health Organization has prioritized the development of a safe and effective vaccine against *S. flexneri*[1]. However, despite promising results with attenuated live vaccine strains, the current vaccine candidates are either not sufficiently attenuated or immunogenic enough[14,15], suggesting that the identification of additional attenuation or protective antigens is warranted.

In order to identify such targets in *S. flexneri*, a profile of immunogenic proteins would be a useful tool. To

date, a proteomic study of detectable immunoreactive proteins from natural *Shigella* infection of humans has not been reported. It is possible to identify antigens from two-dimensional gels using immunoproteomics, where proteins of a particular pathogen are separated by two-dimensional electrophoresis (2D-E) and blotted onto membranes and probed with patients' sera. Subsequent matching and identification of any reactive protein spots generates an immunoproteome for the organism in question. This technique was initially used to visualize the antigens of *Borrelia garinii* and has since been used for a number of microorganisms including *S. aureus*, *H pylori* and *C. albicans*[16-19]. Similar studies have recently been performed with *S. flexneri* protein probed with sera from immunized mice or rabbits[20-22]. However, shigellosis is not a natural disease of mice or rabbits and it remains unclear what use immunoproteome data from laboratory animals will be in the study of human *Shigella* infection. Consequently the generation of an immunoproteome for *S. flexneri* with sera from human shigellosis patients would ultimately be a desirable tool for future vaccine and virulence research.

In this paper, we report the use of immunoproteomics to visualize and identify immunogenic *S. flexneri* 2457T serotype 2a soluble and membrane proteins, which are reactive to sera from *S. flexneri* infected patients.

## MATERIALS AND METHODS

### Bacterial cell culture

*S. flexneri* 2457T was grown in Luria Bertani broth overnight at 37℃ in a shaking incubator. Overnight cultures were diluted 1:100 and shaken at 140 rpm until an $OD_{600}$ of 0.5 was reached.

### Isolation of soluble proteins

Cultures at $OD_{600}$ 0.5 were pelleted by centrifugation at 14 000 *g*, 4℃ for 10 min. Pellets were washed in wash buffer (68 mmol/L NaCl, 2 mmol/L KCl, 1.5 mmol/L $KH_2PO_4$, 9 mmol/L $NaH_2PO_4$) and pelleted again. The pellet was resuspended in lysis buffer (8 mol/L urea, 4% w/v CHAPS, 1% w/v DTT, 0.8% 3/10 ampholytes, 35 mmol/L Trizma, 5 mmol/L EDTA, 1 mmol/L PMSF) and sonicated on ice at constant duty and an output of 8 for 10 × 10 s with 20 s breaks by a Branson 450 Sonifier (Branson Ultrasonics Corporation, Danbury, CT, USA). Unbroken cells and debris were removed by centrifugation at 100 000 × *g* for 1 h at 10℃. The supernatant was collected and stored at -80℃ until required. The protein concentration of the remaining supernatant was determined by Bradford assay using BSA suspended in lysis buffer as the standard.

### Isolation of outer membrane proteins

For the extraction of membrane proteins the method was modified from Molloy *et al*[23], with the following changes. Cultures at $OD_{600}$ of 0.5 were collected by spinning at 1400 × *g* for 10 min. Harvested cells were resuspended in wash buffer (68 mmol/L NaCl, 2 mmol/L KCl, 1.5 mmol/L $KH_2PO_4$, 9 mmol/L $NaH_2PO_4$) and spun again. The pellet was resuspended in 50 mmol/L Tris-HCl pH 7.3 containing 0.2 g/L DNase I. The cells were disrupted by sonication as described above and unbroken cells and cell debris were removed by centrifugation at 6000 *g* for 10 min. The supernatant was collected and the pellet was resuspended in 50 mmol/L Tris-HCl pH 7.3 and submitted to sonication and centrifugation again. The supernatants were pooled, carbonate treated and collected as described by Molloy *et al*[23]. The collected membrane proteins were resuspended in 2% ASB-14, 1% DTT, 7 mol/L urea, 2 mol/L thiourea, 0.5% 3/10 ampholytes and stored at -80℃.

### Two-dimensional electrophoresis

For the first dimension isoelectric focusing, 11 cm Immobiline Dry Strips with the pH gradient 4-7 were used (Amersham Pharmacia Biotech, Uppsala, Sweden). Strips were rehydrated for 16 h in 200 μL of rehydration solution (8 mol/L urea, 0.5% w/v CHAPS, 0.15% w/v DTT, 0.5% v/v Biolyte 3-10 carrier ampholytes and a trace of bromophenol blue). Strips were placed in a Multiphor Ⅱ horizontal electrophoresis system (Amersham Pharmacia Biotech, Uppsala, Sweden) and 200 μL samples (about 1000 μg) of soluble and membrane protein preparations were cup loaded at the anode. Focussing was run at 20℃, 1 mA, 5 W at the following voltage gradients: 1 min 150 V, 20 min 150 V, 20 min 200 V, 2 h 300 V, 2 h on a linear gradient to 3500 V and 20 h at 3500 V. After isoelectric focussing, the strips were treated with equilibration solution I (40% v/v glycerol, 0.05 mol/L Tris-HCl pH 6.8, 6 mol/L urea, 2% w/v SDS and 2% w/v DTT) for 10 min and 10 min in equilibration solution Ⅱ (40% v/v glycerol, 0.05 mol/L Tris-HCl pH 6.8, 6 mol/L urea, 2% w/v SDS and 2% w/v iodoacetamide and 0.005% bromophenol blue). Second dimension SDS-PAGE was performed on a horizontal Multiphor Ⅱ electrophoresis system using precast Excel Gels with 12%-14% acrylamide gradient (Amersham Pharmacia Biotech, Uppsala, Sweden). Twin gels were loaded with a soluble protein and a membrane protein 11 cm Immobiline strip. Low molecular range markers (Amersham Pharmacia Biotech, Uppsala, Sweden) were loaded between the strips. Electrophoresis was performed at 200 V for 1 h 45 min and 600 V for 4-6 h, until the bromophenol blue front reached the edge of the gel. One of the twin gels was stained with CBB G250[24]. Approximately 150 spots were visualized for the soluble protein preparation and 25 for the membrane preparation.

### Immunoblotting

The remaining gel was used for immunoblotting, where the proteins were transferred to PVDF membrane using the horizontal semi-dry electrophoretic Pharmacia LKB NovaBlot system (Amersham Pharmacia Biotech, Uppsala, Sweden) at a constant current of 0.8 $mA/cm^2$. A reference map of proteins was produced by amido black staining of the membrane. The image was scanned before the membrane was destained[16].

The membrane was blocked with 5% skim milk powder/PBS/0.1% Tween-20 overnight at 4℃, washed three times with PBS/0.05% Tween-20 for 10 min each

and incubated overnight at 4℃ with 1:100 dilution of pooled sera from five *S. flexneri* patients (International Centre for Diarrhoeal Disease, Bangladesh) diluted in 1% skim milk powder/PBS/0.1% Tween-20. The serum samples were obtained by the International Centre for Diarrhoeal Disease, Bangladesh, from *S. flexneri* shigellosis patients during the acute stage of shigellosis, 3-6 d after the onset of diarrhoea. Sera were pooled due to the limited volume of patients' sera available. The membrane was washed three times with PBS/0.05% Tween-20 for 10 min and incubated with 1:100 000 dilution of sheep anti-human Ig-HRP (Chemicon International, Temecula, CA, USA) in 1% skim milk powder/PBS/0.1% Tween-20 for 1.5 h, at 4℃. The membrane was washed with PBS/0.05% Tween three times and twice with PBS, before being incubated for 5 min with SuperSignal West Pico Chemiluminescent Substrate (Pierce Biotechnology Inc, Rockford, IL, USA) and developed by autoradiography. Membranes were stripped as per Jungblut and Bumann[25] and reprobed as above using sera from five healthy individuals obtained from the Australian Red Cross Blood Bank.

### Peptide mass fingerprinting

Developed films, scanned pictures of the amido black stained membrane and the Coomassie stained twin gel were superimposed and compared using Adobe Photoshop® and transparencies prepared from the images. Protein spots from the Coomassie gel, which aligned to spots on the film, were excised from the gel. Some samples were sent for matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) analysis at the Australian Proteome Analysis Facility (APAF) at Macquarie University. Alternatively protein spots were digested with trypsin for 16 h at 37℃ and the peptide mixture was mixed (1:1) with a saturated α-cyano-4-hydroxycinnamic acid solution in 30% acetonitrile-0.07% trifluoroacetic acid. One μL of mixture was spotted onto a sample template plate and mass spectra were obtained at the Research School of Biological Sciences (RSBS) Molecular Biology Facility (Australian National University) with an Omniflex mass spectrometer (Bruker Daltonics, Billerica, USA). Autolysing trypsin peptide 2211.1 Da was used as an internal calibration. Post-analysis data processing was done using XMASS™ software (Bruker Daltonics, Billerica, USA). Searches were performed against the MSDB database with the MASCOT peptide mass fingerprint search engine (Matrix Science)[26]. In all significant matches, the top match was a *S. flexneri* protein.

## RESULTS

### Soluble protein profile

Figure 1A shows the CBB G250 stained 2D-E gel of *S. flexneri* membrane and soluble protein preparations. This gel is a representative example of the three independent repeats of protein extraction and 2D electrophoresis performed for this study. Approximately 150 soluble protein spots were visualized from about 1 mg of soluble protein preparation applied to the CBB G250 stained gel. A twin gel was used for electroblotting onto PVDF membrane and membranes were transiently stained with amido black to aid in subsequent spot matching to the CBB gel. The membrane was probed with sera from *S. flexneri* patients and a map of immunoreactive proteins was visualized by chemiluminescence. The pattern of immunogenic proteins is shown on the film in panel B of Figure 1. This film is a representative of the three films from the immunoblot repeats. A total of 6 immunogenic proteins could be matched in each of the three repeats to a corresponding protein spot on the CBB G250 gel. These proteins were identified using peptide mass fingerprinting. The membranes from all three repeats were stripped and reprobed with sera from healthy volunteers. The resulting films had very little signal and displayed only 2 or 3 reactive spots (data not shown), none of which could be aligned to a visible *S. flexneri* protein spot on the CBB G250 gel and were not analysed by peptide mass fingerprinting.

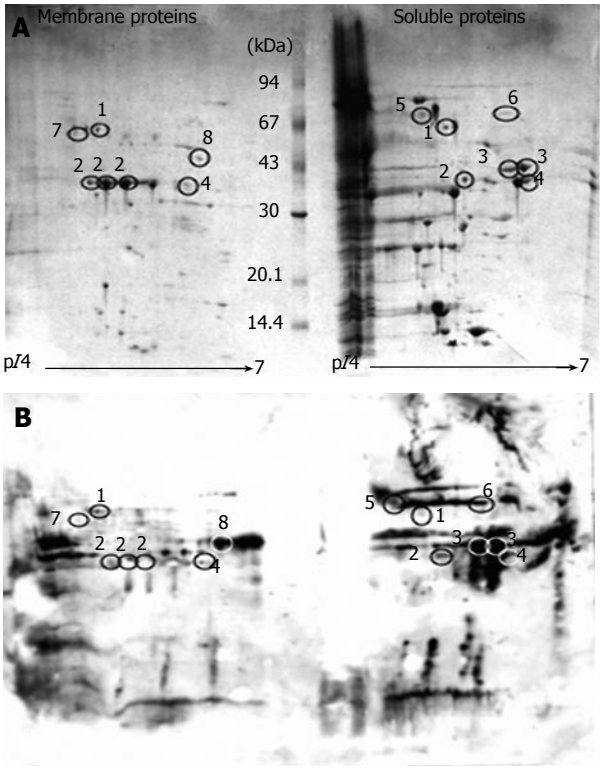


**Figure 1** **A**: Coomassie blue stained 2D gel of *S. flexneri* membrane and soluble protein preparations; **B**: Immunoblot of *S. flexneri* membrane and soluble protein preparations. Circles indicate immunoreactive spots successfully matched and identified from the Coomassie gel (**A**). Numbers correspond to Table 1.

### Outer membrane protein profile

The outer membrane protein extractions, stained by CBB G250, are shown on the representative gel in Figure 1A. Approximately 25 proteins were visualized for each of the three protein preparations. The film displaying the immunoreactive membrane proteins to *S. flexneri* patients' sera is shown in panel B of Figure 1. It was possible to match five immunogenic spots on the film to the corresponding protein spots on the twin CBB G250 gel. Membranes, from each of the three repeats, were stripped and reprobed with control sera from healthy individuals. However, no immunoreactive spots were detected against the membrane protein preparations.

**Table 1 Proteins identified from the soluble and membrane protein immunoblots**

| Spot No. | Accession No. (GenBank) | Name | Description | Obs. pI/$M_r$[1] | Exp. pI/$M_r$[2] | Coverage (%) | *n* (peptides matched) |
|---|---|---|---|---|---|---|---|
| 1 | GI:30042642 | TolC | Outer membrane channel | 5.10/65.0 | 5.35/53.8 | 17 | 7 |
| 2 | GI:38000008 | OmpA | Outer membrane protein A (fragment) | 5.00-5.50/42.0 | 5.47/35.3 | 38 | 8 |
| 3 | GI:13310620 | IpaD | 37 K membrane antigen | 5.80/40.0 | 5.55/36.6 | 39 | 9 |
| 4 | GI:30042535 | AnsB | Periplasmic L-asparaginase | 6.10/38.0 | 5.95/36.8 | 22 | 6 |
| 5 | GI:17380384 | GroEL | 60 kDa chaperonin, protein Cpn60 | 4.80/68.0 | 4.85/57.3 | 18 | 7 |
| 6 | GI:32307045 | Spa33 | Surface presentation of proteins antigen | 5.80/68.0 | 5.67/33.4 | 23 | 4 |
| 7 | GI:30065275 | Ggt | Gamma-glutamyltranspeptidase | 5.00/65.0 | 5.55/61.8 | 15 | 4 |
| 8 | GI:30040341 | TolB | Involved in the tonB-independent uptake of group A colicins | 6.50/46.0 | 7.0/46.0 | 18 | 6 |

[1]The observed pI and molecular weight of protein calculated from the protein spots position on the CBB G250 2D gel; [2]The predicted pI and molecular weight of the protein calculated from MSDB database.

### *Identified immunoreactive proteins*

Protein spots, which corresponded to an immunogenic spot from the immunoblot film were excised and submitted to in-gel tryptic digestion and MALDI-TOF. Of these protein spots, eight proteins were successfully identified by peptide mass matching. All eight proteins were shown as both the protein spot on the 2-DE gel and as the corresponding immunogenic spot on the matching film (Figure 1). Table 1 lists the identified proteins from the soluble and membrane preparations. Five of the eight proteins were detected in the membrane protein extraction: OmpA, AnsB, TolC, Ggt, TolB. However, OmpA, TolC and AnsB were also detected as immunogenic spots in the soluble protein extraction. Three proteins were identified exclusively as immunogenic soluble proteins: GroEL, Spa33 and IpaD. OmpA, TolC, IpaD and Spa33 were detected as immunogenic spots numerous times in the same gel, suggesting they are occurring as protein series, with each variation retaining its antigenic characteristics.

## DISCUSSION

A total of eight proteins were successfully identified in three separate 2D-E gel immunoblots with a pool of *S. flexneri* patients' sera. Of the eight immunogenic proteins, only two are known to be immunoreactive for *S. flexneri*: IpaD and OmpA. IpaD is a secreted protein essential for entry into epithelial cells and has been reported to be antigenic in a number of *S. flexneri* studies[8,10,27-29]. However, it remains unclear whether anti-IpaD antibodies contribute to protective immunity in humans[2]. All four Ipa proteins have previously been reported to be immunogenic in natural infection, yet IpaA, IpaB and IpaC were not detected as antigens in this immunoproteome study[8]. A two-dimensional proteome map of *S. flexneri* 2457T generated by Liao *et al*[30] was also unsuccessful in detecting any of the Ipa proteins besides IpaD. Likewise, all three previous immunoproteomic studies for *S. flexneri* were unsuccessful in detecting any of the Ipa protein series as immunogenic[20-22]. It is possible that IpaA, IpaB and IpaC were not present in the 2-DE gels at sufficient protein concentrations for detection by the CBB G250 staining. The numbers of *S. flexneri* protein spots visualized on the CBB G250 gels were quite low, possibly due to insolubility problems during the protein extractions or low resolution of the pI 4-7 IEF strips used. A number of immunogenic spots detected on the immunoblot X-ray film could not be matched to a corresponding Coomassie stained spot, suggesting that some proteins were simply present at concentrations not detectable by CBB G250. It would be possible to improve the resolution of the 2D-E gels by using silver staining which increases the sensitivity of protein detection by an order of magnitude over CBB. Alternatively, fluorescent stains that are readily compatible with MALDI-TOF could increase the sensitivity of protein detection.

OmpA is a 35 kDa outer membrane protein and was identified a number of times during the immunoproteome analysis. OmpA is most probably a constituent of the major outer membrane proteins described as immunoreactive in *Shigella* patients and thus is known to be immunogenic[8].

The other six proteins have not previously been shown to be reactive to antibodies found in patients with a natural *S. flexneri* infection. However, the *E. coli* L-asparaginase Ⅱ (AnsB) protein which shares 99.1% identity with *S. flexneri* AnsB, and the highly conserved chaperonin GroEL, in a number of bacterial species, have been reported as antigenic[31,32]. The remaining four proteins are potential candidates for *Shigella*-specific antigenic and immunization studies and could ultimately be useful in the development of new *Shigella* vaccines. However, being immunogenic does not necessarily mean that an antigen is capable of producing a protective immune response, with as little as 2.5% of antigens displaying protective properties[33]. Alternatively, because these antigenic proteins are clearly expressed during invasion of the host, as they are recognized by the host immune response, they may be important for pathogenesis. Any such proteins may be potential virulence factors and could be used in bacterial attenuation of live vaccine strains.

Membrane and surface associated proteins are often immunogenic due to their likelihood of being exposed to the host's immune response. A number of the immunogenic proteins identified in this study are membrane or surface displayed proteins. Three outer membrane proteins were identified as immunogenic, OmpA, TolC and TolB. TolC plays a role in outer

membrane permeability and has been used as an epitope carrier in *Salmonella*, while TolB is involved in the import of colicins[34,35]. Spa33 is a surface displayed protein involved in the regulation of the IpaB and IpaC secretion from the cell, an important virulence step during *S. flexneri* invasion[36]. Although Spa33 was identified as a significant match by MASCOT with peptide mass fingerprints produced from the corresponding protein spot, the observed molecular weight was inconsistent with the predicted mass. Spa33 was observed as a 68 kDa protein in Figure 1 but the predicted size was 33.4 kDa. This size discrepancy may be due to Spa33 interacting with itself as a dimer or possibly forming a complex with a protein which was not detected in the consequent peptide mass fingerprinting. There is no evidence in the literature for Spa33 forming homodimers although it is possible that Spa33 interacts with Spa32, which is predicted to be a protein of 35.6 kDa[36].

Often periplasmic proteins are immunogenic, particularly when they are found extracellularly where they are exposed to the host's immune response. In this study, two metabolic periplasmic enzymes, Ggt and AnsB were identified; gamma glutamyltranspeptidase (Ggt) is a periplasmic enzyme, which is essential for the utilization of gamma-glutamyl peptide as an amino acid source and L-asparaginase Ⅱ (AnsB) is a high affinity periplasmic enzyme, induced by anaerobiosis[37,38]. Both AnsB and Ggt were identified from the membrane extraction of *S. flexneri* proteins. It is possible that the membrane preparation contains some contaminating soluble proteins. The protein sequence of Ggt contains a signal peptide and has 99.7% aa identity to *E. coli* K12 Ggt which also contains a signal peptide and localizes to the periplasm[39]. However, the mammalian form of Ggt is linked to the plasma membrane and *Neisseria meningitidis* Ggt associates with the inner membrane, suggesting that the presence of *S. flexneri* Ggt in the membrane fraction may not simply be inefficient separation of membrane proteins from soluble proteins but perhaps due to a membrane association of the protein.

Control immunoblots were performed with each of the three membranes, using sera from five healthy individuals. It can be assumed that these five individuals have previously been exposed to commensal gut microflora, including *E. coli* of which *Shigella* is a clone[40]. Consequently, the control sera should contain antibodies reactive to *E. coli* and other commensal bacterial proteins, which may potentially recognize homologous *Shigella* proteins[41]. Thus, the control immunoblots could be used as a baseline, where any *Shigella* proteins recognized by the control sera were most likely reacting with antibodies raised against the commensal gut microflora. Consequently, immunogenic proteins, which were only detected in the *Shigella* immunoblots and not in control blots could be considered as part of a *Shigella*-specific immune response. The control immunoblots did not generate any spots that aligned to the eight *S. flexneri* protein spots from the *S. flexneri* patients, suggesting that the eight antigenic proteins identified reacted to anti-*Shigella* specific antibodies in the *Shigella* patients' sera.

This is the first *S. flexneri* proteomic study of the immunoreactive proteins expressed during natural *Shigella* infection of humans. A similar study has been performed using immunized mouse sera in 2-DE gel immunoblots of *S. flexneri* outer membrane protein and soluble protein preparations. That study identified 13 seroreactive proteins, none of which correspond with our identified proteins[20]. Another group has visualized immunoproteomes for *S. flexneri* protein probed with sera from immunized rabbits[20-22]. Only two proteins, OmpA and TolC identified as immunoreactive in the present study were also detected in that work. As *S. flexneri* does not naturally infect or cause shigellosis-like symptoms in mice or rabbits and the experimental animals were not immunized mucosally, it remains unclear how relevant these seroreactive proteins are to the natural human anti-*S. flexneri* immune response.

In this study, eight proteins with reactivity to sera from patients with *S. flexneri* infection were identified using immunoproteomics. Six of these identified proteins have not previously been reported as immunogenic in *S. flexneri* natural infection. These immunoreactive proteins could be novel candidates for vaccine development. Additionally, such proteins have great potential for roles in virulence, as it seems likely they are expressed by the bacteria during the infection of the host, making them prospective attenuation targets for live vaccine construction.

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*RAPID COMMUNICATION*

# Analysis of microsatellite instability in stool DNA of patients with colorectal cancer using denaturing high performance liquid chromatography

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## Abstract

**AIM:** To evaluate the usefulness of denaturing high performance liquid chromatography (DHPLC) for analyzing microsatellite instability (MSI) status in stool DNA of patients with colorectal cancer.

**METHODS:** A total of 80 cancer tissues from patients with primary sporadic colorectal tumor (proximal cancer: 27, distal cancer: 53) and matched stool (which were employed for comparison with the tissues) were analyzed for MSI status in BAT 26. DNA samples extracted from stool were evaluated by nested polymerase chain reaction (PCR) and DHPLC for MSI analysis.

**RESULTS:** Six cases (7.5%) of MSI were identified in BAT 26 from 80 cancer tissues. All the stool DNA samples from patients whose cancer tissue showed MSI also displayed MSI in BAT 26.

**CONCLUSION:** As MSI is one of the established fecal DNA markers to screen colorectal cancer, we propose to use DHPLC for the MSI analysis in fecal DNA.



**Key words:** Colorectal cancer; Denaturing high performance liquid chromatography; Fecal DNA; Microsatellite instability; Stool

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## INTRODUCTION

Colorectal cancer, one of major causes of tumor-induced death in the Western population, becomes prevalent in Korea. The mortality rate from colorectal cancer has increased rapidly from 3.9 per 100 000 individuals in 1983 to 11.4 in 2003 in Korea[1]. Due to its orderly natural history, location within readily accessible organs, and high lifetime incidence, colorectal cancer is suitable for mass screening. It has been estimated that more than 50% of mortality due to colorectal cancer has been prevented through screening tests[2]. Fecal occult blood tests are non-invasive and useful, particularly as an adjunct to sigmoidoscopy[3]. However, the relatively high false positive rates and other problems have necessitated a search for more specific non-invasive tests. In this regard, assays for mutations in fecal DNA are particularly promising[4]. Following the initial identification of mutant K-*ras* in stools[5], many investigators have conducted molecular genetic analyses of stools. In view of the heterogeneity of mutations in colorectal cancer, multiple genetic targets have been screened. Dong *et al*[6] found that analysis of a combination of *p53*, BAT 26 and K-*ras* mutations in stools identified 71% cases of colorectal cancer. Ahlquist and colleagues[7] improved the detection sensitivity to 91% by using a panel consisting of *p53*, BAT 26, APC, and K-*ras*.

MSI is caused by a failure of the mismatch repair system to correct errors that occur during DNA replication. This phenomenon is characterized by the presence of novel alleles in DNA of cancer tissues that are absent in matched repetitive sequences in normal tissues designated 'microsatellites'[8,9]. The MSI status has notable biological significance. For instance, colorectal cancer patients with MSI show better prognosis[9-11]. Cancer detection via microsatellite analyses has been reported for several malignancies, including renal cell carcinoma[12], uterine cervical carcinoma[13] and head and neck squamous

cell carcinoma[14]. Koshiji *et al*[15] reported the results of MSI analysis of fecal DNA for colorectal cancer detection, while Traverso *et al*[16] demonstrated the practical application of the fecal BAT 26 assay with sigmoidoscopy.

In view of the importance of MSI analysis, these techniques required substantial improvement[17]. One particular concern was that the database would be limited if optimal high-throughput assays for running and interpreting microsatellite assays were not developed[18]. Thus, simple and automatic methods are required that make MSI analysis more systematic and convenient, and the many clinical approaches to cancer therapy feasible.

DHPLC has emerged as one of the most versatile technologies for the evaluation of genetic variations. The most patent advantage of this technique is the feasibility of automatic high-throughput analysis using computer-controlled systems. We have developed and reported a novel protocol for MSI analysis in cancer tissues that utilizes DHPLC [19]. In this study, we extended the protocol for MSI analysis using DHPLC to stool samples of patients with colorectal cancer.

## MATERIALS AND METHODS

### Patients

Eighty cancer tissues, taken from 27 patients with cancer of the proximal colon (i.e., between the cecum and splenic flexure) and from 53 patients with distal cancer were analyzed. Matched stool samples from the 80 patients were also analyzed. None of the patients investigated had familial adenomatous polyposis or hereditary nonpolyposis colorectal cancer. All cancer tissues and stool were collected from the Center for Colorectal Cancer, National Cancer Center, Korea. The tumor stage was determined using the American Joint Committee on Cancer TNM system[20]. All patients gave informed consent prior to entry into the study.

### DNA preparation

Genomic DNA was extracted from surgical specimens, using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's specifications. Stool samples were obtained before commencing laxative treatment to prepare for surgery or colonoscopy. Solid stools were weighed into 10 g lots, and stored at -70℃. After defrosting, we employed a QIAamp Stool Mini Kit (QIAGEN, Hilden, Germany) for DNA preparation. DNA was extracted according to the manufacturer's instructions. Briefly, 180-220 mg of stool was prepared in a 2 mL microcentrifuge tube, and 1.6 mL Buffer ASL was added for homogenization of samples. Following centrifugation at full speed for 1 min, supernatants were transferred a new 2 mL tubes, and one InhibitEX tablet was added. The tablet was thoroughly dissolved in the stool solution, followed by a second round of centrifugation for 3 min. The supernatant was transferred to a new 1.5 mL tube with 25 μL of proteinase K. AL buffer (600 μL) was added to the prepared solution, and incubated at 70℃ for 10 min. After the addition of 600 μL of ethanol, solutions (600 μL) were applied to the QIAamp spin column, and centrifuged three times. The column was washed with 500 μL of AW1 and AW2 and stool DNA samples eluted using 200 μL of AE buffer.

Primer sequences for amplifying microsatellite marker, BAT 26 were obtained from the GDB Human Genome Database (www.gdb.org). Stool DNA was initially amplified with the outer primers, forward, 5'-TTTAGGTTGCAGT TTCATCA-3', and reverse, 5'-ACCATTCAACATTTTT AACCC-3'. The outer PCR product (1 μL) was employed for a second inner round of amplification. Inner PCR primers for BAT 26 were follows as: forward primer, 5' -ACTGTCTGCGGTAATCAAGT-3'; and reverse primer, 5'-CCATTCAACATTTTTAACCC-3'. Amplification for DHPLC analysis was performed with 1 μL of the first PCR product, 1X PCR buffer supplemented with 1.5 m mol/L $MgCl_2$, 10 pmol/μL of primers, 50 μmol/L dNTPs (each), and 0.25 U of *Taq* polymerase (QIAGEN, Hilden, Germany) in a total volume of 25 μL. For heteroduplex formation, crude PCR products were denatured at 95℃ for 5 min, followed by gradual cooling from 95℃ to 25℃ over a period of 1 h.

### MSI analysis using DHPLC

DHPLC was performed with a fully automated system (WAVE, Transgenomic Inc., Omaha, NE, USA) as previously reported[19]. Amplified products were automatically injected into a DNA Seq® cartridge (Transgenomic Inc., Omaha, NE, USA), and eluted at a flow rate of 0.9 mL/min through a linear gradient of acetonitrile with 0.1 mol/L triethylammonium acetate (TEAA). Buffers A (0.1 mol/L triethylammonium acetate (TEAA) solution) and B (0.1 mol/L TEAA containing 25% acetonitrile) were automatically adjusted to obtain the best running conditions. The portion of Buffer A was 53% and Buffer B was 47%. The sample injection volume per analysis was 5 μL of PCR product. Regardless of the markers and samples, the running temperature was set to 50℃ for double-stranded DNA analysis in WAVEMAKER™ software (Transgenomic Inc., Omaha, NE, USA). The analysis of individual samples took an average of 9 min from sample injection to the finished result. UV detection was performed at 260 nm. DHPLC buffer conditions for the marker were automatically calculated. MSI analysis by DHPLC took 9 min per amplified DNA sample. Since no matched normal tissue DNA was required for quasimonomorphic markers of BAT 26, the MSI status was determined within 9 min. Results were analyzed using WAVEMAKER software (Transgenomic, Omaha, NE, USA.) in real time. No additional adducts (i.e., radioisotope or fluorescent-labeled primers) were employed other than the conventional PCR reaction, and no extra software was required.

### Statistical analysis

Statistical analysis was performed using the Pearson's Chi-square test, Fisher's exact test, or Student's *t* test, depending on the nature of the data. Two-tailed $P < 0.05$ was considered statistically significant.

## RESULTS

We evaluated the status of the microsatellite from surgical

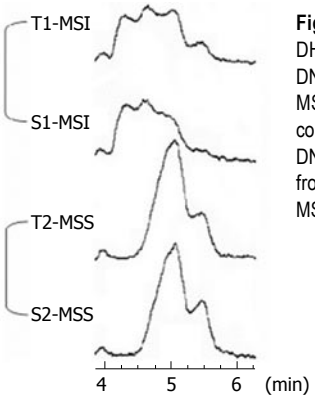


**Figure 1** MSI analysis in BAT 26 using DHPLC. Chromatograms of four different DNA samples are shown. T2-S2 displayed MSS, while T1-S1 exhibited MSI in both colorectal cancer tissue and stool DNA. T, DNA from colorectal cancer tissue: S, DNA from stool: MSI, microsatellite instability; MSS, microsatellite stability.

specimens of all 80 cancers by DHPLC. DNA of adequate quality was recovered from all the lesions. We identified six cases (7.5%) of MSI in BAT 26 from 80 cancer tissues, 4 of 27 proximal colon cancers and 2 of 53 distal colorectal cancers.

To determine the degree of concordance between BAT 26 alterations in cancer tissue and those in matched fecal DNA, we evaluated the status of the microsatellite from matched stool DNA samples of all 80 patients by nested PCR and DHPLC analysis. Seven cases of MSI in BAT 26 were identified from 80 stool DNA samples. Among 7 stool DNA samples with MSI, 6 stool samples corresponded to tissue MSI and the size of the BAT 26 alteration in the tumor and fecal DNA was identical in each patient (Figure 1). Cancer tissue from one proximal colon cancer did not display MSI, but displayed MSI repeatedly in the matched stool DNA.

## DISCUSSION

MSI induced as a result of failure of the mismatch repair system has significant clinical importance in the diagnosis and prognosis of colorectal cancer. Radioactive gel-based electrophoresis and fluorescent-labeled sequencing analysis have been used for MSI analysis. However, these methods have critical limitations for use in high-throughput analysis in a clinical setting. Thus, there is a need for improved MSI analysis techniques. We have developed a novel protocol for MSI analysis in cancer tissues that utilizes DHPLC[19]. In the proposed method, standardized experimental conditions facilitate robust, high-throughput MSI analysis, and consistent results are obtained with high specificity, regardless of sample type or individual researchers' skills. DHPLC can separate PCR products that differ by as little as a few base pairs[21]. This allows the size-dependent separation of double-stranded DNA fragments. In cases where there is a deletion in a microsatellite leading to a shorter allele, the resulting chromatogram is shifted to the left compared with that of the original allele. All the MSI samples in this investigation displayed left-shifted bands, indicating deletion alterations. The main advantage of DHPLC is that automatic high-throughput analysis is feasible using its computer-controlled systems[21].

The BAT 26 marker is employed as an indicator of microsatellite instability, since its mononucleotide tract is altered in nearly all mismatch-deficient tumors[22]. We initially attempted to amplify stool DNA using original BAT 26 markers, but failed to detect mutated DNA in DHPLC. Consequently, we devised a nested PCR analysis method by adding an outer amplification step. A comparison of the outer and inner PCR experiments disclosed that outer PCR followed by BAT 26 amplification led to the best results. Regarding the correlation between MSI status of stools and clinical data, MSI (+) status has been associated with proximal location[23]. Consistent with this, MSI (+) stools were significantly associated with proximal location in our data ($P = 0.040$).

Our data confirm the reliability of stool DNA for MSI analysis of colorectal cancers. Significantly, all 6 cases with BAT 26 mutations in their tissues displayed a positive stool DNA test. In one case with only MSI in stool DNA, it was possible that tumor heterogeneity influenced the result of tissue MSI. We used a part of tissue instead of whole tumor. Baisse *et al*[24] suggested that a molecular heterogeneity in tumors could modify MSI detection.

Main limitation of stool MSI was a low sensitivity. Colorectal cancers with MSI are less frequent even in the proximal colon compared with those without MSI. Using only MSI analysis is not suitable as a screening method. An analysis of a combination of several DNA alterations including MSI status using BAT 26 in stools improved the sensitivity over 90%[7].

In this work, we have extended the DHPLC method for MSI analysis to stool DNA and verified the reliability of stool DNA in the MSI analysis for one of the multiple markers of the genetic screening of colorectal cancers. As MSI is one of the established fecal DNA markers to screen colorectal cancer, we propose to use DHPLC for the MSI analysis in stool DNA.

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*RAPID COMMUNICATION*

# Clinical significance of hepatitis B e antigen level measurement during long-term lamivudine therapy in chronic hepatitis B patients with e antigen positive

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## Abstract

**AIM:** To determine the changes of quantitative hepatitis B e antigen (HBeAg) that predicts early detection of non-response or breakthrough to long-term lamivudine (LAM) therapy.

**METHODS:** Among HBeAg positive chronic hepatitis B patients who failed to achieve HBeAg seroconversion within 12 mo, we retrospectively analyzed 220 patients who had received LAM more than 24 mo.

**RESULTS:** The mean duration of LAM therapy was 36 (range, 24-72) mo. HBeAg seroconversion after the first 12 mo of LAM therapy was achieved in 53 (24.1%) patients. Viral breakthrough was observed in 105 (47.7%) patients. To find out whether the changing patterns of HBeAg levels can predict the outcome of LAM therapy, we analyzed the reduction rates of HBeAg levels during LAM therapy. Using the decrease more than 90% of pretreatment HBeAg levels, the sensitivity and specificity of response were 96.2% and 70.1%, respectively. Patients were divided into 3 groups according to the reduction patterns of the decrease of quantitative HBeAg: decrescendo, decrescendo-crescendo, no change or fluctuating groups. The optimal time to predict non-response or breakthrough was the first 9 mo of therapy. At 9 mo of therapy, 49 (92.5%) of 53 patients who had achieved HBeAg seroconversion were included in the decrescendo group. On the contrary, in the no change or fluctuating group, only four (7.5%) had achieved HBeAg seroconversion. Among patients who did not show the continuous decrease of HBeAg levels at 9 mo, 95.2% (negative predictive value) failed to achieve HBeAg seroconversion.

**CONCLUSION:** Almost all patients who failed to show a continuous decrease of HBeAg levels at 9 mo of LAM therapy were non-response or breakthrough. Therefore, monitoring changes of HBeAg levels during LAM therapy in HBeAg positive chronic hepatitis B may be valuable for identifying patients who are at high risk of non-response or breakthrough.



**Key words:** Hepatitis B e antigen positive chronic hepatitis B; lamivudine; Quantitative HBeAg levels; Non-response; Breakthrough

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## INTRODUCTION

Hepatitis B virus (HBV) infection is a serious public health problem worldwide and a major cause of chronic hepatitis, cirrhosis, and hepatocellular carcinoma[1]. Of the approximately 2 billion people who have been infected worldwide, more than 400 million are chronic carriers of HBV[2]. The goals of therapy in patients with HBV are to limit or reverse progression of disease through sustained suppression of HBV replication[3]. This is usually achieved through treatment-induced suppression of HBV DNA and through hepatitis B e antigen (HBeAg) seroconversion in HBeAg-positive chronic hepatitis B (CHB) patients[4,5]. Clinical trials for LAM have shown it to be effective in HBV DNA suppression, alanine aminotransferase (ALT) normalization, HBeAg seroconversion and for reducing liver inflammation and the progression of hepatic fibrosis and cirrhosis[4,6-9]. Recently, lamivudine (LAM) treatment has shown to prevent the emergence of hepatocellular carcinoma in patients with HBV[10]. However, the optimal duration of LAM therapy is not well established. In view of the long half-life of covalently closed circular DNA (cccDNA) and the variable turnover of infected hepatocytes[11], long-term treatment beyond 1 year is required to achieve complete HBV elimination[12].

Moreover, the HBeAg seroconversion rates gradually continue to increase with extended LAM therapy[5,8,9,13-15]. Unfortunately, long-term LAM therapy is associated with progressively increasing rates of viral resistance due to mutations within YMDD motif of the HBV DNA polymerase[9,13,14,16]. The incidence of viral resistance to LAM therapy is approximately 20% per year reaching approximately 70% of patients after 5 years of therapy[17]. The initial benefits of treatment with LAM seem to be lost among patients who develop YMDD mutations[4,14]. Nevertheless, acute exacerbation of hepatitis may occur after the emergence of YMDD mutant[18,19]. Fatal hepatic failure after emergence of YMDD mutant has also been reported[20,21]. Currently, the appearance of YMDD mutants during LAM therapy is considered an indication for switching or adding the other approved oral agents, adefovir dipivoxil (ADV) or entecavir.

Earlier identification of non-virologic response or viral breakthrough facilitates the change to other drugs or the addition of a second agent, and could help reduce considerably viral breakthrough and treatment cost in patients who can not get HBeAg seroconversion as well. Moreover, this goal would prevent premature discontinuation of treatment in those who would ultimately have achieved HBeAg seroconversion. However, few investigations have been made about the factors that identify earlier the non-response or breakthrough to LAM treatment in HBeAg positive chronic hepatitis B patients.

Compared with qualitative measurements, quantitative HBeAg levels are sensitive, with a high reproducibility and a wide dynamic range and easy to perform[22,23]. Quantification of HBeAg levels have already been found useful in several studies in monitoring and predicting the outcome of interferon treatment[22-26]. More recently, in our previous study, the changing patterns of quantitative HBeAg levels by serial monitoring during LAM therapy may allow not only the prediction of treatment responses, but also an early recognition of a viral breakthrough[27]. Moreover, viral breakthrough by the turning time in the decrescendo-crescendo group was an earlier detection than that in HBV DNA monitoring by a hybridization assay[27].

The aim of this study was to determine the patient-dependent or laboratory variables that predict earlier identification of virologic non-response or breakthrough in patients who had not achieved seroconversion by 12 mo of therapy. Especially, the change of quantitative HBeAg over time during LAM treatment may be valuable for earlier identifying patients who are at high risk of non-response or breakthrough.

## MATERIALS AND METHODS

### Patients

Among HBeAg positive naïve CHB patients who had not achieved HBeAg seroconversion by 12 mo of LAM therapy, we retrospectively analyzed 220 patients (176 men and 44 women; mean age, 38.6 ± 10.2 years) who had received LAM for more than 24 mo. All patients had high upper limit of normal (ULN) serum ALT levels, as well as hepatitis B surface antigen (HBsAg), and HbeAg. The HBV DNA in serum was documented for at least 6 mo before the start of LAM therapy. No patient had a history of previous interferon or nucleoside analogues therapy. None of the patients had clinical cirrhosis, and all were excluded from having hepatitis C, D, HIV infection and autoimmune hepatitis. The diagnosis of CHB was based on histological examination for 84 patients. The remaining 136 patients were diagnosed clinically. The clinical criteria for CHB were elevated serum ALT levels over 6 mo, the absence of clinical evidence of portal hypertension and imaging features suggestive of cirrhosis. Written informed consent was obtained from all patients participating in this study, and the study was approved by the Institutional Review Boards at the Ulsan University Hospital.

### Methods

Serum HBeAg, anti-HBe, HBV DNA and ALT were measured every 1 or 3 mo until HBeAg seroconversion. HBV DNA levels were measured with a hybridization capture assay with the lower limit of detection at 0.5 ng/L (Digene Hybrid Capture Ⅱ, Gaithersburg, MD, USA). Serum quantitative HBeAg and anti-HBe levels were measured by microparticle enzyme immunoassay (AxSYM, Abbott, Chicago, IL, USA). The AxSYM assay calculated a result based on the ratio of the sample rate (S) to the cutoff (CO) for each sample and control. Samples with S/CO value less than or equal to 1.0 were considered negative for HBeAg. In this study, HBeAg seroconversion was defined as the loss of HBeAg accompanied with detection of anti-HBe. Response to therapy was defined as a simultaneous HBeAg seroconversion and HBV DNA negativity on two occasions at least one month apart. A non-response to therapy was defined as persistent presence of HBV DNA during treatment. A viral breakthrough was considered as the reappearance of HBV DNA in serum on two or more occasions after its initial disappearance.

### Statistical analysis

All data was analyzed using the statistical package SPSS (version 12.0: SPSS Inc., Chicago, IL, USA). To identify favorable factors of response among pretreatment variables, we compared variables for response and non-response or breakthrough using $\chi^2$ test or using univariate logistic regression. Cut-off levels of reduction rate of quantitative HBeAg values were determined by univariate receiver operating characteristics (ROC) analysis. The cumulative rates of HBeAg seroconversion and viral breakthrough were calculated by the Kaplan-Meier method. In all cases, a 2-tailed *P* value less than 0.05 was considered statistically significant.

## RESULTS

### Treatment outcome

Baseline demographic and clinical features of the 220 patients who had not achieved HBeAg seroconversion by 12 mo of therapy are shown in Table 1. The mean duration of LAM therapy was 36.4 ± 10.5 (range, 24-72) mo. HBeAg seroconversion after 12 mo of LAM therapy was achieved in 53 (24.1%) patients. The cumulative rates of HBeAg seroconversion at 18, 24, 36, 48, and 60 mo were 9%, 15%, 24%, 31%, and 37%, respectively. Viral

**Table 1 Patient characteristics at baseline and response to LAM therapy ($n$ = 220)**

| Patient characteristics | |
|---|---|
| Age (yr) | 38.6 ± 10.2 (16-68) |
| Sex (M/F) | 176:44 |
| ALT (IU/L) | 232 ± 108 (41-1269) |
| < 2 × ULN | 24 (10.9%) |
| ⩾ 2- < 5 × ULN | 112 (50.9%) |
| ⩾ 5- < 10 × ULN | 52 (23.6%) |
| ⩾ 10 × ULN | 32 (14.6%) |
| AST (IU/L) | 145 ± 126 (35-880) |
| HBV DNA (ng/L) | |
| Mean ± SD, $\log_{10}$ | 2.35 ± 1.07 (0.08-5.00) |
| HBeAg level (S/CO) | 256 ± 161 (10.4-1858) |
| Response | 53 (24.1%) |
| Breakthrough | 105 (47.7%) |
| Non-response | 62 (28.2%) |

Data are expressed as mean ± SE (range) unless otherwise stated. ULN: upper limit of normal; S/CO: sample rate/cutoff rate.

**Table 2 Comparative results according to changing patterns of HBeAg levels during LAM therapy**

| | Response | Non-response or breakthrough | Odds ratio (95% CI) | Sensitivity | Specificity | PPV[1] | NPV[2] |
|---|---|---|---|---|---|---|---|
| At 6 mo | | | 4. 8 (2.2-13.5) | 83 | 49.7 | 34.4 | 90.2 |
| D[3] | 44 | 84 | | | | | |
| N[4] | 9 | 83 | | | | | |
| D-C[5] | 0 | 0 | | | | | |
| At 9 mo | | | 11.0 (3.8-32.0) | 92.5 | 47.9 | 36 | 95.2 |
| D | 49 | 87 | | | | | |
| N | 4 | 67 | | | | | |
| D-C | 0 | 13 | | | | | |
| At 12 mo | | | 10.5 (3.6-30.5) | 92.5 | 46.7 | 35.5 | 95.1 |
| D | 49 | 89 | | | | | |
| N | 2 | 60 | | | | | |
| D-C | 2 | 18 | | | | | |

[1]Positive predictive value. The proportion of patients achieving HBeAg seroconversion among those with decrescendo pattern of HBeAg levels at each time after initiation of therapy; [2]Negative predictive value. The proportion of patients without HBeAg seroconversion among those with the decrescendo-crescendo and no change or fluctuating patterns during treatment; [3]Decrescendo pattern. Continuously decreasing HBeAg levels by more than 90% of pretreatment values; [4]No change or fluctuating pattern. No change or fluctuation of HBeAg levels during treatment; [5]Decrescendo-crescendo pattern. A continuous decrease of HBeAg levels by more than 90% of pretreatment values, and then progressively increasing.

breakthrough was observed in 105 (47.7%) patients at a mean of 19.8 ± 9.5 (range, 7-60) mo after the start of LAM therapy. The cumulative breakthrough rates at 24, 36 and 48 mo were 39%, 49% and 54%, respectively.

### *Changing patterns of quantitative HBeAg levels during therapy as predictive factors for non-response or breakthrough*

To find out whether the changing patterns of HBeAg levels at different times (6, 9, and 12 mo) of therapy can predict non-response or breakthrough, we analyzed the reduction rates of HBeAg levels by serial monitoring during LAM therapy. The cut-off values of reduction rate of HBeAg levels based on ROC curves provided the maximal test efficacy, as defined by the sum of sensitivity and specificity. Therefore, the decrease more than 90% of pretreatment HBeAg levels was chosen to significantly improve the specificity, without reducing the sensitivity, and was adopted as the cut-off level for subsequent subgroup analysis. Using the decrease more than 90% of pretreatment HBeAg levels, the sensitivity and specificity of non-responders were 96.2% and 70.1%, respectively. All the responders, except one, showed the decrease more than 90% of the pretreatment HBeAg values for the maximal reduction rate during LAM therapy. Patients were divided into 3 groups according to reduction patterns, as compared to the pretreatment HBeAg levels, at different times (6, 9, and 12 mo) of LAM therapy: (1) a continuous decrease of HBeAg levels to more than 90% of pretreatment values (decrescendo group), (2) a continuous decrease of HBeAg levels to more than 90% of pretreatment values, and then progressively increasing (decrescendo-crescendo group), (3) no change or fluctuation of HBeAg levels (no change or fluctuating group). The calculation of predictive values of response to therapy was performed using reduction patterns of quantitative HBeAg levels during LAM therapy. A positive predictive value (PPV) at different times of LAM treatment was defined as the proportion of patients achieving HBeAg seroconversion among those with the decrescendo pattern of HBeAg levels each time after the initiation of therapy. A negative predictive value (NPV) was defined as the proportion of patients without HBeAg seroconversion among patients with the decrescendo-crescendo and no change or fluctuating patterns during LAM treatment. Detailed analysis of reduction patterns of quantitative HBeAg at different times had revealed that sensitivity was excellent from 83.0% to 92.5%, while specificity was poor from 46.7% to 49.7% (Table 2). The early prediction of non-responder that optimized capture of potential responders (highest sensitivity) while excluding the largest proportion of non-response or breakthrough (highest negative predictive value) was the changing patterns of HBeAg levels at 9 and 12 mo of treatment (Table 2). Although the changing patterns of HBeAg levels at 9 and 12 mo had similar results, because of increasing rate of LAM mutants and treatment cost, it is desirable to define diagnostic markers that can allow identification of viral non-response or breakthrough as early as possible. Thus, the optimal time to predict non-response or viral breakthrough is month 9 of LAM treatment. According to the changing patterns of HBeAg levels at 9 mo of LAM therapy, the numbers of decrescendo, no change or fluctuating, and decrescendo-crescendo groups were 136, 71, and 13, respectively. Forty-nine (92.5%) of 53 patients who had achieved HBeAg seroconversion were included in the decrescendo group. On the contrary, from the no change or fluctuating group, only 4 (7.5%) had achieved HBeAg seroconversion, and remained patients were non-response or breakthrough. All patients in the decrescendo-crescendo group were non-response or breakthrough. Ultimately, not all patients who had the decrescendo pattern of HBeAg levels achieved HBeAg

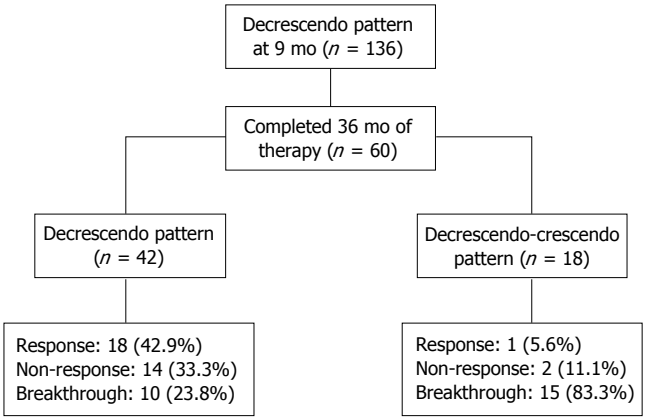


**Figure 1** Clinical courses of HBeAg positive chronic hepatitis B patients who were treated more than 36 mo among 136 patients in decrescendo group at 9 mo of lamivudine treatment. Decrescendo pattern, continuously decreasing HBeAg levels by more than 90% of pretreatment values over time; Decrescendo-crescendo pattern: continuous decrease by more than 90% of pretreatment values, and then progressively increasing.

seroconversion. The PPV at 9 mo was only 36.0%. Among the 136 patients with the decrescendo pattern of HBeAg levels at 9 mo, 106 patients failed to achieve HBeAg seroconversion within 24 mo of treatment. Of these 106 subjects, 60 received repeated quantitative HBeAg level testing at month 36 (Figure 1). According to the changing patterns of HBeAg levels at 36 mo after the start of LAM therapy, there were 42 (70.0%) and 18 (30.0%) patients in the decrescendo and decrescendo-crescendo groups, respectively. HBeAg seroconversion after 24 mo of LAM therapy was achieved in 19 (31.7%). Eighteen (94.7%) of these 19 patients were included in the decrescendo group. Only one of 18 patients in the decrescendo-crescendo group had achieved HBeAg seroconversion.

## DISCUSSION

Most HBeAg positive patients do not have a virologic response after 1 year of LAM treatment, and prolonged treatment beyond 1 year is often necessary. Treatment may be continued in patients who fail to achieve HBeAg seroconversion and have no evidence of viral breakthrough as HBeAg seroconversion may occur with continued treatment. However, treatment beyond 1 year has not been adequately investigated and the benefits of continued treatment must be balanced against the risks of resistant mutants. The current practice for CHB patients with YMDD mutants is options of continuing or stopping LAM or converting to other antiviral drug when LAM resistance develops, depending on the patient's clinical state, ALT and HBV DNA levels[3,12,28]. However, recently, resistance against ADV has been observed as well[29]. Moreover, the emergence of ADV mutation appeared to present earlier and more frequent in LAM-resistant patients than in nucleoside/-tide treatment naïve patients and was associated with reduced antiviral efficacy to ADV therapy[30,31]. In addition, LAM resistance leads to selection pressure for HBV with additional mutations in the DNA polymerase gene that reduces the susceptibility to entecavir[32]. Therefore, earlier prediction of non-response or breakthrough in the course of treatment would not only help reduce the incidence of HBV mutants and treatment cost, but would also allow alternative treatment options to be pursued sooner in the course of treatment. Such goal helps maximize the proportion of patients achieving response, which minimizes emergence of mutant virus in patients who might be ultimately non-response during continuation of LAM therapy.

Serum HBeAg, anti-HBe, and HBV DNA are currently the most important markers for assessing the response to antiviral therapy in patients with HBeAg positive CHB. HBeAg is used typically as a qualitative serological marker for diagnosing the virologic response in HBeAg positive CHB. Therefore, serum quantitative HBeAg levels may reflect levels of the cccDNA in hepatocytes because the mRNAs of HBeAg are transcribed from the cccDNA[33-36]. It is conceivable that patients with decreasing HBeAg levels are on the way to HBV clearance, and reflect the process towards HBeAg seroconversion. The absence of decline for quantitative HBeAg levels may indicate the persistence of ongoing viral replication, despite the antiviral treatment. Furthermore, a resurgence of quantitative HBeAg levels during antiviral therapy may be explained by the reappearance of wild type strains or emergence of mutant strain[27]. To date, available information on the association between the changing patterns of HBeAg levels and early prediction of viral non-response or breakthrough to LAV therapy in patients with HBeAg positive CHB is limited. In the present study, we have retrospectively investigated the utility of monitoring of HBeAg level changes to predict treatment outcomes in naive HBeAg positive CHB patients who had not achieved HBeAg seroconversion by 12 mo of therapy. Based upon the predictive values of a positive test for virologic non-response or breakthrough (NPV), the optimal time to earlier predict non-response or breakthrough is recommended to be month 9 of treatment. Forty-nine (92.5%) of 53 patients who had achieved HBeAg seroconversion after 12 mo of LAM therapy were included in the decrescendo group. On the contrary, from the no change or fluctuating group, only four (7.5%) had achieved HBeAg seroconversion and the remained patients were non-response or breakthrough. All patients in the decrescendo-crescendo group were non-response or breakthrough. That is, almost all patients who did not show a continuous decrease of HBeAg levels at 9 mo during LAM therapy were non-response or breakthrough. That is, among patients who did not have the decrescendo pattern of HBeAg levels at 9 mo during LAM therapy, 95.2% (NPV) failed to achieve HBeAg seroconversion. These patients have very little chance of attaining virologic response even if an additional therapy is administered, and thus it is recommended that other nucleoside analogues or the combination with another antiviral agents be used. However, not all patients who had the decrescendo pattern of HBeAg levels at 9 mo of therapy ultimately achieved HBeAg seroconversion. Positive prediction of virologic response during therapy remains disappointing on the basis of the data available. Indeed, the positive predictive value at 9 mo was only 36.0%. In addition, from 60 patients who were treated more than 36 mo among 136 patients of the decrescendo group at 9 mo of treatment, 19 (31.7%)

had achieved response. Eighteen patients (94.7%) were included in the decrescendo pattern of HBeAg levels at 36 mo of treatment. Only one of responders was included in the decrescendo-crescendo group at 36 mo of treatment. That is, if patient had the decrescendo pattern of HBeAg levels at 9 mo, further treatment should be continued, and serial monitoring of HBeAg levels during treatment would be necessary.

It is therefore imperative that HBeAg levels and HBV DNA are measured during LAM therapy to monitor the response to therapy and facilitate the early detection of drug-resistant strains. HBV DNA testing by PCR and mutant genotyping are very sensitive methods in detecting viral breakthrough during LAM therapy. Unfortunately, our study did not perform the quantitative PCR and assay for YMDD mutants. Also, HBV DNA monitoring by a hybridization assay has a rather low sensitivity, and it was expected to lag behind in the detection of viral breakthrough, as compared to quantitative PCR. Therefore, further studies are needed to compare the quantitative decrease in HBeAg with the HBV DNA level measurement by the sensitive PCR assay. However, these tests do not predict outcomes of treatment before the emergence of HBV mutants. Nevertheless, this is a very important practical point to clinicians because a quantitative HBeAg level test is easy to measure, less expensive, with a wider dynamic range than other molecular tests. Most importantly, it can identify the changing patterns of HBeAg levels during treatment that have the potential to serve as predictors of long-term drug response to LAM therapy. For patients with the decrescendo pattern of HBeAg levels during LAM therapy, continuous LAM therapy without rescue treatment could be expected for achievement of HBeAg seroconversion. On the contrary, for patients who had the decrescendo-crescendo and the no changing or fluctuating patterns of HBeAg levels at 9 mo of LAM therapy, most patients were viral non-response or breakthrough, and HBeAg seroconversion was rarely developed. Therefore, these patients should be monitored more strictly. In the near future, to prevent the development of breakthrough hepatitis, other antiviral agents should be applied when the decrescendo-crescendo pattern and the no changing or fluctuating of HBeAg levels at 9 mo is observed during LAM therapy.

In conclusion, almost all patients who failed to show the continuous decrease of HBeAg levels at 9 mo of LAM therapy were non-response or breakthrough. On-treatment prediction of no-response or breakthrough to long-term LAM therapy in HBeAg positive CHB can be predicted early at mo 9 of treatment based on the changing patterns of HBeAg levels. That is, monitoring of serum quantitative HBeAg levels, in addition to measurement of HBV DNA by PCR, is helpful for evaluating the response to LAM treatment and for the early detection of non-response or LAM-resistant strains as well.

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*RAPID COMMUNICATION*

# Carcinoid tumor of the appendix: A consecutive series from 1237 appendectomies

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## Abstract

**AIM:** To report the experience of the CHU Sart Tilman, University of Liège, Belgium, in the management of appendiceal carcinoid tumor.

**METHODS:** A retrospective review of 1237 appendectomies performed in one single centre from January 2000 to May 2004, was undertaken. Analysis of demographic data, clinical presentation, histopathology, operative reports and outcome was presented.

**RESULTS:** Among the 1237 appendectomies, 5 appendiceal carcinoid tumors were identified (0.4%) in 4 male and 1 female patients, with a mean age of 29.2 years (range: 6-82 years). Acute appendicitis was the clinical presentation for all patients. Four patients underwent open appendectomy and one a laparoscopic procedure. One patient was reoperated to complete the excision of mesoappendix. All tumors were located at the tip of the appendix with a mean diameter of 0.6 cm (range: 0.3-1.0 cm). No adjuvant therapy was performed. All patients were alive and disease-free during a mean follow-up of 33 mo.

**CONCLUSION:** Appendiceal carcinoid tumor most often presents as appendicitis. In most cases, it is found incidentally during appendectomies and its diagnosis is rarely suspected before histological examination. Appendiceal carcinoid tumor can be managed by simple appendectomy and resection of the mesoappendix, if its size is $\leqslant$ 1 cm.



**Key words:** Surgery; Digestive cancer; Laparoscopy;

Review

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## INTRODUCTION

The appendix is one of the most common single site for carcinoid tumor[1]. Histopathologically, appendiceal carcinoid tumor is mostly enterochromaffin (EC) cell type and derives from a subepithelial cell population, which is different from neuroendocrine tumor in other sites[2]. Although rare and usually detected incidentally in appendectomy, it is considered the most common type of appendiceal primary malignant lesion, and is found in 0.3%-0.9% of patients undergoing appendicectomy[3]. This tumor rarely presents with metastases[4,5]. Despite the fact that it is among the most frequently occurring carcinoids, in surgical practice most surgeons may encounter only one or two such lesions during their career[6]. Therefore, it is important to define a correct management of such a rare tumor. The authors report here a series of 5 appendiceal carcinoid tumors found during appendectomy in a single centre and compared this experience with the recent literature on this subject.

## MATERIALS AND METHODS

A retrospective review of all appendectomies was performed from January 2000 to May 2004 in a single department of abdominal surgery in a tertiary referral center (CHU Sart Tilman, University of Liège, Belgium). The data of the patients who were histologically reported to have carcinoid tumor of the appendix were further reviewed for demographic characteristics, clinical presentation, histopathology, operative reports and follow-up.

## RESULTS

A total of 1237 patients underwent appendectomy during the study period (991 for acute appendicitis or appendicular syndrome and 246 during other abdominal procedures). Out of these 1237 patients, 5 (0.4%) were found to have

histological evidence of carcinoid tumor of the appendix. There were 4 male and 1 female patients with a mean age of 29.2 years (range: 6-82 years). Acute appendicitis was the clinical presentation for all patients. Open appendectomy was performed in 4 patients and laparoscopic appendectomy in one. Histologically, all tumors were located at the tip of the appendix with a mean diameter of 0.6 cm (range: 0.3-1.0 cm). One patient was reoperated two months later to complete the excision of mesoappendix after histological analysis. All patients were alive and disease-free during a mean follow-up of 33 mo (range: 21-49 mo). Our results are summarized in Table 1.

**Table 1 Characteristics of 5 patients with appendiceal carcinoid tumor**

| | |
|---|---|
| Incidence (compared to appendectomy) | 0.4% |
| Sex ratio (M/F) | 4/1 |
| Mean age (range) (yr) | 29.2 (6-82) |
| Location | Tip of the appendix (100%) |
| Mean diameter (range) (cm) | 0.6 (0.3-1.0) |
| Clinical feature | Appendicitis |
| First procedure | Appendectomy |
| Second procedure | Resection of mesoappendix (1/5) |
| Mean follow-up (range) (mo) | 33 (21-49) |

## DISCUSSION

Appendiceal carcinoid tumor is a unique carcinoid tumor and differs from those encountered elsewhere in the gastro-intestinal system characterized by a relatively common frequency, small size of the appendix, usually indolent behavior, occurrence in younger patients and a trend towards female predominance[6,7]. It accounts for 32%-57% of all appendiceal tumors[6,8] in patients with a reported mean age of 42 years[9]. Surprisingly, there is a male predominance in our series (male/female: 4/1). Appendiceal carcinoid tumor lacks specific clinical features and its clinical presentation may not differ from that of acute appendicitis. It is usually diagnosed incidentally during surgery for acute appendicitis and occasionally during other abdominal procedures (colectomy, cholecystectomy, salpingectomy)[5,10]. In our series, the diagnosis of all patients with symptoms of acute appendicitis was made only after histological analysis of the surgical specimen. Appendiceal carcinoid tumor exhibits little metastatic potential and therefore rarely presents with metastases[4,5]. Characteristics of the tumor predicting aggressive behavior include size, histological subtype and mesoappendiceal involvement[3]. The predictive value of tumour size is supported by many studies[4,11,12]. The calculated risk of metastasis from tumors $\leqslant$ 1 cm is zero, while a definite increase of risk occurs with tumor size $\geqslant$ 2 cm, the rate of metastasis ranges from 20%[13] to almost 85%[14]. In the present study, all the tumours were less than 1cm (mean diameter of 0.6 cm) and localized at the tip of the appendix with no evidence of regional or distant metastases. Appendiceal carcinoid tumor usually metastasizes to the regional lymph nodes rather than to the liver[15,16]. Carcinoid syndrome occurs in less than 10% of patients with carcinoid tumor[17]. Clinically, this syndrome develops when vasoactive substances produced by carcinoid tumor escape hepatic degradation and gain access into the systemic circulation. Its clinical features include cutaneous flushing, bronchoconstriction, diarrhea, and right-sided cardiac valvular fibrosis. This syndrome can be seen in appendiceal carcinoid tumor patients with liver metastases (< 2% of all appendiceal carcinoid tumors)[10].

The vast majority of appendiceal carcinoid tumor patients do not require any further procedure or investigation following appendectomy. Those who may benefit from additional screening are patients with high-grade malignant tumor though smaller than 1 cm, patients with tumor of between 1 cm and 2 cm or larger than 2 cm, and patients with incomplete resections and metastatic disease[3]. Plasma chromogranin A is the currently available most accurate blood marker, with its level raised in 80%-100% of neuroendocrine tumor patients[18,19]. Other investigations include 24-h urinary levels of 5-hydroxyindoleacetic acid, computed tomography and $^{111}$In-labelled octreotide scintigraphy.

Appendix tumor less than 1 cm in diameter is unlikely to be metastatic and may be managed with simple appendectomy. Our five appendiceal carcinoid tumor patients underwent appendectomy. Reoperation was performed in 1 patient two months later to complete the resection of mesoappendix, since histological analysis of the surgical specimen showed an incomplete mesoappendix. Therefore, consideration should be given to perform a complete mesoappendix resection during appendectomy. Appendiceal carcinoid tumor greater than 2 cm in size demonstrates an increased incidence of regional and distant spread and should be managed with a formal right hemicolectomy[17]. Controversy exists over the management following appendectomy, especially with regard to the role of right hemicolectomy in patients with tumor 1 to 2 cm in size[3]. Acceptable indications for right hemicolectomy in controversial cases have been recently suggested by Goede *et al*[3], including histological evidence of mesoappendiceal extension, tumor at the base of the appendix with positive margins or involvement of the caecum, high-grade malignant carcinoid tumor with a raised tumor prognostic index as measured by mitotic index and Ki67 levels.

The management of metastatic tumors and carcinoid syndrome has focused on cytoreductive chemotherapy and pharmacologic control of the bioactive substances produced by these tumors[17]. Response rates less than 40% have been reported for combined chemotherapy with streptozotocin and 5-fluorouracil or doxorubicin but the response time is short and chemotherapy regimen has significant side effects[20]. Thus, chemotherapy is not the first choice of treatment for patients with carcinoid tumor. Its use has to be discussed in a multidisciplinary approach related to other available treatments. The limited efficacy of systemic chemotherapy underlines the need to restrict its use in progressive cases or in patients with uncontrolled hormone-mediated symptoms despite biological or local treatment[20,21]. Octreotide, a somatostatin analog, is the most effective pharmacologic agent available to improve symptoms associated with carcinoid syndrome with a clinical and biochemical response rate of up to 60%. Available

data on growth control indicate that stabilisation of tumor growth seems to be the most beneficial anti-proliferative effect occurring in up to 50% of patients with slowly growing tumor. Partial tumor regression is marginal and occurs in only 3%-5% of cases. The long-acting somatostatin analogs are actually preferred[22].

Several studies on the antiproliferative effect of interferon-$\alpha$ in metastatic neuroendocrine tumor patients have reported that index hormone is reduced more than 50% in 40%-60% of patients with concomitant improvement of flushing and diarrhea. Stabilisation of tumor growth is observed in 20%-40% and a reduction in tumour size in 12%-15% of patients[23,24]. However, this therapeutic gain must be outweighed by the frequency and severity of toxic reactions[25]. Other treatment modalities such as local therapy and tumor-targeted radiotherapy are available for metastatic carcinoid tumor. Hepatic arterial chemoembolization (HACE) for non-resectable diffuse liver metastases could represent a good therapeutic option if the disease is not under control with other available treatment modalities[26,27]. HACE can be sued as a first line treatment. By targeting somatostatin receptor-positive tumor, it is possible to deliver a tumoricidal dose of radiation with $^{111}$In or $^{90}$Y coupled to somatostatin analogs. Preliminary studies in end-stage neuroendocrine tumor patients have shown promising results[28,29]. This treatment might be effective in patients refractory to conventional strategies and merits further development.

The prognosis of appendiceal carcinoid tumor is much better than midgut carcinoid tumor[17]. The 5-year survival rate of patients with local disease is reported to be 92% and 81% of those with regional metastases, and 31% of those with distant metastases, respectively[1]. All the patients in our series were alive and disease-free during a mean follow-up of 33 mo.

In summary, appendiceal carcinoid tumor occurs most often as acute appendicitis. In most cases, it is found incidentally during appendectomies and its diagnosis is rarely suspected before histological examination. Although appendiceal carcinoid tumor has an excellent overall prognosis, consideration should be given to screening this group of patients, since the frequency of associated synchronous and metachronous colorectal cancer is high (13%-33%)[1,4,6,8].

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*RAPID COMMUNICATION*

# Hepatitis B virus genotypes in chronic liver disease patients from New Delhi, India

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## Abstract

**AIM:** To study the Hepatitis B virus (HBV) genotypes and their effect on the progression and outcome in patients with chronic liver diseases from New Delhi, India.

**METHODS:** Sera from 100 HBV-related chronic liver disease (CLDB) cases were tested for HBV genotype using Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) and Type-specific primers-based PCR (TSP-PCR) targeting to the surface (S) gene encoding hepatitis B surface antigen.

**RESULTS:** Only genotypes A and D were present and genotype D was dominant. Genotype D was present in all CLDB patient categories. The genotype distribution for the 100 patients with CLDB was as follows: genotype A, 16/100 (16%) (7/40- 17% chronic hepatitis B (CHB); 8/47, 17%, HBV-related cirrhosis (CRB); 1/13, 7.6%, HBV-related hepatocellular carcinoma (HCCB); genotype D- 84/100 (84%) (32/40- 80% CHB; 38/47- 81%, CRB; 11/13, 85%, HCCB); genotype A + D, 3/100 (3%) (1/40- 3% CHB; 1/47- 2%, CRB; 1/13, 7.6%, HCCB); C, 0; B, 0; E, 0; F, 0; G 0, H 0; ($P < 0.01$, genotype D *vs* A).

**CONCLUSION:** Only HBV genotypes A and D were present in patients with CLDB from New Delhi, India. Compared with genotype D, genotype A patients had no significant clinical or biochemical differences ($P > 0.05$). Mixed infection with genotype A and D were seen in 3% of the cases. Genotype D was the dominant genotype prevalent in all patient categories.



**Key words:** HBV-related chronic liver disease; Hepatitis B virus genotypes; PCR-RFLP; Type-specific primer-based PCR

## INTRODUCTION

Approximately, two billion people in the world have been infected by Hepatitis B virus (HBV), 350 million of whom are chronic carriers of the virus[1,2]. Worldwide HBV isolates have been classified into eight genotypes: A, B, C, D, E, F, G and H[1,3]. The eight genotypes have a characteristic geographical distribution[1]. Several studies have revealed the association of HBV genotypes with the severity of chronic liver disease, but the results are not consistent[1, 4-6]. In Europe, most patients with genotype D are reported to have acute hepatitis B, while most patients with genotype A have chronic hepatitis B[5]. In northern Taiwan, 52% of asymptomatic carriers have genotype B, while 60% of patients with cirrhosis harbor genotype C; genotype B is more common in patients less than 50 years of age with HCC[7]. In Japan, HCC patients with genotype B younger than 60 years of age are rare[8]. Thus, the influences of HBV genotypes on clinical outcomes need to be clarified. Recent studies have shown that virological characteristics and clinical manifestations may differ even among HBV isolates of the same genotype[1]. Two subgroups of genotype B can be differentiated: a Japanese subgroup Bj with no indication for recombination events and an Asian subgroup called Ba which shows a recombination of the precore and core region from HBV genotype C. Response to antiviral therapies and the prevalence of hepatitis B e antigen (HBeAg) differ among patients with chronic liver diseases who are infected with HBV/Ba and HBV/Bj. Also, among isolates of HBV genotype A (HBV/A), two subtypes have been reported, one of which is distributed widely in European countries and the USA (Subtype A), while the other prevails in sub-Saharan Africa (Subtype A'). Subtype A' seems to be virologically distinct from the original genotype A and is associated with reduced serum levels of HBV DNA and a low capacity to encode HBeAg. In addition, subtype A' tends to induce hepatocellular carcinoma[1]. HBV genotype C isolates from Australian Aboriginees shows several features of a separate subgroup called genotype $C_{australia}$. The South American genotype F segregates into two clades F1 and F2[1].

Scanty information exists regarding the prevalence of HBV genotypes, their clinical relevance and therapeutic impact within CLDB category from India[9-12]. We have used recently developed two genotyping methods based on restriction fragment length polymorphism (RFLP)[13] and polymerase chain reaction (PCR) with type-specific primers[14] both targeting to the S-gene encoding the hepatitis B surface antigen.

## MATERIALS AND METHODS

### Patients

The study subjects included HBV DNA positive patients belonging to different clinical categories: (1) The diagnosis of HBV-related cirrhosis (CRB) was made when the patients presented with features of HBV-related chronic liver disease associated with portal hypertension, had biochemical evidence of hepatocellular failure and barium swallow or endoscopy showed evidence of esophageal varices (When possible confirmation was done by histology). Other causes of portal hypertension were excluded on the basis of clinical features, liver function tests, and liver biopsy. The severity of the cirrhosis was quantified using the Childs-Pugh Classification strategy[15]. Forty-seven CRB cases were selected on the basis of the above-mentioned criteria. (2) Patients with infection of HBV showing symptomatic, biochemical (alanine aminotransferase more than upper limit of normal) or serological (hepatitis B surface Antigen (HBsAg), Hepatitis B e Antigen (HBeAg), IgG antibodies to hepatitis B core antigen (IgG- Anti- HBc) positivity), evidence of continued liver disease of more than 3 to 6 mo without steady improvement were diagnosed as suffering from Chronic Hepatitis B (CHB)[9-12,15]. Forty CHB cases were selected based on the above-mentioned criteria. (3) HBV related hepatocellular carcinoma (HCCB) was diagnosed in those HBV related cases when a percutaneous liver biopsy showed histological features of a primary malignancy of hepatocytes and/or alfa-fetoprotein levels of more than 30 nanogram/mL[1,12,15]. Thirteen HCCB cases were selected on the basis of the above-mentioned criteria.

### Sample size estimation

With the assumption of prevalence of HBV, based on Indian Council of Medical Research (ICMR), Government of India, multicenter data[10-12], to be 70 % in chronic hepatitis (CH), 80% in liver cirrhosis (CRR), 60% in hepatocellular carcinoma (HCC), the minimum sample size required at 80% power level and 5% level of significance would be 30 cases each of HBV-related liver cirrhosis (CRB) and chronic hepatitis B (CHB) and 11 HBV-related hepatocellular carcinoma (HCCB). This sample size was achieved and some more cases were included by consecutive screening of 900 cases of various chronic liver disease patients over a period of 42 mo (June 2002 to December 2005). The study was approved by the institutional ethical committee of Maulana Azad Medical College, New Delhi. Study protocol was explained to all eligible participants and informed consent was obtained before subject enrollment.

### Serological tests

Serological status was investigated by the third generation enzyme immuno-assay (EIA) method using the following commercially available EIA kits: hepatitis B surface antigen (HBsAg), hepatitis B e antigen (HBeAg), IgG antibodies to hepatitis B core antigen (IgG- Anti- HBc), IgG antibodies to hepatitis D (IgG anti-HDV) (Abbott Laboratories, Chicago, IL, USA) and anti-HCV by Innotest HCV AB Ⅲ (Innogenetics NV, Ghent, Belgium).

### Biochemical investigations

Serum aspartate aminotransferase (AST), serum alanine aminotransferase (ALT), alkaline phosphatase (ALP), total bilirubin (T. Bil.), Prothrombin time (PT) tests were done weekly when the patients were admitted to the wards, while routine biochemical tests were done at monthly follow-up after the patients were discharged from the hospital.

### Lamivudine therapies for CHB patients

In all CHB cases, Lamivudine monotherapy was provided. The drug was given at a dose of 100 mg per day for 18 mo. End of therapy response (ETR) in HBeAg positive patients was defined as loss of HBeAg (with or without the appearance of anti-HBe), reduction of viral DNA to undetectable level and normalization of ALT. However, in HBeAg negative/anti-HBe positive patients it was defined as loss of viral DNA accompanied with the normalization of ALT[16]. Sustained virological response (SVR) was considered when the status of ETR persisted for six months after the therapy was stopped[16].

### DNA extraction and amplification of S-gene of HBV by PCR

Total nucleic acid from 100 μL serum was isolated using standard Proteinase-k/ phenol/ chloroform method[7]. Part of the S-gene was amplified by nested PCR as described earlier[13].

### Determination of HBV genotypes by RFLP analysis of S-gene

The second round PCR Product with a length of 485 bp was subjected to digestion with five kinds of restriction enzymes i.e. *Ear* Ⅰ, *Alw* Ⅰ, *Nci* Ⅰ, *Hph* Ⅰ, *Nla* Ⅳ[13].

### Determination of HBV genotypes by PCR using type-specific primers

Genotyping was done on the basis of PCR based type-specific DNA bands as performed earlier by Naito H *et al* 2001[14].

### Sensitivity of TSP-PCR

The amount of DNA corresponding to 100 μL of serum was dissolved in 25 μL of sterile deionised water and 10 μL was subjected to PCR. A $10^{-1}$ to $10^{-6}$ dilution of this sample was used to determine the sensitivity of the PCR protocol. Products were obtained up to $10^{-5}$ dilution. The detection limit of the PCR was calculated to be 200 HBV genomes/mL serum.

### HBV Genotype analysis based on nucleotide sequencing

Representative PCR amplified products of S-gene (485

**Table 1 Demographic and clinical characteristics of patients in the three study groups**

| Parameters | CRB | CHB | HCCB |
|---|---|---|---|
| *n* | 47 | 40 | 13 |
| Male: Female | 41:6 | 36:4 | 11:2 |
| | (6.8:1) | (9:1) | (5.5:1) |
| Mean age (yr) | 38.36 ± 4.13[a] | 37.65 ± 9.49 | 47.80 ± 18.70 |
| ALT (nKat/ L) | 1333 ± 483 | 1200 ± 579.5 | 908.5 ± 363.4 |
| AST (nKat/L) | 1152 ± 620 | 1283.6 ± 483 | 971.4 ± 360 |
| Total Bil. (μmol/ L) | 0.00016 ± 0.00012 | 0.00147 ± 0.00218 | 0.0028 ± 0.00032 |
| IgG anti-HBc | 91% | 94% | 93% |
| Risk factors | | | |
| Transfusions | 27% | 27% | 24% |
| Tattoo | 7% | 5% | 6% |
| Operation | 8% | 4% | 6% |
| Intravenous drug abuse | 5% | 6% | 3% |
| Unknown | 53% | 58% | 61% |
| Genotype A | (8/47) 17% | (7/40) 17% | (1/13) 7.6% |
| Genotype D[b] | (38/47) 81% | (32/40) 80% | (11/13) 85% |
| Genotype A+D | (1/47) 2% | (1/40) 3% | (1/13) 7.6% |
| HBeAg +ve (%) | 77%[a] | 82%[a] | 51% |
| Sustained virological response to therapy | | Genotype A: (2/ 7) 28.5% Genotype D: (12/32) 37.5% ($P$ = NS) | |

[a]$P < 0.05$ *vs* HCCB; [b]$P < 0.01$ *vs* Genotype A; NS: Not significant.

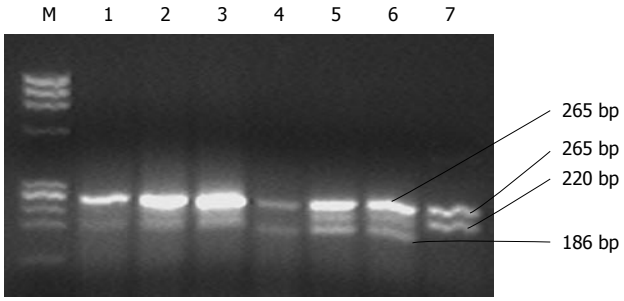


**Figure 1** Restriction Digestion of 485 bp PCR products into two fragments by *Nla* Ⅳ. M: *Hae* Ⅲ digested *Phi* X 174 DNA ladder, Lanes 1- 4: Restriction Digestion of 485 bp into 265 bp and 186 bp fragments showing the presence of Genotype D; lanes 5,6: Restriction Digestion of 485 bp into 265 bp, 220 bp and 186 bp fragments showing the presence of mixed Genotypes A and D; lanes 7: Restriction digestion of 485 bp into 265 bp and 220 bp fragments showing the presence of genotype A.

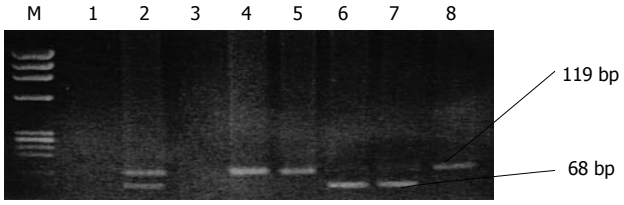


**Figure 2** Agarose gel electrophoresis of HBV genotypes A and D, (Using Mix-A, Mix-B, PCR with type-specific primers based genotyping strategy). PCR Product of 119 base pairs (type D) and 68 base pairs (type A). Lane M- (*Phi* X 174*Hae* Ⅲ) Molecular weight marker, lane 1, 3- Negative Control, lane2-Positive Controls, lanes 4-8: Samples; Lanes 4, 5, 8: Type D; Lanes 6, 7: Type A.

bases) were sequenced using the dideoxy chain termination method in an automated DNA sequencer (ABI 377-18 sequencing system, Perkin Elmer). Nucleotide sequences were aligned with CLUSTALW software. DNA sequences of Genotypes A-H were downloaded from the Gene bank according to the accession numbers for the respective genotype and phylogenetic analysis was carried out with the CLUSTALW algorithm.

### *Statistical analysis*

Quantitative data was expressed as mean ± SD (Standard Deviation). Statistical significance was determined by Chi-square test with Yates' correction (wherever needed) or by 2-sided Fisher exact test and Student's *t* test using SPSS-10 software. The p values of less than 0.05 were considered significant throughout.

## RESULTS

### *Demographic and clinical profile of the patients*

The S-gene of HBV isolates from 100 various CLDB patients were analyzed for HBV genotypes. The demographic and baseline clinical characteristics of the patients belonging to the three different groups (CHB, CRB, HCCB) is shown in Table 1. In all the three groups, the frequency of male patients was more than the female patients (Table 1). None of the patients was anti-HCV or anti-HDV positive as revealed by EIA tests.

### *HBV genotyping by PCR-RFLP*

The PCR products of 100 HBV DNA positive subjects were digested with restriction enzymes specific for genotypes B, C, E and F and no characteristic fragment for each genotype was observed. Finally, by digestion with *Nla* IV, characteristic fragments of genotype A were observed in 16 subjects, characteristic fragments of genotype D were found in 81 subjects (Figure 1). Genotypes A+D mixed infections were found in three cases (Table 1).

### *HBV genotyping by PCR with type specific primers*

As explained in the methodology two sets of the 2nd PCR was carried out for detection of genotypes A to F. Sixteen (16%) of the 100 subjects had a PCR product of 68 bp that was characteristic of genotype A (Figure 2). The other 81/100 (81%) had PCR product a length of 119 bp and were classified into genotype D. Genotype A + D mixed infections were found in three cases (Table 1).

### *Verification of RFLP and TSP-PCR genotyping methods by sequencing*

Twenty representative sequences were selected randomly from both genotype A ($n$ = 5) and D ($n$ = 15) samples. The 20 sequences were recorded in Gen Bank (Accession numbers: DQ875877 and DQ885267-DQ885285). A phylogenetic tree was constructed based on 410 bp of the S-gene of the HBV genome using CLUSTALW software. Out of the 20 isolates, 15 isolates were clustered with

genotype D, and five were clustered with genotype A, validating both the genotyping strategies. The genotype A and D sequences were further classified into subtype A1 and $D_1$ by blast analysis. None of the sequences was clustered with the sequences of the genotypes B, C, E, F, G or H.

### *Virological response in CHB patients*

Sustained virological response on treatment with lamivudine was seen in 28.5% of patients of genotype A while it was seen in 37.5% of patients of genotype D (*P* value is not significant) (Table 1). The clinical data of CHB patients on therapy harboring either genotype A or genotype D did not differ significantly.

### *Comparison of clinical profiles between genotypes A and D in northern India*

The majority of the cases turned out to be genotype D; 81/100 (81%), followed by genotype A; 16/100 (16%) using both the genotyping methods. Genotypes A + D mixed infections were found in three cases (Table 1). The ALT and AST levels, mean age, and HBeAg, IgG anti-HBc positivity were compared between genotypes A and D within each of the study groups and no significant differences were observed (Table 1). However, ALT, AST, HBeAg and IgG-anti-HBc positivity were significantly different between HCCB and CHB/ CRB cases ($P < 0.05$ CHB, CRB *vs* HCCB, Table 1).

## DISCUSSION

There have been no studies from northern India comparing the clinical outcome of CLDB patients infected with different HBV genotypes using the TSP-PCR method. We have attempted to validate two most commonly used methods originally described by Naito *et al* and Mizokami *et al*[13,14]. Genotypes determined by both the methods were concordant in all the subjects.

The present study demonstrates that HBV genotype D is highly prevalent. This result, however, differs from that of two earlier studies on chronic liver diseases where genotype A and D were found to be prevalent in equal proportions[9,17]. The major reason of the different results might be due to the differential demographic distribution of the HBV-genotypes. The previous study was carried out in Lucknow; a city situated 600 km away from New Delhi, India. Also, the majority of the cases in Lucknow come from semi-urban and rural areas. The patients in New Delhi are more or less from urban or semi-urban areas[17]. Another reason could be selection bias, as earlier studies were conducted on small series of non-consecutive chronic cases [9,17].

In this study, mixed infection of genotypes A and D of HBV were found in 2% CRB cases, 3% CHB and 7.6% HCCB cases. This is in accordance with what has been reported earlier[17]. Interestingly, all the cases (3/3) with mixed infections with genotype A and D had histories of blood transfusions. In our study, genotype A was found only in the minority of the patient categories (Table 1). The impact of HBV genotypes on response to lamivudine therapy has been studied in various countries[18]. A study in Germany suggested that the rate of resistance to lamivudine was higher in patients with HBV genotype A infection than in patients with genotype D infection. No difference in the risk of lamivudine resistance is found between patients with genotype B and patients with genotype C. In patients with genotype C infection, however, virological response is worse during lamivudine therapy, and is also less durable after the discontinuation of therapy than in patients with genotype B infection[18]. The response to lamivudine is poorer in patients infected with subtype Ba, which contains a recombination with genotype C, than in those with subtype Bj without such a recombination[19]. Influence of genotypes on therapeutic response needs to be examined in patients infected with the other genotypes, particularly in those with genotype A or D infection[19]. In the present study, a significantly higher percentage of patients with CLDB were infected with genotype D and they did not influence the therapeutic response. Sustained virological response on treatment with lamivudine was seen in 28.5% of patients of genotype A while it was seen in 37.5% of patients of genotype D (*P* value is not significant). Determining the genotype could be helpful for predicting the outcome of antiviral therapy in patients with chronic hepatitis B. Transfusion was associated as the most important risk factor of HBV transmission in all the patient categories (Table 1).

Twenty representative samples from the study population were sequenced for the S-gene which led to confirmation of the RFLP and TSP-PCR results. Based on the nucleotide sequence of the S-region, genotype D emerged as the predominant genotype (15/20, 75%). Indian strains belonging to genotype A and D differed from each other by about 5.2% in the S-gene (410 bases).

In conclusion, genotype D appears to be the dominant genotype circulating in north Indian population with CLDB. Genotype A appears to be the minor genotype.

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*RAPID COMMUNICATION*

# Levels of serologic markers of celiac disease in patients with reflux esophagitis

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## Abstract

**AIM:** To investigate the prevalence of celiac disease serologic markers (antigliadin IgA, IgG, and anti-endomysial IgA) in patients with reflux esophagitis and to detect the relationship between reflux esophagitis and celiac disease (CD).

**METHODS:** This study was performed prospectively between January 2003 and January 2004. Sixty-eight adult reflux esophagitis patients and 40 people as control group for symptoms related with gastrointestinal system were enrolled in this study. The diagnostic work-up included an accurate medical history with gastrointestinal symptoms, routine laboratory measurements, the detection of antibodies against gliadin (IgA and IgG) and endomysium (IgA), and an upper endoscopy with postbulbar biopsy.

**RESULTS:** IgA-AGA and IgG-AGA were positive at 8.8% and 10.3% in patients with reflux esophagitis. In control group, it was found that 10% people had positive IgA-AGA, and 7.5% people had positive IgG-AGA. There was no significant relationship between patients and control group regarding positive IgA-AGA and IgG-AGA. The patients and persons in control group had no positive IgA-EMA. On postbulbar biopsies, no finding was detected concerning celiac disease. There were no symptoms and signs for gluten enteropathy in patients and control group.

**CONCLUSION:** This review supports that an association does not exist between celiac disease and reflux esophagitis. We think these diseases exist independently from each other.



**Key words:** Celiac disease; Reflux; Esophagitis

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## INTRODUCTION

Celiac disease (CD) is a chronic inflammatory disorder characterized by damage of the mucosa of the small intestine[1,2]. CD is induced in sensitive individuals by the ingestion of gluten and may range from overt malabsorption to few or no symptoms when only malabsorption of selective nutrients present[3,4]. Clinical manifestations of CD vary markedly with the age of the patient, the duration and extent of disease[1,5]. Clinical and histological improvement occurs with withdrawal of gluten from the diet[6]. The diagnostic criteria of CD are based on the finding of small intestinal mucosal villous atrophy with crypt hyperplasia. Small intestinal histology is the current gold-standard diagnostic test for celiac disease[7].

Subtle and atypical symptoms often make the diagnosis difficult, and, therefore serological screening tests have proved essential in the diagnostic approach[8]. Serological studies currently in clinical use include IgA endomysial antibody (IgA-EMA), IgA tissue transglutaminase antibody (IgA-tTG) and antigliadin antibodies IgA and IgG (AGA). Serology has a limited value in diagnostic procedures and a negative result does not rule out the diagnosis of CD[9].

CD patients have been demonstrated to be associated with a number of motor abnormalities of upper gastrointestinal tract[10-14]. The patients with untreated CD show a significant decrease in low esophageal sphincter (LOS) pressure[13]. However, whether adult celiac patients are more susceptible to reflux esophagitis is still unknown. A few reports support that patients with CD are more susceptible to reflux esophagitis, whereas others do not support this connection[16,17].

So far, no data are available on the relationship between reflux esophagitis and CD. Therefore, we aimed in this study to investigate the prevalence of celiac disease serologic markers (antigliadin IgA, IgG, and antiendomisyum IgA) in patients with reflux esophagitis and to detect the relationship between these two gastrointestinal pathologies.

## MATERIALS AND METHODS

This study was performed prospectively in Gastroenterology Department of Gulhane Military Medical Academy between January 2003 and January 2004. Sixty-eight adult reflux esophagitis patients (20 women and 48 men, median age 41 years, range 20-77) were enrolled in this study (Table 1). The diagnostic work-up included an accurate medical history with gastrointestinal symptoms (heart burn, abdominal pain, regurgitation, dysphagia, odynophagia, pulmonary symptoms, diarrhea, meteorism), routine laboratory measurements, the detection of antibodies against gliadin (IgA and IgG) and endomysium, and an upper endoscopy with postbulbar biopsy (Table 2). Upper gastrointestinal endoscopy was performed in the standard way, after a 6-h fast. The severity of esophagitis was assessed according to the Los Angeles Criteria[18].

Forty people (12 women and 28 men, median age 38 years, range 20-68) formed the control group for symptoms related with gastrointestinal system. The control groups comprised patients suffering from dyspepsia and no evidence of gastro-oesophageal reflux disease. The other exclusion criteria were age < 20 or > 80 years and use of any drugs. CD was eliminated by small-intestinal biopsy in all controls. There was no statistical difference of the gender between the two groups.

Small intestinal biopsy specimens were taken from each patient, and the diagnosis of celiac disease was made based on the findings of total or subtotal small intestinal mucosal villous atrophy, crypt hyperplasia, and lymphoplasmacellular infiltration[7]. Enzyme-linked immunosorbent assay (ELISA) technique was used for the detection of serum level of antigliadin IgA and IgG. Serum IgA endomysial antibodies were fixed by indirect immunofluorescence.

The aim of this study was explained to all patients and control subjects and informed consent form was obtained from all. The study was applied according to the principles of the Declaration of Helsinki and was approved by the Ethical Committee of Gulhane Military Medical Academy.

### *Statistical analysis*

All of the statistical analyses were performed using statistical software package SPSS 11.5 (SPSS Inc., Chicago, IL, USA). The results were shown as the median (min-max). Odds ratios and their 95% confidence intervals were calculated, also. Relationships between the controls and patients were investigated with Chi-square test. *P* values less than or equal to 0.05 were considered as statistically significant.

**Table 1 Demographic data of patients and controls**

| Demographic data | Patients | Controls |
|---|---|---|
| Gender (M/F) | 20/48 | 12/28 |
| Median age (min-max) | 41 (20-77) | 38 (20-68) |
| Esophagitis | | |
| Grade A | 80.90% | - |
| Grade B | 14.70% | - |
| Grade C | 4.40% | - |
| Grade D | - | - |

**Table 2 Esophageal symptoms in patients with reflux disease (*n* = 68)**

| Symptoms | *n* (%) |
|---|---|
| Heartburn | 48 (70.6) |
| Chest pain | 40 (58.8) |
| Regurgitation | 48 (70.6) |
| Bleeding | 5 (7.4) |
| Dysphagia | 3 (4.4) |
| Odynophagia | 2 (3.0) |
| Pulmonary symptoms | 10 (14.7) |
| Others (diarrhea…) | 11 (16.2) |

**Table 3 Prevalence of positive serologic markers for coeliac disease according to the esophagitis grade (*n* = 68)**

| Grades | Antigliadin IgA | | Antigliadin IgG | | Antiendomysium IgA | |
|---|---|---|---|---|---|---|
| | *n* | % | *n* | % | *n* | % |
| Grade A (*n* = 55) | 4 | 7.2 | 3 | 5.4 | - | - |
| Grade B (*n* = 10) | 1 | 10 | 2 | 20 | - | - |
| Grade C (*n* = 3) | 1 | 33.3 | 2 | 66.6 | - | - |

**Table 4 Prevalence of positive serologic markers for coeliac disease in control subjects (*n* = 40)**

| Serologic markers | Control subjects (*n* = 40) | |
|---|---|---|
| | *n* | % |
| Antigliadin IgA | 4 | 10 |
| Antigliadin IgG | 3 | 7.5 |
| Antiendomysium IgA | - | - |

## RESULTS

Antigliadin IgA (IgA-AGA) was found positive in 6/68 (8.8%) patients with reflux esophagitis. Four of 6 patients were grade A (4/55, 7.2%), one was grade B (1/10, 10%) and one was grade C (1/3, 33.3%). Seven (7/68, 10.3%) patients had positive antigliadin IgG (IgG-AGA); 3/55 (5.4%) were grade A, 2/10 (20%) grade B, and 2/3 (66.6%) grade C (Table 3). No patient had positive antiendomysium IgA (IgA-EMA) and in addition, there were no patients with both positive IgA-AGA and positive IgG-AGA.

On endoscopy in control cases, the macroscopic appearance of the esophageal mucosa was normal. In control group, it was found that 4 (10%) people had positive IgA-AGA, and 3 (7.5%) people had positive IgG-AGA (Table 4), while none in control group had positive IgA-EMA.

On postbulbar biopsies, no finding was detected

concerning celiac disease. In 3 of 6 patients with positive IgA-AGA, histologically slightly chronic non-specific duodenitis was seen and the others had normal duodenal mucosa. The number of moderate chronic nonspecific duodenitis, slightly chronic nonspecific duodenitis, and normal duodenal mucosa were 2, 2, and 3, respectively, in 7 patients with positive IgG-AGA. All other patients had normal duodenal mucosa on histology.

There was no statistically significant relationship between controls and patients for IgA-AGA ($\chi^2 = 0.041$; $P = 0.839$; OR = 0.871, 0.230-3.294). The similar result was obtained for IgG-AGA ($\chi^2 = 0.234$; $P = 0.629$; OR = 1.415, 0.345-5.813).

## DISCUSSION

Gastroesophageal reflux disease (GERD) is a motor disorder involving lower esophageal sphincter (LES) and esophageal peristalsis. The mean basal pressure of the LES and peristaltic waves are significantly lower in patients with GERD. These abnormalities are responsible for an ineffective removal of refluxed contents, longer contact of acid with the esophageal mucosa, and possibly esophagitis[19].

The increased frequency of reflux esophagitis in CD was reported in some studies[13,15]. In our study, CD was not seen in patients suffering from reflux esophagitis and in control group suffering from unspecific upper gastrointestinal symptoms. Epidemiological studies using serological tests with biopsy verification have revealed higher prevalence of 1:300 to 1:500 for CD in most countries[20]. In primary care practice it is not recommended that cases with reflux esophagitis must be searched for CD. Moreover, the diagnosis depending GERD is difficult and invasive procedures are needed, including endoscopy, biopsy and pathology. Investigations available include flexible oesophagoscopy (with biopsy), ambulatory or static pH manometry, and radiological assessment. A perfect method for diagnosing reflux disease does not yet exist. For this reason, finding a specific marker for reflux esophagitis is necessary.

The results of our study are apparently in contrast with those of Iovino *et al* who suggested that celiac patients with steatorrhea present a higher prevalence of esophageal symptoms and a lowered esophageal sphincter pressure compared with celiac patients without steatorrhea and control subjects[13]. Cuomo *et al* found a twofold increase in the prevalence of endoscopic esophagitis in adult patients who had been diagnosed with CD compared with control non-celiac subjects[15]. All patients in our study were investigated in primary-care setting and all of them were reflux esophagitis patients. None of our patients had severe esophagitis. The most important finding was that none of the patients had CD. The apparent discrepancy with our study might be due to the fact that subjects enrolled in those studies had CD.

Recently, Collin *et al* evaluated the occurrence of esophagitis in CD. In this study, 0.9% of patients with esophagitis and 0.6% of those with esophageal reflux symptoms had CD[17]. They interpreted that the association between these two conditions was weak. This supports our findings and makes weak the claims that CD may play a role in the pathogenesis of GERD.

The abnormalities in LES and esophageal peristalsis associated with GERD are the important factors in the presence of reflux esophagitis. Some gastrointestinal hormones appear contributing to these dysfunctions. One of these, plasma enteroglucagon, which decreases LES pressure and delays gastric emptying was significantly higher in CD patients than controls[21,22]. Despite of this finding supporting the association between reflux esophagitis and CD, we believe that it needs further clarification.

It is known that reflux esophagitis can be seen in celiac patients, but reflux esophagitis is not accepted as a basic finding in CD. This review suggests that an association does not exist between CD and reflux esophagitis. We think these two diseases exist independently from each other. The negative results are possibly due to the small sample size. Since the correlation between celiac disease and reflux esophagitis may be very weak, much bigger sample size is needed to support the null hypothesis.

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*RAPID COMMUNICATION*

# Monocytes in systematic inflammatory response syndrome: Differences between sepsis and acute pancreatitis

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## Abstract

**AIM:** To unravel the differences between systematic inflammatory response syndrome (SIRS) of acute pancreatitis compared to the same syndrome in sepsis.

**METHODS:** Twenty-five patients were enrolled, 12 with sepsis and 13 acute pancreatitis. After diagnosis 20 mL blood was sampled. Half were assayed for isolation of monocytes and 10 mL was centrifuged for serum test of tumor necrosis factor alpha (TNFα) and interleukin-6 (IL-6). Half of monocytes were incubated in the presence of patients' serum and supernatants were collected. The other half was treated for estimation of optical photometry under caspase-3 inhibition. TNFα and IL-6 were estimated by an enzyme immunoassay.

**RESULTS:** median ± SE of serum IL-6 in septic patients and acute pancreatitis patients was 192.30 ± 35.40 ng/L and 21.00 ± 16.05 ng/L, respectively ($P < 0.01$). Respective values of caspase-3 were 0.94 ± 0.17 pmol/min $10^4$ cells and 0.34 ± 0.09 pmol/min $10^4$ cells ($P < 0.05$). IL-6 of monocyte supernatants of patients with sepsis was significantly increased after addition of patients' serum, while that of patients with acute pancreatitis did not show significant difference.

**CONCLUSION:** The data have shown that monocyte activity is different between acute pancreatitis and sepsis. This phenomenon might be explained as a different pathway to the pro-inflammatory cytokines release or could be a novel anti-inflammatory response in acute pancreatitis.



## INTRODUCTION

Although the knowledge about the underlying pathogenic mechanisms in acute pancreatitis and sepsis has been considerably enriched, comparative studies are lacking. There is evidence that pro-inflammatory cytokine cascade plays a crucial role in both acute pancreatitis and sepsis[1]. Acute pancreatitis is an inflammation often complicated with systematic inflammatory response syndrome (SIRS). During the evolution to sepsis microbial components trigger serum monocytes, which release pro-inflammatory cytokines; and the latter leads to SIRS[2].

Septic cascade is thought to be initiated from pro-inflammatory cytokines released from blood monocytes[3]. Acute pancreatitis is one major cause of systemic inflammation attributed to the secretion of pro-inflammatory mediators but the contribution of monocytes to the biosynthesis of these mediators is not yet defined at least with data of the current literature. The aim of the present study was to unravel the differences of the *ex vivo* activity of monocytes upon occurrence of SIRS between patients with acute pancreatitis and sepsis.

## MATERIALS AND METHODS

Twenty-five patients were enrolled in a prospective study from January 2003 to June 2003; 13 patients were diagnosed with acute pancreatitis and 12 with sepsis. All were hospitalized in the 4th Department of Internal Medicine of the Medical School of Athens during that period. The study was conducted in accordance to the Helsinki Declaration.

Exclusion criteria for the study were the presence of (1) neutropenia (< 500 neutrophils/μL), (2) HIV infection, (3) corticosteroids administration for more than one month, defined as more than 1 mg/kg daily of equivalent

prednisone, and (4) protein C administration at least five days before admission.

SIRS was defined by the presence of at least two of the followings[4]: (1) pulse rate > 90/min, (2) breath rate > 20/min or $P_{CO2}$ < 32 mmHg, and (3) leukocytosis [white blood cells (WBCs) > 12000/μL] or leucopenia (WBCs < 4000/μL) or more than 10% bands.

Inclusion criteria were the following for acute pancreatitis[5]: (1) SIRS, (2) initiation of a typical epigastric pain radiating to the back over the last 12 h before admission; (3) serum amylase at least three times above normal values, (4) urine amylase at least three times above normal values, (5) findings of acute edematous pancreatitis on ultrasound or on computed tomography, and (6) the absence of any primary liver disease.

Inclusion criteria for sepsis were the following in accordance to ASCP/SCCM 1997 classification[4]: (1) clinically proven infection with fever as its first manifestation over the last 12 h before admission, and (2) SIRS. Primary infections for sepsis were acute pyelonephritis, lower respiratory tract infection (LRTI) and acute cholangitis.

The diagnosis of acute pyelonephritis was made on the basis of the following criteria[6,7]: (1) a case history compatible with acute pyelonephritis comprising at least two spikes of fever above 38℃ and presence of lumbar tenderness on examination, (2) pyuria defined as presence of more than 10 polymorphs per high power field under a light microscope, and (3) positive urinary culture with the colony count exceeding $10^4$ cfu/mL for a single Gram-negative bacterial species. Diagnosis of LRTI was established in any patient presenting with the following signs: (1) core temperature > 38℃, and (2) new or persistent consolidation on lung X-ray[8]. Diagnosis of acute cholangitis was based on the following criteria: (1) core temperature > 38℃, (2) acute pain of upper right quadrant of abdomen elicited on palpation, and (3) ultrasound findings compatible with cholangitis[9].

Upon admission, a total of 20 mL of blood were collected after puncture of one forearm vein. Ten milliliters of blood were collected in a sterile heparinized syringe and the remaining in a sterile tube. Blood was centrifuged and the supernatant was kept at -70℃ until assayed.

For the isolation of blood monocytes[10], the collected heparinized venous blood was layered over Ficoll Hypaque (Biochrom, Berlin, Germany) and centrifuged. Isolated mononuclear cells were washed three times with PBS (pH 7.2) (Merck, Darmsadt, Germany) and incubated with RPMI 1640 enriched with 10% FBS and 2 mmol/L of glutamine in the presence of 100 U/mL of penicillin G and 0.1 g/L of streptomycin (Sigma Co, St. Louis, USA) in flasks of 25 $cm^3$. After one hour of incubation at 37℃ in 5% $CO_2$, non-adherent cells were removed; adherent monocytes were thoroughly washed with Hanks' solution (Biochrom). Monocytes were then harvested with the application of a 0.25% trypsin/0.02% EDTA solution (Biochrom) and counted in a Neubauer plate. Their purity was more than 95% as determined after staining with the anti-CD14 monoclonal antibody at the fluorocolor FITC (emission 520 nm, Immunotech, Marseille, France) and reading through the EPICS XL/MSL flow cytometer (Beckman Coulter Co, Miami, Florida).

Half of the isolated monocytes were treated with an ice-cold cell lysis buffer (50 mmol/L HEPES, 0.1% CHAPS, 5 mmol/L DTT, 0.1 mmol/L EDTA, pH 7.4). After centrifugation for 10 min at 10000 × *g*, and 4℃, activity of caspase-3 was estimated in the cytosolic extract by an enzymatic chromogenic assay (BIOMOL Research Laboratories, Plymouth, PA). It was based on the rate of hydrolysis at 37℃ of a substrate releasing p-nitroaniline over-time, as assessed by sequential photometry at 405 nm. The assay was also performed in the presence of a caspase-3 inhibitor. The activity of caspase-3 in cell lysates was expressed as pmol/min $10^4$ cells.

The remaining half of monocytes were distributed in two wells of a 12-well plate; they were incubated with RPMI 1640 supplemented with 10% FBS and 2 mmol/L of glutamine for 18 h at 37℃ in 5% $CO_2$ in the absence/presence of 0.1 mL of serum of the patient. The total volume of the added growth medium was 2.4 mL where patients' sera represented 4.1%. After incubation, cell supernatants were collected and kept refrigerated at -70℃ until assayed.

Concentrations of TNFα and IL-6 in sera and monocyte supernatants were estimated by an enzyme immunoabsorbent assay (Diaclone, Paris, France). Lowest limits of detection were 0.5 pg/mL for TNFα and 6.25 ng/L for IL-6. Their concentrations in supernatants were expressed as pg/$10^4$ monocytes. TNFα and IL-6 and activity of caspase-3 were selected as parameters since the former two represented the considerable burden of pro-inflammatory cytokines secreted from monocytes in an early course of events leading to inflammation and the latter an index of apoptosis.

### Statistical analysis

Concentrations of TNFα, IL-6 in sera and monocyte supernatants and caspase-3 intracellular activity were expressed as their median ± 95% confidence intervals of the mean (CI) or standard error of the mean (SE). Comparison between groups was made by Mann-Whitney *U* test. Correlations between concentrations of TNFα and IL-6 in serum, intracellular activity of caspase-3 and concentrations of TNFα and IL-6 of monocyte supernatants were performed according to Spearman's rank of order. According to the estimated intracellular monocytic activity of caspase-3, patients were divided into those with activity lower and higher than 100 pmol/min $10^4$ cells. Comparisons between sepsis and acute pancreatitis were performed by Pearson's chi-square test. Any *P* value less than 0.05 was considered as significant.

## RESULTS

mean ± SD age of enrolled patients with acute pancreatitis was 59.6 ± 18.5 years and of patients with sepsis 61.5 ± 17.6 years. Predisposing factors of pancreatitis were gallstones in eleven patients and hypertriglyceridemia in two patients. Primary infection for sepsis was acute pyelonephritis in six patients, LRTI in four patients and

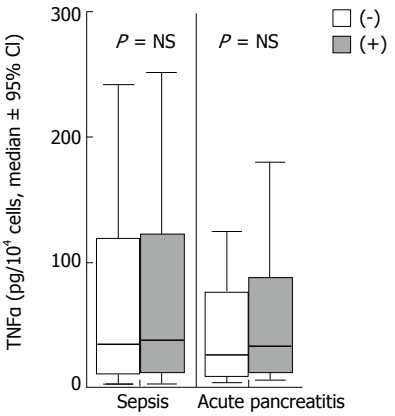


**Figure 1 Comparisons of the concentrations of tumor necrosis factor-alpha (TNFα) in the supernatants of monocytes isolated from patients with sepsis and acute pancreatitis.** (-): incubation in the absence of patients' serum; (+): incubation in the presence of patients' serum.

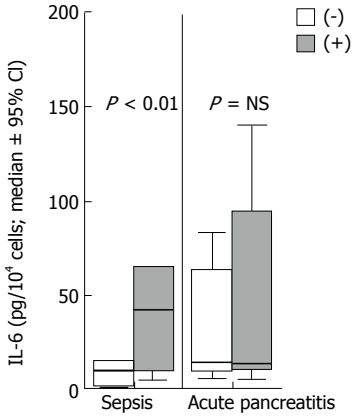


**Figure 2 Comparisons of the concentrations of inteleukin-6 (IL-6) in the supernatants of monocytes isolated from patients with sepsis and acute pancreatitis.** Circles denote outliers. (-): incubation in the absence of patients' serum, (+): incubation in the presence of patients' serum.

acute cholangitis in two patients.

mean ± SD APACHE Ⅱ scores in patients with acute pancreatitis and sepsis were 7.68 ± 1.22 and 8.23 ± 1.48 respectively ($P$ = NS). mean ± SD of Ranson Index score in patients with pancreatitis on admission and during the first 48 h after admission was 1.34 ± 0.32 and 1.69 ± 0.82 respectively.

median ± SE TNFα in serum was 11.61 ± 7.57 pg/mL and 17.01 ± 8.64 pg/mL, in patients with sepsis and acute pancreatitis, respectively ($P$ = NS). Respective values for IL-6 were 192.30 ± 35.40 pg/mL and 21.00 ± 16.05 pg/mL ($P < 0.01$) and for intracellular activity of caspase 3, 0.94 ± 0.17 pmol/min $10^4$ cells and 0.34 ± 0.09 pmol/min $10^4$ cells, respectively. Occurrence of high-level caspase-3 monocytic intracellular activity was greater in patients with sepsis than in patients with acute pancreatitis ($P < 0.05$).

Concentrations of TNFα of monocyte supernatants of patients with sepsis and acute pancreatitis incubated in the absence or presence of patients' sera are shown in Figure 1. No differences were found between yielded concentrations in the absence and presence of sera from either patients with sepsis or pancreatitis.

Concentrations of IL-6 of monocyte supernatants of patients with sepsis and acute pancreatitis incubated in the absence or presence of patients'sera are shown in Figure 2. The ratios of concentration of IL-6 in the presence of patients' sera to concentration in the absence of patients' sera were estimated. The ratio was significantly higher in sepsis than in acute pancreatitis ($P < 0.01$).

No correlation was found between intracellular activity of caspase-3 and TNFα and IL-6 of serum from either patients with acute pancreatitis or sepsis. Neither was correlation found between the intracellular activity of caspase-3 and either TNFα or IL-6 concentrations of monocyte supernatants in patients with either acute pancreatitis or sepsis.

## DISCUSSION

Acute pancreatitis and sepsis represent two significant causes of mortality; subsequent multiple organ failure is thought to be the most severe complication of both[2]. SIRS is considered to result from pro-inflammatory cytokines released by the inflamed tissues or cells of the innate immune system. Recent data evidence for probable alterations of the function of cells of the innate immunity in both acute pancreatitis and sepsis. These changes are expressed as decreased expression of HLA-DR on cell membranes of monocytes[11,12]. Based on the latter evidence, the present study was focused on the comparative *ex vivo* activity of monocytes between patients with sepsis and acute pancreatitis.

It has been observed that most of the patients with acute pancreatitis develop organ dysfunction 48-72 h after initiation of their first clinical symptoms[13]. To our knowledge, the present study is the first attempting to investigate the probable role of monocytes in acute pancreatitis compared to their respective role in sepsis at an early time course; blood was sampled within 12 h after the first clinical symptom in both study groups[14].

Our results revealed that serum concentrations of IL-6 in patients with sepsis were significantly increased compared with that in patients with acute pancreatitis; similar finding was not observed for TNFα. The lack of similarities between TNFα and IL-6 might be attributed to the earlier release of TNFα in the pro-inflammatory cascade than that of IL-6[15].

TNFα of monocyte supernatants did not differ between sepsis and acute pancreatitis; it remained unaffected by the addition of patients' serum (Figure 1). The lack of difference of TNFα release by monocytes between sepsis and pancreatitis might also be explained by its early production in the inflammatory cascade.

On the other hand, IL-6 secretion by monocytes was significantly increased in the presence of serum of patients with sepsis compared to that of patients with acute pancreatitis (Figure 2). Presence of serum in monocyte supernatants represented only 4% of the total culture volume; that percentage might be considered minimal to produce any confounding. A limitation of the study is the lack of evidence to explain the effect of serum and only hypotheses may be made. It might be presumed that

an anti-inflammatory mediator was present in serum in patients with acute pancreatitis inhibiting further release of IL-6 from monocytes. That response might be implicated in sepsis also, but probably was inadequate to inhibit secretion of IL-6. However, it is of great significance that monocytes preserve secretion activity in acute pancreatitis; a finding seen for the first time.

Caspase-3 is involved in the intracellular apoptotic process[16]. Its intracellular activity was significantly increased in patients with sepsis compared to those with acute pancreatitis. Increased apoptosis of monocytes in sepsis may constitute an attempt of the host to reduce inflammatory responses. In patients with acute pancreatitis, apoptosis of monocytes was minimal. However, it might designate a late activation of monocytes in the evolution of acute pancreatitis compared to sepsis, as described by others[17].

The presented data reveal a different *ex vivo* activity of monocytes between sepsis and acute pancreatitis. This phenomenon might be explained by the existence of either of different pathways for the release of pro-inflammatory cytokines or of a novel anti-inflammatory activity of serum of patients with acute pancreatitis.

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*RAPID COMMUNICATION*

# Efficacy of thymosin alpha-1 and interferon alpha in treatment of chronic viral hepatitis B: A randomized controlled study

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## Abstract

**AIM:** To observe the efficiency and safety of thymosin-α1 treatment in patients with hepatitis B e antigen (HBeAg) and HBV DNA positive chronic hepatitis.

**METHODS:** Sixty-two patients were randomly divided into groups A and B. The patients in group A received subcutaneous injection of 1.6 mg thymosin-α1, twice a week (T-α1 group) for six months, and the patients in group B received 5 MU interferon alpha (IFN-α) each day for fifteen days, then three times weekly (IFN-α group) for six months. The results between two groups treated with and the group untreated with IFN-α which was followed up for 12 mo (historical control group consisting of 30 patients) were compared, and three groups were comparable between each other ($P > 0.05$) at baseline (age, sex, clinical history, biochemical, and serological parameters).

**RESULTS:** At the end of treatment, complete response, which was defined as alanine aminotransferase (ALT) normalization and HBV DNA and HBeAg loss, occurred in 9 of 29 (31.0%) patients in the T-α1 group and in 15 of 33 (45.5%) patients in the IFN-α group ($\chi^2 = 1.36$, $P > 0.05$). After a follow-up period of six months, a complete response was observed in 14 of 29 (48.3%) patients in the T-α1 group and in 9 of 33 (27.3%) patients in the IFN-α group ($\chi^2 = 2.93$, $P > 0.05$). Compared with the results observed in the historical control (HC) group untreated with IFN-α which was followed up for 12 mo, the rate of complete response was significantly higher in IFN-α group at the end of therapy (1 of 30 *vs* 15 of 33, $\chi^2 = 14.72$, $P < 0.001$) and in the T-α1 group at the end of follow-up (1 of 30 *vs* 14 of 29, $\chi^2 = 15.71$, $P < 0.001$). In T-α1 and IFN-α treatment groups, the area under (the plasma concentration time) curve (AUC) of negative HBV DNA and HBeAg was 34%, 17%, 31% and 19% smaller than that in the HC group. By the end of the follow-up period, the proportions of ALT normalization and negative HBV DNA in the T-α1 group were significantly higher than those in the IFN-α and HC groups. The odds of ALT normalization and negative HBV DNA at the end of the follow-up was three-fold higher in the T-α1 group than in the IFN-α group. Unlike IFN-α, T-α1 was well tolerated by all patients, and no side effects appeared in T-α1 group.

**CONCLUSION:** The results suggest that a 6-mo course of T-α1 therapy is effective and safe in patients with chronic hepatitis B. T-α1 is able to reduce HBV replication in patients with chronic hepatitis B. Furthermore, T-α1 is better tolerated than IFN-α and can gradually induce more sustained ALT normalization and HBV DNA and HBeAg loss. However, a response rate of 48.3% is still less ideal. A more effective therapeutic approach warrants further study.



**Key words**: Chronic hepatitis B; Efficacy; Interferon-alpha; Thymosin alpha-1

You J, Zhuang L, Cheng HY, Yan SM, Yu L, Huang JH, Tang BZ, Huang ML, Ma YL, Chongsuvivatwong V, Sriplung H, Geater A, Qiao YW, Wu RX. Efficacy of thymosin alpha-1 and interferon alpha in treatment of chronic viral hepatitis B: A randomized controlled study. *World J Gastroenterol* 2006; 12(41): 6715-6721

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## INTRODUCTION

Chronic hepatitis B virus (HBV) infection is a serious problem worldwide and may result in adverse sequelae, such as cirrhosis and hepatocellular carcinoma (HCC)[1-2], which is becoming more prevalent worldwide, especially in HBV-endemic areas[3-4]. According to the World Health Organization estimation, more than 350 million people are infected with HBV worldwide, of them up to 20% will become chronic HBV carriers, and 50% would develop chronic liver disease with a significant risk of developing liver cirrhosis and HCC[4]. Despite the introduction of universal vaccination against hepatitis B in over 100 countries, persistent HBV infection is still a serious problem worldwide, causing one million deaths each year[4-6]. It may take several decades to reduce its transmission and morbidity by vaccination. Meanwhile, patients with persistent HBV infection require better antiviral therapeutic modalities than the currently available ones[4]. The ultimate goal of therapy for chronic hepatitis B is to prevent its progression to cirrhosis and development of HCC. Recent prospective studies from Taiwan indicate that higher plasma HBV DNA levels in persons infected with HBV are associated with increased risk of developing HCC, reinforcing the importance of controlling viral replication among patients with chronic hepatitis B[7-10].

Over the past 20 years, many antiviral or immunomodulatory agents have been used in the treatment of chronic HBV infection[11-20]. Among them, interferon alpha (IFN-α), has been shown to be effective against chronic HBV infection, and induces an apparent initial response in approximately 30%-40% of treated patients[21-23]. However, the response rate is far from satisfactory, particularly in Asian patients. The relapse rate after treatment withdrawal is high[24].

Lamivudine suppresses viral replication, resulting in HBeAg seroconversion in 16%-17% of patients and histologic improvement in 52%-56% patients after one year of therapy[25,26]. However, within four years, the rate of emergence of lamivudine-resistant HBV mutants approaches 70%[27-30], which is usually associated with a rebound in viral load and exacerbation of hepatitis[31-34]. Pegylated-interferon alfa-2a (IFNα- 2a) appears to have a better efficacy than lamivudine HBeAg seroconversion rate was 32%, 27% and 19%, respectively in pegylated-IFNα-2a monotherapy, plus Lamivudine and lamivudine monotherapy; alanine aminotransferase (ALT) normalization was 41%, 39% and 28%, respectively in HBeAg-positive patients 6 mo after 48-wk treatment. However, the rate of adverse events associated with pegylated IFNα-2a is higher than that of lamivudine[35]. Patients with HBeAg-negative chronic hepatitis B treated with pegylated IFNα-2a have a significantly higher rate of response which could sustain for 24 wk after the therapy (monotherapy or in combination with lamivudine) than those treated with lamivudine monotherapy. After 24 wk of follow-up, the percentages of patients with ALT normalization or HBV DNA level below 20 000 copies/mm$^3$ are 59%, 43%, 60% and 44% *vs* 44% ($P$ = 0.004 and $P$ = 0.003) and 29% ($P$ = 0.007 and $P$ = 0.003)[36].

Thymosin-α1 (T-α1) is an immune modifier that can trigger maturational events in lymphocytes, augment T-cell function, and promote reconstitution of immune defects[37]. T-α1 has been shown to promote disease remission and cessation of HBV replication in patients with HBeAg-positive chronic hepatitis B without significant side effects[38,39]. Clinical trials using T-α1 in the treatment of patients with immunodeficiency or cancer indicate that this agent is nontoxic, enhances immune responsiveness and augments specific lymphocyte functions, including lymphoproliferative responses to mitogens, maturation of T-cells, antibody production, and T-cell-mediated cytotoxicity[40-42]. On the basis of these observations, we conducted a randomized, controlled trial to compare the efficacy and safety of T-α1 *vs* INF-α therapy in patients with chronic hepatitis B.

## MATERIALS AND METHODS

### Patients

Sixty-two Chinese patients were enrolled in the study. All met the following criteria for entry: age 18-60 years, presence of hepatitis B surface antigen (HBsAg) and hepatitis B e antigen (HBeAg) in serum for at least 12 mo, positive serum tests for HBV DNA documented on at least two occasions and at least 3 mo apart 12-mo before entry, aminotransferase levels higher than 1.5 times the upper normal limit for at least 12 mo, and liver biopsy taken within 3 mo before enrollment showing chronic hepatitis. Eligible patients with evidence of cirrhosis were also included. Patients treated with immunosuppressive or antiviral therapy within 1 year before entry, and those with concurrent hepatitis C virus, hepatitis delta virus, and human immunodeficiency virus infections, causes of liver disease other than HBV, intravenous drug abuse, pregnancy, malignancy, chronic renal failure, or other serious medical illness that might interfere with this trial were excluded.

Another 30 patients with the same virological and clinical characteristics, never treated with IFN-α and followed up for at least 12 mo were used as a historical control (HC) group to evaluate the efficacy of the therapies.

### Methods

The 62 patients were randomly divided into two groups to receive either subcutaneous injection of T-α1 twice a week for 6 mo or of 5 MU IFN-α once a day for 15 d, then 3 times weekly for 6 mo. The patients in HC group were followed up without specific treatment. All patients were assigned to receive general liver-protecting medication in the first month.

Efficacy and safety analysis was performed for all randomized patients who received both thymosin-α1 and interferon-α medication dose in the study. Two predetermined primary measures of efficacy assessed after 24 wk of treatment-free follow-up were HBeAg seroconversion (defined by the loss of HBeAg and the presence of anti-HBe antibody) and suppression of HBV DNA to levels below 1000 copies per milliliter. Secondary efficacy mea-

**Table 1 Characteristics of the patients at entry to the study[1]**

| Characteristics | HBeAg-positive CHB | | |
|---|---|---|---|
| | T-α1 group | IFN-α group | HC group |
| Number | 29 | 33 | 30 |
| Male: Female | 26:3 | 27:6 | 21:9 |
| Age (yr)[2] | 45 ± 8 | 42 ± 11 | 44 ± 10 |
| Duration of infection (yr)[2] | 10.8 ± 5.6 | 9.3 ± 4.7 | 9.6 ± 5.5 |
| Cirrhosis at entry | 2 | 3 | 2 |
| Previous 1FN therapy | 2 | 0 | 0 |
| Serum ALT (U/L)[2] | 177.79 ± 58.82 | 189.94 ± 63.08 | 183.5 ± 68.32 |
| Serum AST (U/L) | 167.72 ± 34.19 | 154.27 ± 73.34 | 141.8 ± 49.85 |
| Albumin (g/L)[2] | 44 ± 9 | 47 ± 8 | 45 ± 8 |
| Total bilirubin (μmol/L) | 18.57 ± 9.65 | 17.92 ± 9.22 | 16.68 ± 8.72 |
| Serum HBV DNA (copies/mL)[3] | | | |
| $< 5.0 \times 10^5$ copies/mL | 10 (34%) | 15 (45%) | 13 (43%) |
| $\geqslant 5.0 \times 10^5$ copies/mL | 19 (66%) | 18 (55%) | 17 (57%) |

[1]No significant differences were observed among groups. [2]Data are expressed as mean ± SD. [3]Data are expressed as *n* (%). Normal values: ALT < 40 IU/L; AST < 40 IU/L; albumin = 35-55 g/L; total bilirubin < 17 μmol/L. ALT: Alanine aminotransferase; AST: aspartate aminotransferase; HBV: hepatitis B virus; T-α1: thymosin - α1; IFN-α: interferon -α; HC: historical control.

sures assessed after 24 wk of treatment-free follow-up included the combined response (HBeAg seroconversion, normalization of ALT levels, and suppression of HBV DNA levels to below 1000 copies per milliliter). Measures of safety included adverse events, vital signs, hematologic measurements, clinical and chemical measurements, and routine urinalysis. The severity of adverse events was graded on a three-point scale (mild, moderate, and severe), and causality was determined by the investigator. These safety parameters were assessed at wk 0, 1, 2, 4, 6, 8, and every four weeks thereafter throughout the treatment and during the follow-up.

All patients were assessed biweekly for the first 2 mo, and then monthly for 12 mo. Clinical and laboratory assessments consisted of a detailed history, including post injection symptoms and physical examination, routine serum biochemical tests [serum ALT, aspartate transaminase (AST), r-glutamine transpeptidase (r-GT), alkaline phosphatase (AKP), albumin, globulin, bilirubin, *etc*], complete cell count, markers of HBV replication and urine analysis. All biochemical and hematological tests were performed with routine automated techniques. HBV-markers (HBsAg, HBsAb, HBeAg, HBeAb, HBcAb, and Igm HBcAb) were measured at virological laboratory by using enzyme linked immunosorbent assay (ELISA). Serum HBV DNA was detected by polymerase chain reaction (PCR).

Responses were evaluated both at the end of therapy and at the end of follow-up. A complete virological response was defined as a sustained loss of serum HBeAg in association with the disappearance of serum HBV DNA during the 12-mo study period. A biochemical response was defined as sustained normalization of serum ALT. At the end of treatment and follow-up, a complete response was defined as HBV DNA and HBeAg clearance from the serum and normalization of ALT activity. Relapse was assessed on the basis of ALT flare and/or HBV DNA/HBeAg reappearance during the follow-up period.

**Table 2 Response to treatment at the end of therapy *n* (%)**

| | T-α1 (*n* = 29) | IFN-α (*n* = 33) | HC (after 6 mo of follow-up) (*n* = 30) |
|---|---|---|---|
| ALT normalization | 11 (37.9) | 16 (48.5)[b] | 5 (16.7) |
| Negative HBV DNA | 16 (55.2)[c] | 22 (66.7)[c] | 2 (6.7) |
| ALT normal/nagative HBeAg & HBVDNA | 9 (31.0)[b] | 15 (45.5)[c] | 1 (3.3) |

[b]$P < 0.01$ *vs* HC; [c]$P < 0.001$ *vs* HC.

**Table 3 Response to treatment at the end of follow-up *n* (%)**

| | T-α1 (*n* = 29) | IFN-α (*n* = 33) | HC (after 12 mo of follow-up) (*n* = 30) |
|---|---|---|---|
| ALT normalization | 17 (58.6)[c,f] | 10 (30.3) | 3 (10) |
| Negative HBV DNA | 21 (72.4)[d,f] | 13 (39.4)[b] | 2 (6.7) |
| ALT normal/negative HBeAg & HBV DNA | 14 (48.3)[f] | 9 (27.3)[a] | 1 (3.3) |

[a]$P < 0.05$ *vs* HC; [c]$P < 0.05$ *vs* IFN; [b]$P<0.01$ *vs* HC; [d]$P < 0.01$ *vs* IFN; [f]$P < 0.001$ *vs* HC.

### *Statistical analysis*

Initial calculation was performed with a sample size of 20 patients per treatment group, which provided a statistical power of 80% at the 0.025 level of significance to detect the difference in negative HBV DNA (suppression below 1000 copies per milliliter) rates of 45% *vs* 6.7% or HBeAg seroconversion rates of 40% *vs* 3.3%. The sample size increased in 25 patients to allow for withdrawals.

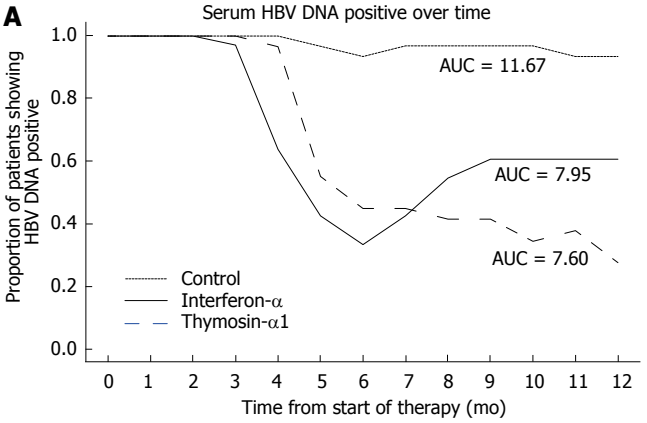
Differences in the final virological and biochemical response rates of two treatment groups and the non-treatment group were compared using the chi square test or Fisher's exact test when the expected number in the cells was below 5. Odds ratio and corresponding 95% confidence intervals were also computed. $P \leqslant 0.05$ was considered statistically significant.

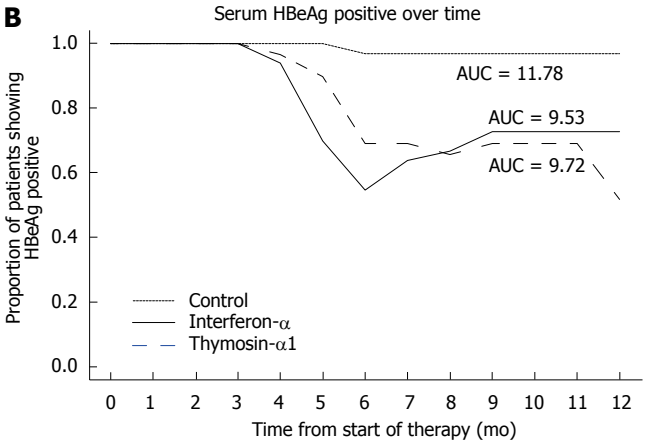
## RESULTS

Of the 92 patients enrolled in the study, 30 were never treated with IFN-α for HBV infection, 2 were non-responders to earlier IFN-α therapy who received T-α1 in the study, 29 patients were randomized to receive T-α1 and 33 to receive IFN-α. All patients completed the 6-mo follow-up period. No significant difference in age, sex, clinical history, biochemical, histological and serological parameters was found among the 3 groups (Table 1).

The biochemical and virological modifications at the end of treatment and follow-up period in the two treated groups and the biochemical and virological events in the HC group are listed in Tables 2 and 3 and Figure 1.

At the end of treatment, IFN treatment group had the highest ALT normalization followed by the T-α1 and HC groups, with no statistical significance. The IFN treatment group also had the highest rate of negative HBV DNA and other combined favorable results, differing from those



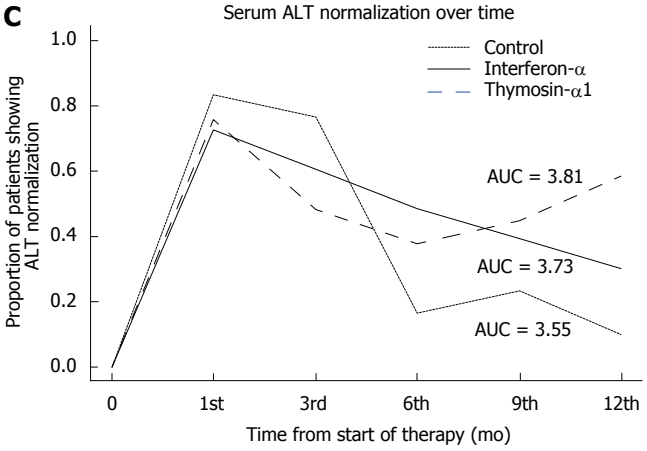




**Figure 1** Rates of virologic response (**A**, **B**) and biochemical response (**C**) during the 12-mo study period. A virologic response was defined as a serum HBV DNA loss (HBV DNA level of less than 1000 copies per milliliter) and a serum HBeAg loss. A biochemical response was defined as the normalization of alanine aminotransferase levels.

of the HC group but not from those of the T-α1 group. During the follow-up period, the results were however more favorable in the T-α1 group. This group had seven additional patients showing HBV DNA loss (at mo 2-4 in 4 patients and at mo 6 in 3 patients, respectively), whereas HBV DNA reappeared in two patients (at mo 3 and 5, respectively). In the group receiving IFN-α, HBV DNA reappeared in 9 patients (in 3 patients at mo 1, 4 at mo 2 and 2 at mo 3), while no one lost HBV DNA. In the HC group, HBV DNA became negative in 3 of 30 patients (at mo 5, 6, and 11, respectively), whereas HBV DNA reappeared in one patient (at mo 7).

**Table 4 Response to treatment between thymosin-α1 & IFN-α groups**

| | At the end of therapy OR (95% CI) | At the end of follow-up OR (95% CI) |
|---|---|---|
| ALT normalization | 0.65 (0.21, 2.01) | 3.19 (1.02, 10.61)[a] |
| HBVDNA-negation | 0.62 (0.19, 1.94) | 3.94 (1.23, 13.69)[b] |
| HBeAg-negation | 0.55 (0.17, 1.72) | 2.45 (0.77, 8.23) |
| ALT normal/ HBeAg & HBVDNA-negation | 0.55 (0.17, 1.72) | 2.45 (0.77, 8.23) |

[a]$P < 0.05$, [b]$P < 0.01$ *vs* IFN-α.

HBV DNA loss was significantly higher in the T-α1 and IFN-α groups than in the HC group at the end of therapy ($\chi^2 = 16.36$, $P < 0.001$ and $\chi^2 = 23.99$, $P < 0.001$, respectively) and follow-up period ($\chi^2 = 26.81$, $P < 0.001$ and $\chi^2 = 9.28$, $P < 0.01$, respectively). The rates of seroconversion of HBeAg antibody in the T-α1, IFN-α and HC groups were 31.0% (9/29), 45.5% (15/33) and 3.3% (1/30), respectively at the end of treatment, and 48.3% (14/29), 27.3% (9/33) and 3.3% (1/30), respectively at the end of follow-up. Serum ALT levels returned to the normal range in 11 of 29 patients given T-α1, in 16 of 33 patients in IFN-α group at the end of treatment and in 5 of 30 of the HC group after 6 mo of follow-up. During the follow-up period, 7 additional patients receiving T-α1 had normal ALT and one patient showed ALT flare, whereas 6 patients of the IFN-α group showed ALT flare, and no one had normal ALT. In the HC group, two patients had normal ALT during months 6 and 12 of follow-up and ALT flare was seen in 4 patients who had normal ALT during the first 6 mo of follow-up. At the end of the study period a complete response (ALT normalization and HBV DNA/HBeAg loss) was observed in 14 of 29 (48.3%) patients treated with T-α1, in 9 of 33 (27.3%) receiving IFN-α, and in 1 of 30 (3.3%) in HC group (T-α1 *vs* IFN-α and HC, $\chi^2 = 2.93$, $P > 0.05$ and $\chi^2 = 15.71$, $P < 0.001$).

As shown in the graphs (Figure 1), the AUC of HBV DNA and negative HBeAg was 31% and 19% smaller in IFN-α-treated subjects than in HC subjects. The AUC of ALT normalization was 1.5% greater in IFN-α-treated subjects than in HC subjects. The AUC of HBV DNA and negative HBeAg was 34% and 17% smaller in T-α1 treated subjects than in HC subjects, and was 2.2% greater in IFN-α-treated subjects than in HC subjects. The AUC of HBV DNA and negative HBeAg in T-α1 group was 2.9% and 1.6% smaller than in IFN-α-treated subjects.

By the end of the follow-up period, the proportions of ALT normalization and negative HBV DNA in the T-α1 group were significantly higher than those in the IFN-α and HC groups. The odds of ALT normalization and negative HBV DNA at the end of the follow-up was more than three-fold higher in the T-α1 group than in the IFN-α group (Table 4).

Typical side effects of IFN-α treatment, such as flu-

like syndrome, fatigue, irritability, and headache, were seen in most of the patients treated with IFN-α. However, no serious or long-term side effects were noted and no patients discontinued the treatment. Therapy with T-α1 was not associated with significant side effects. Three patients reported local discomfort at injection sites. No systemic or constitutional symptoms were observed after T-α1 administration.

## DISCUSSION

The results of the present randomized, controlled trial have shown that T-α1 therapy at a dose of 1.6 mg *via* subcutaneous injection, twice a week for 6 mo, is effective and safe in patients with chronic hepatitis B. Compared with IFN-α, T-α1 has a slower speed but more sustainable and better end results of virological clearance. Nearly 50% of the treated patients became seronegative for HBeAg and HBV DNA 6 mo after the therapy. This response rate was not only significantly higher than that of the spontaneous seroconversion rate (3.3% in this study), but also obviously higher than the response to IFN-α therapy alone (27.3%) assessed 6 mo after the therapy, suggesting that T-α1 has the same efficacy as IFN-α in inducing clinical and virological remission of chronic hepatitis. The rate of response in terms of ALT normalization and/or HBV DNA and/or HBeAg loss was not significantly different in the T-α1 group compared with the IFN-α group at the end of the treatment ($P > 0.05$). However, there was a significant difference in the rate of response between the two groups at the end of the follow-up period ($P < 0.05$). The normalization of serum ALT and loss of HBV DNA and HBeAg was observed more frequently in the IFN-α group at the end of therapy and in the T-α1 group at the end of the follow-up period. Furthermore, the response to the treatment was observed also in the T-α1 group during the follow-up period, but not in the IFN-α group. On the basis of these results and considering that ALT normalization and negative HBV DNA and HBeAg may spontaneously occur in untreated patients, we retrospectively compared the two treated groups with a untreated group which was followed up for at least 12 mo. The results showed that a significant higher rate of complete response occurred in the IFN-α group at the end of therapy and in the T-α1 group at the end of follow-up period than that in the HC group.

The benefit of T-α1 was not immediately significant at the end of therapy and complete virological response had a tendency to increase or accumulate gradually after the therapy in our study. In contrast, the effect of IFN-α was relatively more quick but less sustainable. The beneficial effect of T-α1 has been reported in a multicenter American trial in which 5 of the 12 responders to T-α1 therapy showed a delayed response[43]. This is in contrast to therapy with IFN-α, in which responses usually occur during the first 4 mo of treatment. These contrasting patterns of response have been demonstrated in a recent Italian study involving HBeAg-negative, HBV DNA-positive, interferon-naive patients with a higher ALT level (181 ± 159 U/L), in which the complete response (ALT normalization and HBV DNA loss) rate increased gradually from 29.4% at the end of therapy to 41.2% 6 mo after the T-α1 therapy, the response to IFN therapy decreased from 43.8% at the end of therapy to 25% 6 mo after the therapy[44]. This delayed effect of T-α1 has also been reported by Chien *et al*[45,46] in patients with chronic hepatitis B recently.

The reasons for the delayed effect of T-α1 are not clear. The delayed response is not likely a result of direct antiviral effects similar to those of interferon. T-α1 may exert an immunoregulatory function that promotes the endogenous antiviral immune response, improves the effectiveness and coordination of the host cellular immune mechanisms in clearing HBV-infected hepatocytes. It was reported that patients treated with T-α1 have a higher peripheral blood helper T cell count (CD4) and IFN- r production by peripheral blood mononuclear cells during and after the T-α1 therapy[39]. In view of the immune mechanisms involved in the pathogenesis of liver injuries in chronic HBV infection, it is possible that T-α1 may activate viral-specific helper T cells, result in the amplification of humoral immune response to viral proteins, induce viral antigen-specific cytotoxic T lymphocytes through secreting endogenous IFN-α, IFN-r, interleukin-2, and tumor necrosis factor, and increase lymphocyte interleukin-2 receptor expression[47-58]. Moreover, T-α1 is able to stimulate natural killer activity by acting synergistically with endogenous IFN-α and IFN-β[41]. Although T-α1 is not known to possess antiviral properties, it was reported that this agent is able to inhibit woodchuck hepatitis virus replication[59,60], suggesting that the delayed effect after T-α1 therapy in the present study is possibly caused by the immunomodulating effect of T-α1 that induces persistently higher helper T-cell function. Because noncytolytic inhibition of HBV RNA, nucleocapsid particles, and replicative DNA intermediates by cytotoxic T lymphocytes has been described in the transgenic mouse model[61,62], it is also possible that viral clearance after T-α1 therapy, particularly preceding ALT flare, is mediated by noncytolytic antiviral effects of cytotoxic T lymphocytes. Further studies are needed to elucidate the possible mechanisms.

In addition to having better HBV clearance, T-α1 is also more tolerable than IFN-α and has no side effects. These together with a small number of weekly injections could favor better patient compliance.

In conclusion, the results of this trial indicate that a 6-mo T-α1 therapy is safe and effective in arresting HBV replication and reducing lobular activity in patients with chronic hepatitis B. Furthermore, T-α1 is better tolerated than IFN-α, and may gradually induce more sustained ALT normalization and HBV DNA/HBeAg loss. A more effective therapeutic approach warrants further study.

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*RAPID COMMUNICATION*

# Hyperbilirubinemia after extracorporeal circulation surgery: A recent and prospective study

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## Abstract

**AIM:** To clarify the incidence and nature of postoperative hyperbilirubinemia in patients after modern extracorporeal circulation, to analyze possible perioperative risk factors, and to elucidate the clinical significance of postoperative hyperbilirubinemia associated mortality and morbidity.

**METHODS:** Between March 2005 and May 2006, three hundred and eighty six consecutive patients undergoing extracorporeal circulation surgery due to a variety of cardiac lesions were investigated prospectively. The incidence of postoperative hyperbilirubinemia was defined as a serum total bilirubin concentration of more than 51 μmol/L. Several perioperative parameters were compared by logistic regression between hyperbilirubinemia and non-hyperbilirubinemia patients to determine possible risk factors contributing to postoperative hyperbilirubinemia and mortality.

**RESULTS:** Overall incidence of postoperative hyperbilirubinemia was 25.3% (98/386). In patients with postoperative hyperbilirubinemia, 56.2% reached peak total bilirubin concentration on the first postoperative day, 33.5% on the second day, and 10.3% on the seventh day. Eighty percent of the increase of total bilirubin resulted from an increase of both conjugated and unconjugated bilirubin. Development of postoperative hyperbilirubinemia was associated with a higher mortality ($P < 0.01$), longer duration of mechanical ventilation ($P < 0.05$) and longer ICU stay time ($P < 0.05$). Preoperative total bilirubin concentration, preoperative right atrium pressure, numbers of valves replaced and of blood transfusion requirement were identified as important predictors for postoperative hyperbilirubinemia.

**CONCLUSION:** Early postoperative hyperbilirubinemia after modern extracorporeal circulation is mainly caused by an increase in both conjugated and unconjugated bilirubin, and is associated with a high mortality. Important contributing factors are the preoperative total bilirubin concentration, preoperative severity of right atrial pressure, numbers of valve replacement procedures, and the amount of blood transfusion requirement during and shortly after surgery. We suggest that postoperative hyperbilirubinemia is a multifactorial process, which is caused by both the impaired liver function of bilirubin transport and the increased production of bilirubin from haemolysis.



**Key words:** Extracorporeal circulation; Open-heart surgery; Hyperbilirubinemia; Liver function

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## INTRODUCTION

It has long been recognized that early hyperbilirubinemia or transient jaundice could occur after extracorporeal circulation surgery. According to earlier studies, overall incidence of postoperative hyperbilirubinemia ranges from about 8.6% to even as high as 40%[1-3]. Despite improvements in perioperative management, as well as in surgical and anaesthetic techniques, early hyperbilirubinemia after modern extracorporeal circulation surgery is still seen quite often. The high frequency of hyperbilirubinemia after cardiac surgery in our department prompted us to start a prospective study recently. It is aimed to clarify the incidence and nature of postoperative hyperbilirubinemia in patients following modern extracorporeal circulation, to analyze possible perioperative risk factors, and to elucidate the clinical significance of postoperative hyperbilirubinemia associated mortality and morbidity.

## MATERIALS AND METHODS

### *Patients' backgrounds*

Three hundred and eighty six patients older than 17 years undergoing extracorporeal circulation surgery from March 2005 to May 2006 in our department were consecutively

**Table 1 Comparison of perioperative changes of liver function in two groups (mean ± SD)**

| Variable | HB group | | | | NHB group | | | |
|---|---|---|---|---|---|---|---|---|
| | Preop | d1 | d2 | d7 | Preop | d1 | d2 | d7 |
| TB (mmol/L) | 26.8 ± 2.6[d] | 72.7 ± 9.1[b,d] | 82.8 ± 10.4[b,d] | 63.8 ± 7.5[b,d] | 14.5 ± 1.4 | 33.8 ± 5.5[b] | 20.8 ± 4.5 | 17.1 ± 3.7 |
| UCB (mmol/L) | 9.9 ± 1.9 | 47.6 ± 6.5[b] | 40.9 ± 6.7[b,d] | 32.6 ± 5.6[b,d] | 6.5 ± 1.7 | 18.6 ± 3.9[b] | 11.9 ± 3.2 | 9.7 ± 1.9 |
| CB (mmol/L) | 16.7 ± 1.5[d] | 24.7 ± 5.1[b,d] | 39.6 ± 6.1[b,d] | 31.2 ± 5.4[b,d] | 8.4 ± 1.3 | 15.5 ± 3.1[b] | 9.4 ± 2.1 | 7.6 ± 1.6 |
| ALT (IU/L) | 40.6 ± 3.9[d] | 112.6 ± 11.6[b,d] | 176 ± 15.6[b,d] | 56.6 ± 5.5[d] | 28.5 ± 2.6 | 84.6 ± 11.6[d] | 60.6 ± 5.7 | 50.2 ± 5.1 |
| AST (IU/L) | 40.5 ± 3.6 | 102.6 ± 10.7[d] | 184 ± 17.1[b,d] | 53.2 ± 5.2[d] | 42.6 ± 3.3 | 80.5 ± 10.5[d] | 55.1 ± 5.5 | 48.3 ± 4.9 |

Preop: Preoperative; d1, d2, d7: first, second, and seventh postoperative days, respectively. [b]$P < 0.01$ versus Preop: [d]$P < 0.01$ HB group versus NHB group at the same time point.

enrolled into the study. One hundred and eighty were male and 206 were female. Their ages ranged from 17 to 78 years (mean, 47 years). The disease categories comprised valve disease (Valve, 220 cases), congenital heart disease (CHD, 140 cases), coronary atherosclerotic heart disease (CAHD, 16 cases) and valve combined with coronary disease (Valve + CAHD, 10 cases). The protocol was approved by the Committee of Human Study of our institution. Patients were not selected with any other major diseases, such as viral hepatitis, HIV, and syphilis. Patients with preoperative hyperbilirubinemia were defined as a total bilirubin concentration of more than 2 mg/dL (34 μmol/L). Postoperative hyperbilirubinemia was defined as a total bilirubin level over 3 mg/dL (51 μmol/L) within the first postoperative week, and accordingly, patients were divided into two groups: postoperative hyperbilirubinemia group (HB group) and non-postoperative hyperbilirubinemia group (NHB group).

### Surgery and parameters tested

Anesthesia was induced with fentanyl (40 to 100 μg/kg), etomidate (0.3 to 0.4 mg/kg) or vecuronium (0.1 to 0.15 mg/kg). All patients had cardiopulmonary bypass (CPB), with isovolemic haemodilution, moderate hypothermia (28℃ ± 5℃), roller pump and bubble or membrane oxygenators. The perfusion flow was kept over 2.2 L/m$^2$ during northemia and over 1.8 L/m$^2$ during hypothermia in every patient. The mean arterial pressure (MAP) was kept between 50 to 80 mmHg with dopamine, nitroglycerin, and nitroprusside. Arterial blood gas was monitored routinely every half an hour or on any occasion when considered necessary. The priming solution contained 1.5 to 2.0 L of lactated Ringer's solution, heparin (2000 U/L) and 1/2 to 1 unit of packed red cells if blood cardioplegic solution was indicated or predicted hematocrit level during CPB was blow 20%. Systemic heparin was given through the right atrium at a dose of 300 U/kg just before cannulation. For valvular replacements operations, either a Carbomedics (Carbomedics, Inc) or a St, Jude Medical (St, Jude Medical, Inc) Mechanical prosthesis was used.

### Parameters tested

**Preoperative**: Blood samples were collected within 2 d before operation by vein puncture. Serum was stored at -70℃ for future biochemical and virological studies. Preoperative right atrial pressure was monitored through the preoperative cardiac catheterization recorder. Full blood-count, urea and electrolytes, alanine amino transferase (ALT), aspartate amino transferase (AST), total bilirubin (TB), unconjugated bilirubin(UCB), and conjugated bilirubin (CB) were tested. In all patients, serological viral studies were performed to rule out infection with hepatitis A (HAV IgG + IgM), hepatitis B (HBsAg and HBcAb), hepatitis C (HCV - IgM) and HIV virus.

**Perioperative**: In every operation, the routine clinical monitors including lead electrocardiogram, the radial arterial line, the pulse oximeter, end-tidal carbon dioxide, nasopharyngeal and rectal temperatures, urine output via Foley catheter, the central venous pressure line, pulmonary artery catheters, the operating time, CPB time, aortic cross-clamp time, types of oxygenator, quantity of packed red cells primed during CPB, and the types and numbers of mechanical valves replaced were recorded.

**Postoperative**: We recorded right atrial pressure, minimum MAP, and minimum arterial oxygen tension ($mPO_2$) daily. Blood samples were obtained through the central venous line or venopuncture on the first, second and seventh postoperative days and analyzed for concentrations of albumin, globulin, haemoglobin, ALT, AST, TB, UCB, CB, and reticulocyte count by an automated biochemical analyzer (Beckman Array 3000, USA). Serum bile acids (cholyglycine) were tested using stored serum (RIA). Reticulocyte count, plasma haemoglobin, haptoglobin levels, urinary haemosiderin, and urobilinogen were examined to detect haemolysis. The blood transfusions shortly after surgery, the days of hospitalization in the intensive care unit (ICU), mechanical ventilation days, and the numbers of patients who died during hospitalization were registered.

### Statistical analysis

Statistical analysis was carried out using SPSS version 10.0 software (SPSS, inc, USA). Data were presented as mean ± SE of the mean. The chi-squared test and Student's *t* test were used to compare categorical variables. Stepwise logistic regression was employed for multivariate analysis. Values of *P* less than 0.05 were considered significant.

## RESULTS

The perioperative changes of liver function are shown in Table 1. The incidence of the postoperative hyperbilirubinemia in these patients is shown in Figure 1.

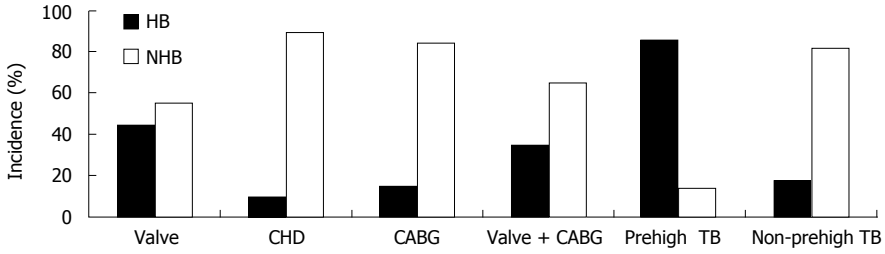


**Figure 1** Incidence of postoperative hyperbilirubinemia among different disease categories, and in patients with and without preoperative hyperbilirubinemia; CHD: Congenital heart disease, CABG: Coronary artery bypass grafting.

**Table 2 Comparison of mortality, ventilation time and ICU stay time in two groups (mean ± SD)**

| Variable | HB group | NHB group | *P* value |
|---|---|---|---|
| Mortality | 4.10% | 0.3%[b] | $P < 0.01$ |
| Ventilation time (h) | 25.5 ± 13.3 | 16.5 ± 9.2[a] | $P < 0.05$ |
| ICU stay time (d) | 11.5 ± 6.5 | 4.1 ± 3.9[a] | $P < 0.05$ |

[b]$P < 0.01$ *vs* HB group; [a]$P < 0.05$ *vs* HB group.

Among the 386 patients enrolled in this study, 36 had preoperative hyperbilirubinemia. The overall incidence of the postoperative hyperbilirubinemia was 25.3%. For patients with preoperative hyperbilirubinemia, the incidence of postoperative hyperbilirubinemia was 86.1%.

In patients with postoperative hyperbilirubinemia, 56.2% reached a peak total bilirubin concentration on the first postoperative day, 33.5% on the second day, and 10.3% on the seventh day. Among the patients with preoperative hyperbilirubinemia, 69% had severe postoperative hyperbilirubinemia with highest TB concentration greater than 171 μmol/L; whereas, in other patients, only 18% had a highest TB concentration greater than 171 μmol/L ($P < 0.01$). The highest TB concentration was significantly greater in patients with preoperative hyperbilirubinemia than in patients without (210.8 ± 28.4 μmol/L *vs* 62.8 ± 9.6 μmol/L, $P < 0.01$). On the first postoperative day, the TB, UCB, and CB concentrations increased in both the patients in HB and NHB groups compared with preoperative levels ($P < 0.01$, Table 1). For the patients with postoperative hyperbilirubinemia, 80.2% of the increased TB was from an increase of the mixture of CB and UCB.

With regard to the effects of the disease and operation category, postoperative hyperbilirubinemia occurred more frequently in patients receiving valvular replacements than in patients undergoing coronary artery bypass grafting (CABG) or operation for CHD($P < 0.01$, Figure 1). The incidence of postoperative hyperbilirubinemia was significantly higher in patients with valvular replacements with mechanical prostheses than in the patients without valvular replacements ($P < 0.01$, Figure 1). The result was similar when patients with preoperative hyperbilirubinemia were excluded.

The comparison of mortality, ventilation time and ICU stay time are shown in Table 2. Only one patient undergoing CABG died in NHB group. He died on 12th d after the operation of respiratory failure. Among four operative mortality cases in the HB group, three had a progressive increase in TB after operation. The highest TB concentration level (over 400 μmol/L) was reached on the seventh postoperative day. One had an increase in TB immediately on the first postoperative day. Two of the four died from hepatic failure, and another two died from multiple organic function failure. Development of postoperative hyperbilirubinemia was associated with a higher mortality ($P < 0.01$), longer duration of mechanical ventilation ($P < 0.05$), and longer ICU stay time ($P < 0.05$).

Analysis of possible risk factors for the postoperative hyperbilirubinemia is shown in Table 3. The results of stepwise logistic regression are shown in Table 4. Preoperative total bilirubin concentration, preoperative

**Table 3 Analysis of possible risk factors for the postoperative hyperbilirubinemia (mean ± SD)**

| Possible risk factors | HB group | NHB group | *P* |
|---|---|---|---|
| Age (yr) | 47 ± 1.16 | 46. ± 1.02 | > 0.25 |
| Body surface area ($m^2$) | 1.66 ± 0.01 | 1.63 ± 0.03 | > 0.10 |
| Preoperative TB (μmol/L) | 26.8 ± 2.6 | 14.5 ± 2.3 | < 0.01 |
| Preoperative CB (μmol/L) | 16.7 ± 1.4 | 8.4 ± 1.5 | < 0.01 |
| Preoperative TB/CB | 1.69 ± 0.01 | 1.71 ± 0.02 | > 0.30 |
| Preoperative ALT (IU/L) | 40.6 ± 3.9 | 28.5 ± 2.6 | < 0.05 |
| Preoperative AST (IU/L) | 40.5 ± 3.6 | 42.6 ± 3.3 | > 0.10 |
| Right atrial pressure (mmHg) | 9.5 ± 0.6 | 4.9 ± 0.5 | < 0.01 |
| Pulmonary artery pressure (mmHg) | 35.5 ± 1.5 | 23.5 ± 1.3 | < 0.01 |
| Blood transfusion requirement (u) | 3.56 ± 0.02 | 2.15 ± 0.03 | < 0.01 |
| Operation time (min) | 220.5 ± 6.0 | 205.5 ± 5.3 | > 0.15 |
| CPB time (min) | 130.3 ± 4.2 | 112.2 ± 3.1 | < 0.01 |
| Aortic crossclamp time (min) | 76.6 ± 3.1 | 68.6 ± 2.3 | < 0.01 |
| Lowest nasopharyngeal temperature (℃) | 26.7 ± 0.4 | 26.3 ± 0.3 | > 0.25 |
| Lowest MAP (mmHg) | 68.6 ± 1.3 | 65.9 ± 1.5 | > 0.15 |
| Lowest $PO_2$ (mmHg) | 66.6 ± 1.4 | 68.7 ± 1.5 | > 0.20 |

**Table 4 Results of logistic regression for the risk factors of postoperative hyperbilirubinemia**

| Variable | β | SE | Significance | R |
|---|---|---|---|---|
| Preoperative TB | 1.274 | 0.312 | 0.000 | 0.205 |
| Right atrial pressure | 0.113 | 0.032 | 0.006 | 0.147 |
| Number of valves replaced | 0.658 | 0.302 | 0.025 | 0.152 |
| Blood transfusion requirement (u) | 0.109 | 0.033 | 0.006 | 0.118 |
| Constant | -3.453 | 0.466 | 0.000 | |

β: Regression coefficient; SE: Standard error; R: Partial correlation coefficient.

right atrial pressure, numbers of valves replaced and blood transfusion requirement were identified as important predictors for the postoperative hyperbilirubinemia. Combination of these four perioperative risk factors could predict development of postoperative hyperbilirubinemia in 81.2% of the patients.

## DISCUSSION

It has been well known that early jaundice and transient liver damage could occur after extracorporeal circulation surgery[1,2]. The mortality can reach 85%-90% once the postoperative jaundice progresses to hepatic failure[4-7]. On the other hand, for patients with preoperative cirrhosis of liver, mortality of CPB surgery can reach 31%[8]. Thus recently, more attention has been paid to the studies of jaundice and liver injuries following CPB.

This prospective study showed that the overall incidence of the postoperative hyperbilirubinemia was 25.3%, similar to the literature report[3]. Despite advances in techniques of perioperative management in cardiac operations, CPB, and cardiac anaesthesia, early hyperbilirubinemia after modern extracorporeal circulation surgery still occurs frequently. With regard to the effects of the disease and operation category, controversies exist about whether incidence differs among different disease category[2,3]. The current results showed that postoperative hyperbilirubinemia occurred more frequently in patients receiving valvular replacements than in patients undergoing CABG or operation for CHD ($P < 0.01$). The incidence of postoperative hyperbilirubinemia was significantly higher in patients with valvular replacements with mechanical prostheses than in the patients without receiving valvular replacements ($P < 0.01$). For patients with preoperative hyperbilirubinemia, the incidence of the postoperative hyperbilirubinemia was 86.1%. The decreased hepatic capacities for bilirubin disposal and bile transport, in addition to the haemolysis from CPB, cardiotomy suction, and mechanical prosthesis result in the postoperative hyperbilirubinemia. Both Weber[1] and Lockey[2] proposed that haemolysis contributed to postoperative jaundice; in contrast, Collins[3] and Chu[4] reported that serum TB concentration mainly resulted from increased CB, i.e., hepatic cellular jaundice. However, for the patients with postoperative hyperbilirubinemia in our study, 80.2% of the increased TB was due to an increase of both CB and UCB; 56.2% reached peak TB concentration on the first postoperative day, 33.5% on the second day and 10.3% on the seventh day. Among the patients with preoperative hyperbilirubinemia, 69% had severe postoperative hyperbilirubinemia with highest TB concentration greater than 171 μmol/L, while in other patients only 18% had a highest TB concentration greater than 171 μmol/L ($P < 0.01$). Development of postoperative hyperbilirubinemia was associated with a higher mortality ($P < 0.01$), longer duration of artificial ventilation ($P < 0.05$), and longer ICU stay time ($P < 0.05$). Furthermore, the mortality of the patients with postoperative hyperbilirubinemia was higher in patients whose TB concentration reached the peak level on the seventh postoperative day than in patients whose TB concentration increased to peak level on the first two days after operation. The occurrence of postoperative hyperbilirubinemia should alert the doctor to the possibility of higher morbidity and mortality, though it might not be considered as a high risk indicator.

Results of the logistic regression showed that preoperative total bilirubin concentration, preoperative right atrium pressure, numbers of valves replaced, and blood transfusion requirement were identified as the important predictors for the postoperative hyperbilirubinemia. Combination of these four perioperative risk factors could predict development of postoperative hyperbilirubinemia in 81.2% of all patients. Patients with severe preoperative cardiac failure may have higher right atrial pressure and preoperative hyperbilirubinemia, both reflecting the degree of liver congestion. The capacity of both bilirubin disposal and bile transport may be impaired[6], which also can lead to a higher preoperative TB level. Collins *et al* had suggested that severe heart failure predisposes the patients to the development of clinical jaundice after CPB[3]. Multiple valve replacements with mechanical prostheses are associated with more haemolysis as a result of CPB, cardiotomy suction, and mechanical prosthesis itself. Haemolysis of transfused blood is a common cause for postoperative jaundice. Our patients who developed postoperative jaundice had received significantly more blood transfusions, which would certainly cause an increased bilirubin load to the liver[9]. The immediate occurrence of postoperative hyperbilirubinemia and rapid decline thereafter may reflect the transient damaging effects on the blood and hepatic function following CPB; whereas, steady progressive increase of TB level to reach its peak on the seventh day indicates liver injury and hepatic dysfunction. CPB can lead to a severe and complicated change of pathophysiology of the liver. Operation stress, reperfusion injury, endotoxemia, and inflammatory reaction may contribute to the liver injury[6,10,11], however, the complex functional and metabolic effects of hepatocyte in CPB require further investigation.

In summary, early postoperative hyperbilirubinemia often occurs after modern extracorporeal circulation. It is mainly caused by an increase in both conjugated and unconjugated bilirubin, and is associated with a higher mortality. Important contributing factors are the preoperative total bilirubin concentration, preoperative severity of right atrial pressure, numbers of valve replacement procedures, and the frequencies of blood transfusion requirement during and shortly after surgery. We suggest that postoperative hyperbilirubinemia is a multifactorial process, which is caused by both the impaired liver function of bilirubin transport and increased production of bilirubin because of haemolysis.

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*CASE REPORT*

# Hepatocellular carcinoma metastasizing to the skull base involving multiple cranial nerves

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## Abstract

We describe a rare case of HCV-related recurrent multiple hepatocellular carcinoma (HCC) metastasizing to the skull base involving multiple cranial nerves in a 50-year-old woman. The patient presented with symptoms of ptosis, fixation of the right eyeball, and left abducens palsy, indicating disturbances of the right oculomotor and trochlear nerves and bilateral abducens nerves. Brain contrast-enhanced computed tomography (CT) revealed an ill-defined mass with abnormal enhancement around the sella turcica. Brain magnetic resonance imaging (MRI) disclosed that the mass involved the clivus, cavernous sinus, and petrous apex. On contrast-enhanced MRI with gadolinium-chelated contrast medium, the mass showed inhomogeneous intermediate enhancement. The diagnosis of metastatic HCC to the skull base was made on the basis of neurological findings and imaging studies including CT and MRI, without histological examinations. Further studies may provide insights into various methods for diagnosing HCC metastasizing to the craniospinal area.



**Key words:** Hepatocellular carcinoma; Distant metastasis; Skull base; Cranial nerve

## INTRODUCTION

Hepatocellular carcinoma (HCC), the most common primary tumor of the liver, is estimated to cause more than a quarter of a million deaths throughout the world each year. Metastasis is one of the most significant factors affecting prognosis. In HCC, intrahepatic metastasis is frequent, and extrahepatic metastasis to the lung, bone and adrenal glands is detected sometimes, while cranial nerves are not likely to be involved in metastatic HCC. We describe a case of a 50-year-old woman with symptoms of ptosis, fixation of the right eyeball, and left abducens palsy caused by HCC metastasizing to the skull base, diagnosed through neurological findings and imaging studies.

## CASE REPORT

A 50-year-old woman was admitted to Kobe Asahi Hospital in October 2002 with symptoms of ptosis, fixation of the right eyeball and left abducens palsy. Since 1989, multiple HCCs which had occurred repeatedly with the background of type C liver cirrhosis were treated more than ten times with percutaneous ethanol injection (PEI) and transcatheter embolization (TAE). Neurological findings were normal except for disturbances of the right oculomotor and trochlear nerves and bilateral abducens nerves. Laboratory studies on admission disclosed the following abnormal values: aspartate aminotransferase 84 kIU/L (normal 5-35 kIU/L), alanine aminotransferase 38 IU/L (normal 4-43 IU/L), hemoglobin 103 g/L (normal 113-152 g/L), platelets 14.7 $\times 10^{10}$/L (normal 13.0-36.9 $\times 10^{10}$/L), thymol turbidity 5.0 $\times 10^{6}$ IU/L (normal 0-4.0 $\times 10^{6}$ IU/L), zinc surface turbidity 15.1 $\times 10^{6}$ IU/L (normal 2.0-12.0 $\times 10^{6}$ IU/L), and γ-globulin 266 g/dL (normal 105-203 g/dL). The levels of tumor markers were 184 mg/L (normal 0-0.02 mg/L) for alfa-fetoprotein (AFP), 6.4% (normal 0%-10%) for lens culinaris agglutinin A-reactive fraction of AFP (AFP L3) and 22.3 kAU/L (normal

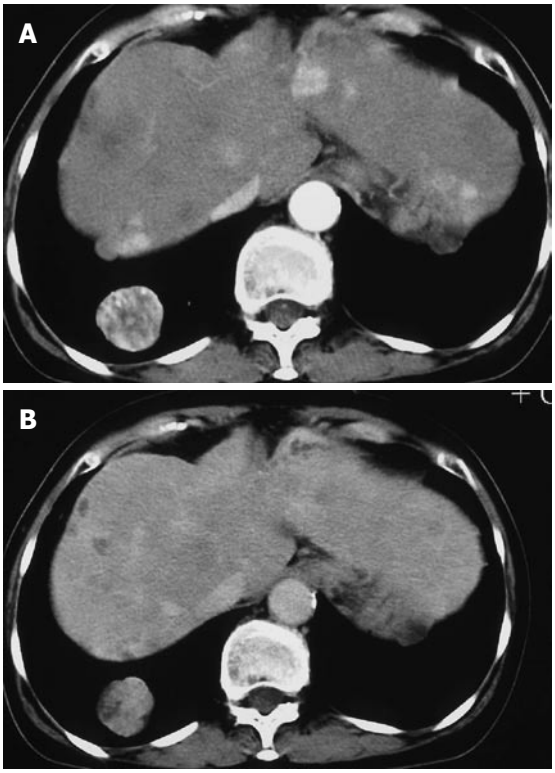


**Figure 1** Abdominal incremental computed tomography (CT) (**A**) showing multiple contrast-enhanced tumors (arterial phase) and abdominal incremental CT scan (**B**) showing low density tumors (portal phase).

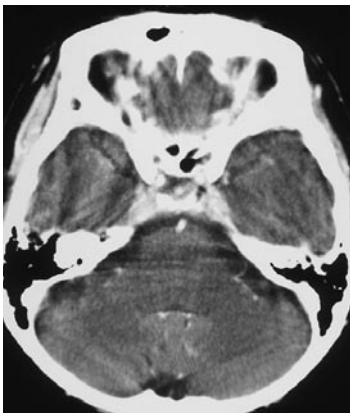

**Figure 2** Brain contrast-enhanced CT image. Abnormal enhancement around the sella turcica is observed.

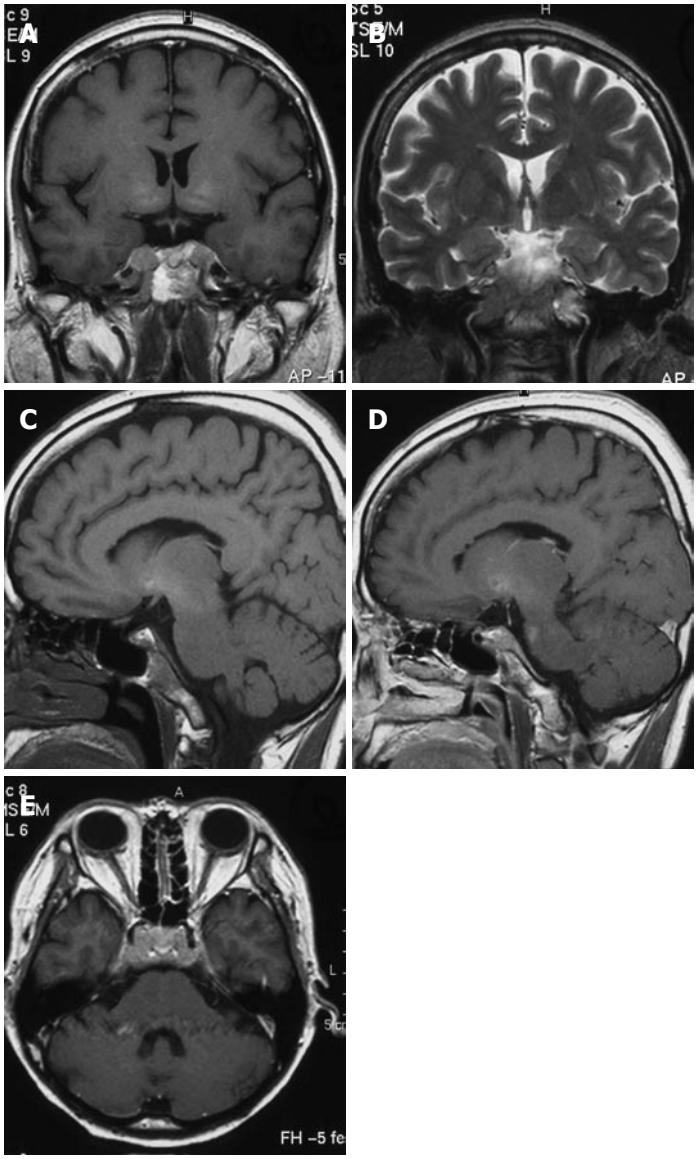


**Figure 3** Brain MRI image. **A**: Unenhanced coronal T1-weighted image reveals ill-defined mass around the sella turnica. The mass shows intermediate signal intensity; **B**: A mass reveals high signal intensity on coronal T2-weighted image; **C**: Unenhanced saggital T1-weighted image reveals ill defined mass involving the sella turcica. **D**, **E**: On contrast-enhanced saggital (**D**) and transverse (**E**) T1-weighted images, the mass shows inhomogeneous intermediate enhancement and involves the clivus, cavernous sinus and petrous apex. Meningeal thickening between the posterior sella turcica and the clivus and dilation of the superior carotid vein are observed.

0-0.04 kAU/L) for protein induced by vitamin K absence (PIVKA Ⅱ). Incremental dynamic computed tomography (CT) revealed multiple HCC nodules in both lobes (Figure 1). Chest X-ray showed extrahepatic metastases to both lungs; however, imaging studies did not show metastases to the bone or adrenal gland. Brain contrast-enhanced CT (Figure 2) revealed an ill-defined mass with abnormal enhancement around the sella turcica. On unenhanced brain magnetic resonance imaging (MRI) the mass was shown to involve the clivus, cavernous sinus, and petrous apex**.** Inhomogeneous intermediate enhancement of the mass was confirmed by contrast-enhanced MRI with gadolinium-chelated contrast medium. Meningeal thickening from the posterior of the sella turcica to the clivus and dilation of the superior carotid vein were observed (Figure 3).

## DISCUSSION

The third, fourth, and sixth cranial nerves may be involved in lesions affecting their nuclei or fibers of efferent in the pons or the mesencephalon, or in the course of nerves through the subarachnoid space, the cavernous sinus, and the superior orbital fissure. Different syndromes occur with lesions at each of these sites. From neurological findings such as ptosis, fixation of the right eyeball, and left abducens palsy, we suspected the involvement of the multiple cranial nerves. Brain CT and MRI with contrast medium revealed abnormal enhancement around the sella turcica, and brain MRI revealed that the mass involved the clivus, cavernous sinus, and petrous apex, therefore we made the definite diagnosis of HCC metastasizing to the

skull base.

Metastasis appears to be a late event in the natural history of HCC, and most patients die of liver failure attributed to liver replacement by the tumor. Extrahepatic metastases are commonly found at autopsy in over half of the cases[1-3], the lung, being the most frequent (approximately 50%) and the regional lymph nodes often being the sites of metastasis. In addition, bone metastases are common, and patients may show a bony metastasis as the initial presentation of occult HCC. Adrenal metastases are found in up to 15% of autopsies, and some cases of pedunculated HCC have been reported as actual metastasis to the right adrenal gland from a nearby tumor. Conversely, the brain is less common metastatic sites. Incidental extrahepatic lesion at such more uncommon site is less confidently viewed as potential metastases when metastatic disease is not detected at the more common sites (the lungs, lymph nodes, and bone). According to neurological findings and imaging studies of brain CT and MRI, our case was confirmed as metastatic HCC to the skull base involving multiple cranial nerves including the third, fourth, and sixth.

The central nervous system is an unusual site of metastatic HCC[4-12]. Seven cases of HCC presenting as cranial metastasis without obvious hepatic involvement have been described: metastatic spread of HCC to the cerebrum in one case, and to the cranium in six cases[8], all manifesting mildly abnormal liver function when first evaluated, leading to the conclusion that in cranial metastasis of unknown origin in a geographical area where HCC is a common disease, HCC should be considered in the differential diagnosis[8]. In Japan as in Taiwan, where HCC is a common disease, however, metastatic HCC to the skull base involving multiple cranial nerves has not been reported so far, except for a case of skull metastasis of HCC associated with acute epidural hematoma[9].

Before 1990, the diagnosis of metastatic HCC to the craniospinal area was confirmed by histological examinations of biopsy, surgical and autopsy specimens. Recently diagnosis is reached by neurological findings and imaging studies, such as CT and MRI because of improvements in these modalities. With the recent advances in the nonsurgical treatment of HCC, such as percutaneous ablation including percutaneous ethanol injection therapy (PEIT), radiofrequency ablation (RFA) and transcatheter embolization, longer-term survival has been attained, even in cases of advanced HCC compared with older autopsy populations. This may be due to current treatment regimens of chemotherapy or chemoembolization, or both, that result in the longer survival of patients diagnosed with HCC, as was in our case. Although HCC metastasizing to the skull base involving multiple cranial nerves is very rare, clinicians need to be vigilant and would do well to conduct imaging studies such as CT and MRI, when neurological findings reveal suspected cranial nerve involvement.

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*CASE REPORT*

# Endoscopic management of a new entity-plastobezoar: A case report and review of literature

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## Abstract

Foreign bodies and bezoars are commonly encountered in children. We describe a child aged 11 years who ingested large amounts of plastic material used for knitting chairs and charpoys. The conglomerate of plastic threads, entrapped food material and other debris, formed a huge mass occupying the whole stomach. Chronic irritation of the gastric antral mucosa led to ulceration and formation of hyperplastic polyps. We labeled this new entity as a "plastobezoar". The entire bezoar could be removed endoscopically.



**Key words:** Bezoar; Stomach; Gastric; Endoscopy; Foreign body; Gastric polyps; Plastic

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## INTRODUCTION

Bezoar is adapted from Arabic "bazahr" or "badzehr" which means an antidote or counter-poison due to the fact that till the 19th century, bezoars obtained from sacrificed animals were widely used as antidote[1]. Foreign bodies and bezoars are commonly encountered in children. We describe a child aged 11 years who ingested large amounts of plastic material used for knitting chairs and charpoys. The conglomerate of plastic threads, entrapped food material and other debris, formed a huge mass occupying the whole stomach and even caused ulceration and polyp formation due to irritation of the stomach wall. It is debatable as to whether to call this a bezoar or a foreign body. The appearance, behavior and management of the gastric mass was more like a bezoar than that of a foreign body, and therefore we strongly feel that it should be called a "plastobezoar".

## CASE REPORT

An 11-year-old boy was admitted with complaints of vomiting and abdominal pain of six months duration and swelling over the face and feet for the last one month.

On clinical examination the child was pale with puffiness over the face. Pedal edema was present. Abdominal examination found a firm lump palpable in the epigastrium, which moved with respiration. No crepitus was felt over the lump. There was mild tenderness present over the mass.

The hemoglobin was 5.6 g/dL, total and differential leukocyte counts were normal. The serum urea was 36.4 mmol/L, creatinine 314 μmol/L, and serum albumin was 2 g/dL. Serum globulin, fasting and post-prandial blood glucose, serum amylase, bilirubin and transaminases were normal. Urine examination did not reveal any abnormality. Serum sodium was 135 mmol/L, potassium 2.8 mmol/L and calcium 1.8 mmol/L.

Just after admission, the patient developed typical grandmal epileptic attack, which was managed by intravenous diazepam injection. Intravenous and oral supplements of potassium and calcium were given and the child did not have any further episodes of epilepsy.

The patient was considered to have pre- renal failure due to severe vomiting and dehydration and was managed conservatively with intravenous fluid. Two packs of whole blood were transfused and 100 mL of 20% albumin was given intravenously. Gradually, the serum creatinine and urea became normal. Clinically the puffiness over the face and pedal edema disappeared and the patient started feeling better. Dialysis was not required.

A contrast enhanced CT scan revealed an irregular, large, non-attached mass of varying attenuation with pockets of air, occupying the stomach (Figure 1). A portion of the mass was also encroaching the duodenum. A few polypoidal masses were seen in the antrum of the stomach near the pylorus. There was no post-contrast enhancement of the mass lesion.

UGI endoscopy revealed a large bezoar occupying nearly the whole stomach (Figure 2). It was black and dark green in color and appeared to be composed of flat plastic thread/grass like material along with entrapped food

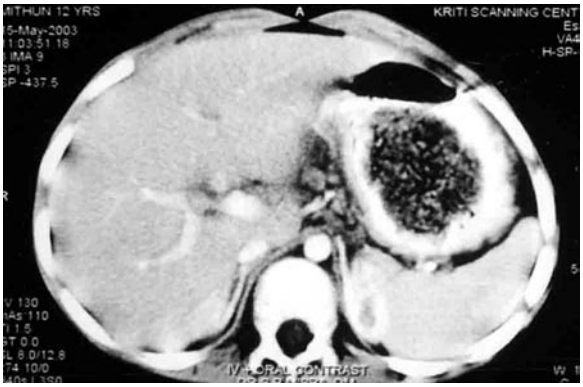


**Figure 1** Contrast-enhanced CT scan showing a large unattached mass (bezoar) in the stomach with air trapped in it. There was no post-contrast enhancement of the mass.

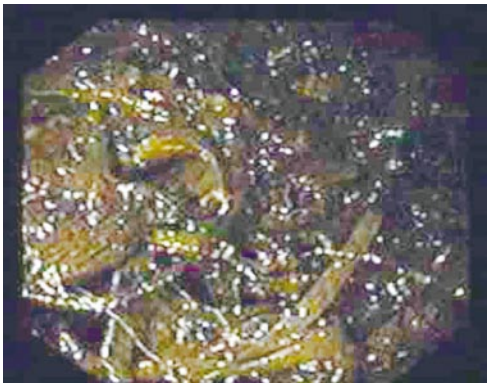

**Figure 2** Endoscopic appearance of the plastobezoar. The bezoar was made of hundreds of plastic strands.

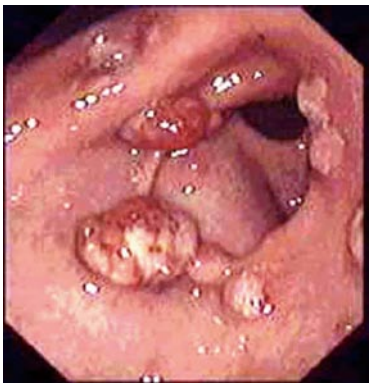

**Figure 3** Hyperplastic gastric polyps in the pre-pyloric region of the stomach.

particles. A small "tail" of the bezoar was also entering the pylorus into the first and second parts of the duodenum. There were several polypoid, masses in the peri-pyloric area of the antrum. Some of the polypoid masses had superficial ulcerations (Figure 3). Ulcerations were also seen at the incisura of the stomach. A few strands of the material forming the bezoar were removed using a rat-toothed forceps. The material was found to be flat plastic threads used in knitting chairs and charpoys. At places, flat cables used for receiving television signals were also found. Attempts to entrap the bezoar in conventional polypectomy snare failed. It could be snared with a large-diameter improvised snare but it could not be delivered through the cardio-esophageal sphincter as one piece due to its size.

On inquiring from the parents of the child, the father said that he had occasionally seen the child peeling off plastic material from chairs and charpoys and eating it for the last three years. It happened when the child was confined to bed (made of plastic) after surgery for an abscess, three years ago, and during this period he started peeling off bits and pieces and strands off the charpoy and swallowing them. Gradually he developed a taste for the plastic material, and even when he recovered he continued to chew and swallow plastic from the charpoys.

The next day, under conscious sedation using injection pentazocine, diazepam and propofol, an overtube was passed into the esophagus over a tightly fitting Savary-Gilliard dilator. Thereafter, the whole bezoar was removed in pieces using rat-toothed forceps, snare and Dormia basket, in an endoscopy session lasting over three hours. Some of the strands from the bezoar measured more than 50 cm. Biopsies were obtained from the polyps in the antrum. The patient was kept nil p.o. overnight. Intravenous pantoprazole was given. A barium meal examination was performed the next day, which did not reveal any abnormality. No obvious delay in the transit time was observed either in the stomach or in the small and large intestines. Psychiatric evaluation did not reveal any abnormality. Histological examination of the biopsy obtained from the polyps in the pre-pyloric region of the stomach revealed the polyps to be hyperplastic in nature. The patient was started on oral pantoprazole and discharged. He is on a regular follow-up about recurrence of the bezoar and disappearance of the hyperplastic polyps.

## DISCUSSION

The word bezoar is a word derived from the Arabic "bazahr" or "badzehr" which means an antidote or counter-poison. Till the 19th century, bezoars obtained from sacrificed animals had been widely used as antidote.[1] The origin of most bezoars is thought to be due to delayed gastric emptying as a result of either vagotomy, antral resection, gastroparesis or gastric outlet obstruction[2,3]. However, in a Japanese study, no evidence of delayed gastric emptying was observed[4]. Phytobezoar formation has also been reported to form in an adolescent with achalasia cardia and hypertrophic pyloric stenosis[5] and in one patient each having intestinal pseudo-obstruction[6] and scleroderma[7]. However, our patient did not have any of the above mentioned conditions. Ingestion of large amounts of indigestible solids may also precipitate bezoar formation[2]. The case in discussion had swallowed a large amount of plastic material and since plastic cannot be digested, a bezoar formed in the stomach. Stomach phytobezoars are also known to occur in uremic patients[8] and although in the present case, serum creatinine rose at the time of hospitalization, it was due to protracted vomiting and hypovolemia leading to pre-renal failure and

not due to any renal disease per se. The renal functions became normal within a few days by conservative management.

Bezoars are conventionally defined as retained concretions of animal or vegetable material in the intestinal tract[1]. Therefore, in the truest sense, our patient did not have a bezoar but will be said to have a foreign body in the stomach. However, the appearance, behavior and management of the gastric mass was exactly like a bezoar and therefore it may aptly be called a "plastobezoar." Apart from common phytobezoars, trichobezoars and diospyrobezoars, cotton bezoars[9], paper bezoars[10], bezoars due to medication (pharmacobezoars)[11-12], synthetic glue[13] and even iron bezoars[14] have been reported. Considering the fact that even non-organic substances have been responsible for producing bezoars, the case in discussion is a fit case to be considered as a bezoar and not a foreign body.

Our patient had a unique bezoar due to eating disorder in which he was eating plastic material used for knitting chairs and charpoys. This abnormal eating disorder of plastikophagia has been reported earlier too[15]. However, the formation of a giant bezoar and its consequences were unique in this patient.

CT scan in our patient revealed a non-attached gastric mass lesion with air pockets. There was no post-contrast enhancement. This appearance was very similar to that described in a patient with trichobezoar[16,17] and therefore it appears that all bezoars may give a similar appearance on CT scan, irrespective of the contents. The fact that all the bezoars are retained substances of foreign origin gives the typical CT picture of a non-attached mass that does not enhance after intravenous contrast injection. Since the bezoar forms umpteen number of pockets in which air is trapped, this is also visualized on the CT scan.

Surgery is frequently resorted to for the removal of bezoars, especially the larger ones[2,3,18]. However, laparoscopic removal[19,20] and endoscopic extraction of bezoars have also been successful. Use of lithotriptor[21], laser ignited mini-explosives[22], mono- polar diathermy knife[21] and electrohydraulic lithotripsy[23] have been successful in removing the bezoars of varying etiologies. In our patient, surgery was considered to be an option but was abandoned in view of his poor general condition and the presence of renal failure. Since the bezoar was made up of hundreds of thread-like strands of plastic and food debris, we were unable to use the above-mentioned endoscopic techniques. The bezoar could be trapped as one piece in a large self-made snare, which has been used by us earlier for removal of large foreign bodies such as a mango kernel from the esophagus[24], but due to its large size it could not be negotiated across the cardio-esophageal sphincter. Therefore, it had to be removed the hard way in bits and pieces using an overtube, a rat-toothed forceps, a Dormia basket and a polypectomy snare. We did not have a large channel endoscope, which has been used to remove gastric phytobezoars of 500, 700 and 1000 ml successfully in one go by sucking them from the stomach[25].

The hyperplastic gastric polyps (Figure 3), in the prepyloric region of the stomach, probably occurred because of chronic irritation of the gastric mucosa by the bezoar. This was also the reason for the ulcerations seen in the stomach. It is logical to think that these polyps may have caused further stasis in the stomach by deforming the pyloric opening and impeding the flow of the gastric contents. It is interesting in this regard to find a couple of case reports where gastric polyposis[26] and hyperplastic gastric polyps[27] were noted to be associated with gastric bezoar formation[4].

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*CASE REPORT*

# A case report of hepatic veno-occlusive disease after ingesting dainties

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## Abstract

Hepatic veno-occlusive disease (HVOD) is rarely encountered and easily misjudged as Budd-Chiari syndrome. It is often related to stem cell transplantation in recent years. We report a case of HVOD that is related to ingestion of some palatable local dishes. The diagnosis was confirmed by liver biopsy pathology with specific observation of inflammatory changes and fibrosis of venules intima, dilated sinusoids and central veins. Chronic diarrhea is unique for this case as a result of ingesting harmful stuffs. This case demonstrated that supervision and instruction of food recipe and traditional medicine are crucial, and prompt diagnosis, supportive care and specific treatment are essential to decreasing the morbidity and mortality of HVOD.



**Key words:** Hepatic veno-occlusive disease; Diagnosis; Management; Biopsy

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## INTRODUCTION

Hepatic veno-occlusive disease (HVOD) is a rarely encountered ailment in the literature[1,2]. The establishment of the diagnosis of this condition can be very difficult because there is no specificity in the clinical manifestations and some common findings are similar to Budd-Chiari syndrome (BCS), a peculiar condition with many pitfalls leading to misdiagnosis[3]. Until now, the mechanism of etiology has not been clearly identified and only considered by some authors[2,4] correlated to the non-thrombotic occlusion of the central veins of hepatic lobules, certain inflammatory factors, detoxification of the liver, *etc.* This is a case of HVOD that is related to ingestion of some palatable local dishes.

## CASE REPORT

A 17-year-old female was admitted with a complaint of escalating abdominal pain, increased abdominal circumference and recurrent diarrhea. The symptoms started 2 mo ago, mild at first, but now became more and more obvious and bothersome. Physical examination revealed mild jaundice in sclera, no skin petechia, no edema, no superficial varices, and no positive findings in the heart and lungs. The abdomen was distended, no tenderness or rebound pain. The liver and spleen were not palpable. The sign of ascites was positive. Laboratory results showed WBC 6.82 × $10^9$/L, Hb 122 g/L, BPC 81.30 × $10^9$/L, HbsAg (-), ALT 66 U/L, AST 81 U/L, serum total bilirubin 39.7 μmol/L, direct bilirubin 10.6 μmol/L, and ALB 32.8 g/L. Abdominal paracentesis aspirated clear ascites, with a total cell count of 6400, WBC 5/mL, and ALB 15.3 g/L. CT scan of the abdomen revealed moderate ascites, hepatomegaly, with areas of inhomogeneously reduced density, delayed enhancement of parenchyma, and obscure hepatic veins.

Considering the symptom of repeated diarrhea, the physicians inquired time and again that if she had eaten anything harmful, but nothing was determined. At last, the details of the patient's food consumption found that she had enjoyed a "cuisine recipe for longevity" for a dozen of times. The dish was delicious, the patient admitted, and all the local people thought that it is one of the valuable dainties. The content of the ingredients proved to consist of the seeds of a plant called "wild sesame" by the local people, whereas actually the entity of it is not barbate deadnettle but rattlebox[5], *Crotalaria sessiliflora.*

Venography by catheterization revealed patent inferior vena cava (IVC) and main hepatic veins (HV). Finally, a percutaneous puncture of liver biopsy was completed with the pathological findings of inflammatory changes and fibrosis of venules intima, dilated sinusoids and central veins, and no obvious degeneration of hepatocytes was found. Then the diagnosis of HVOD was established. The patient was given supportive and hepatoprotective treatment. Her symptoms were relieved and she was completely recovered within two weeks.

## DISCUSSION

In recent years, HVOD often occurs secondary to stem cell transplantation as the most common regimen-related toxicity[6]. However, herbs or plants are occasionally reported to cause HVOD, especially those used in traditional medicine[7].

The mechanism of liver damage caused by plants has not been elucidated, only a hypothesis of "conflict of plant versus animal or plant-animal interaction" seems more acceptable[8]. The defence system of many plants are used to produce compounds such as alkaloids and polypeptides against the animals that ingest them. Such animals are also self-protected by efflux transporters in the gut and detoxification of the liver, herbivore countermechanism to plant chemical defenses, and multidrug resistance-associated protein isoform, *etc.*

Senecio plants[7,9] as well as *Crotalaria* and *gynura segetum* are reported potentially hepatoxic if consumed over a period of weeks. Poisoning can occur through ingestion of especially the seeds, but also leaves and stems. After ingestion of the plants, the major toxic components pyrrolizidine alkaloids (PAs) are absorbed and converted to highly reactive alkylating pyrroles that cause hepatocellular necrosis, biliary hyperplasia, fibrosis, and hepatocytomegaly.

The clinical manifestations of HVOD fall into the categories of mild, moderate and severe according to its final outcome[4,6,10] or acute, subacute and chronic according to its onset and course. Typical findings include abdominal pain, ascites with elevated ALB, jaundice and hepatomegaly. Chronic diarrhea is unique for this case as a result of ingesting harmful stuffs. Imaging diagnoses including gray-scale US, Doppler US, CT and MRI have been reported as convenient and useful. Venography often reveals patent IVC and main outflow of HV.

Pathology of liver biopsy definitely establishes the diagnosis of HVOD, with the hallmark of fibrous obliteration of terminal hepatic venules and small lobular veins. Both percutaneous and laparoscopic liver biopsies[4] are helpful, and transvenous (transjugular) approach[11] as well. The latter has the advantages of possible hemorrhage to be drained intravascularly and the feasibility of measuring hepatic venous pressure gradients with the upper limit of 10 mmHg for the establishment of HVOD diagnosis, and the higher, the more severe. Unfortunately, this case failed to go through this procedure.

It is very important to differentiate the diagnosis of HVOD from that of BCS[2-4] as both of them present the common signs of abdominal distention, jaundice and ascites with elevated ALB. Several points to identify BCS for this differentiation should be kept in mind: (1) superficial varices of the trunk and lower extremities with edema and pigmentation; (2) stricture or obstruction of IVC and /or HV outflow by venography; (3) thromboses in hepatic venules by liver biopsy pathology.

Supportive care remains the therapy available to date. For some severe cases, ascites must be drained in order to allow sufficient pulmonary ventilation. The drug defibrotide[4,12] has been selected for the treatment of severe HVOD, and in a large, FDA-approved, pivotal, prospective, multi-institutional, global phase Ⅲ trial, seems to have few significant side effects and well-tolerated. Transjugular intrahepatic portosystemic shunting (TIPS) was evaluated[4] for chronic cases with serious obstruction of outlet of main HVs, but should be indicated with discretion. In some severe cases, charcoal hemofiltration[13] has been shown to be effective for adsorbing circulating bilirubin and other protein-bound toxins and for supporting patients in hepatic failure.

This case demonstrated that supervision and instruction of food recipe and traditional medicine are crucial[14], and that prompt diagnosis, supportive care and specific treatment are essential to decreasing the morbidity and mortality of HVOD.

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# Meetings

## MAJOR MEETINGS COMING UP

First Biennial Congress of the Asian-Pacific Hepato-Pancreato-Biliary Association
March, 2007
Fukuoka, Japan
http://www.congre.co.jp/1st-aphpba

American College of Gastroenterology Annual Scientific
20-25 October 2006
Las Vegas, NV

14th United European Gastroenterology Week, UEGW
21-25 October 2006
Berlin, Germany

APDW 2006: Asian Pacific Digestive Week 2006
26-29 November 2006
Lahug Cebu City, Philippines

## EVENTS AND MEETINGS IN THE UPCOMING 6 MONTHS

Falk Symposium 151: Emerging Issues in Inflammatory Bowel Diseases
24-25 March 2006
Sydney - NSW
Falk Foundation e.V.
symposia@falkfoundation.de

10th International Congress of Obesity
3-8 September 2006
Sydney
Event Planners Australia
enquiries@ico2006.com
www.ico2006.com

Easl 2006 - the 41st annual
26-30 April 2006
Vienna, Austria
Kenes International

Prague hepatology 2006
14-16 September 2006
Prague
Foundation of the Czech Society of Hepatology
veronika.revicka@congressprague.cz
www.czech-hepatology.cz/phm2006

12th International Symposium on Viral Hepatitis and Liver Disease
1-5 July 2006
Paris
MCI France
isvhld2006@mci-group.com
www.isvhld2006.com

Falk Symposium 152: Intestinal Disease Part I, Endoscopy 2006 - Update and Live Demonstration
4-5 May 2006
Berlin
Falk Foundation e.V.
symposia@falkfoundation.de

Falk Symposium 153: Intestinal Disease Part II, Immunoregulation in Inflammatory Bowel Disease - Current Understanding and Innovation
6-7 May 2006
Berlin
Falk Foundation e.V.
symposia@falkfoundation.de

ILTS 12th Annual International Congress
3-6 May 2006
Milan
ILTS
www.ilts.org

Internal Medicine: Gastroenterology
22 July 2006-1 August 2006
Amsterdam
Continuing Education Inc
jbarnhart@continuingeducation.net

6th Annual Gastroenterology And Hepatology
15-18 March 2006
Rio Grande
Office of Continuing Medical Education
cmenet@jhmi.edu
www.hopkinscme.net

World Congress on Gastrointestinal Cancer
28 June 2006-1 July 2006
Barcelona, Spain
c.chase@imedex.com

International Conference on Surgical Infections, ICSI2006
6-8 September 2006
Stockholm
European Society of Clinical Microbiology and Infectious Diseases
icsi2006@stocon.se
www.icsi2006.se/9/23312.asp

7th World Congress of the International Hepato-Pancreato-Biliary Association
3-7 September 2006
Edinburgh
Edinburgh Convention Bureau
convention@edinburgh.org
www.edinburgh.org/conference

Society of American Gastrointestinal Endoscopic Surgeons
26-29 April 2006
Dallas - TX
www.sages.org

Digestive Disease Week 2006
20-25 May 2006
Los Angeles
www.ddw.org

Annual Postgraduate Course
25-26 May 2006
Los Angeles, CA
American Society of Gastrointestinal Endoscopy
www.asge.org/education

American Society of Colon and Rectal Surgeons
3-7 June 2006
Seatlle - Washington
www.fascrs.org

## EVENTS AND MEETINGS IN 2006

10th World Congress of the International Society for Diseases of the Esophagus
22-25 February 2006
Adelaide
isde@sapmea.asn.au
www.isde.net

Falk Symposium 151: Emerging Issues in Inflammatory Bowel Diseases
24-25 March 2006
Sydney - NSW
Falk Foundation e.V.
symposia@falkfoundation.de

10th International Congress of Obesity
3-8 September 2006
Sydney
Event Planners Australia
enquiries@ico2006.com
www.ico2006.com

Easl 2006 - the 41st annual
26-30 April 2006
Vienna, Austria
Kenes International

VII Brazilian Digestive Disease Week
19-23 November 2006
www.gastro2006.com.br

International Gastrointestinal Fellows Initiative
22-24 February 2006
Banff, Alberta
Canadian Association of Gastroenterology
cagoffice@cag-acg.org
www.cag-acg.org

Canadian Digestive Disease Week
24-27 February 2006
Banff, Alberta
Digestive Disease Week Administration
cagoffice@cag-acg.org
www.cag-acg.org

Prague Hepatology 2006
14-16 September 2006
Prague
Foundation of the Czech Society of Hepatology
veronika.revicka@congressprague.cz
www.czech-hepatology.cz/phm2006

12th International Symposium on Viral Hepatitis and Liver Disease
1-5 July 2006
Paris
MCI France
isvhld2006@mci-group.com
www.isvhld2006.com/

Falk Seminar: XI Gastroenterology Seminar Week
4-8 February 2006
Titisee
Falk Foundation e.V.
symposia@falkfoundation.de

European Multidisciplinary Colorectal Cancer Congress 2006
12-14 February 2006
Berlin
Congresscare
info@congresscare.com
www.colorectal2006.org

Falk Symposium 152: Intestinal Disease Part I, Endoscopy 2006 - Update and Live Demonstration
4-5 May 2006
Berlin
Falk Foundation e.V.
symposia@falkfoundation.de

Falk Symposium 153: Intestinal Disease Part II, Immunoregulation in Inflammatory Bowel Disease - Current Understanding and Innovation
6-7 May 2006
Berlin
Falk Foundation e.V.
symposia@falkfoundation.de

14th United European Gastroenterology Week
21-25 October 2006
Berlin
United European Gastroenterology Federation
www.uegw2006.de

World Congress on Controversies in Obesity, Diabetes and Hypertension
25-28 October 2006
Berlin
comtec international
codhy@codhy.com
www.codhy.com

Asia Pacific Obesity Conclave
1-5 March 2006
New Delhi
info@apoc06.com
www.apoc06.com/

ILTS 12th Annual International Congress
3-6 May 2006
Milan
ILTS
www.ilts.org

XXX Panamerican Congress of Gastroenterology
11-16 November 2006
Cancun
www.panamericano2006.org.mx

Internal Medicine: Gastroenterology
22 July 2006-1 August 2006
Amsterdam
Continuing Education Inc
jbarnhart@continuingeducation.net

6th Annual Gastroenterology And Hepatology
15-18 March 2006
Rio Grande
Office of Continuing Medical Education
cmenet@jhmi.edu
www.hopkinscme.net

Hepatitis 2006
25 February 2006-5 March 2006
Dakar
hepatitis2006@mangosee.com
mangosee.com/mangosteen/hepatitis2006/hepatitis2006.htm

World Congress on Gastrointestinal Cancer
28 June 2006-1 July 2006
Barcelona, Spain
c.chase@imedex.com

International Conference on Surgical Infections, ICSI2006
6-8 September 2006
Stockholm
European Society of Clinical Microbiology and Infectious Diseases
icsi2006@stocon.se
www.icsi2006.se/9/23312.asp

5th International Congress of The African Middle East Association of Gastroenterology
24-26 February 2006
Sharjah
InfoMed Events
infoevent@infomedweb.com
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7th World Congress of the International Hepato-Pancreato-Biliary Association
3-7 September 2006
Edinburgh
Edinburgh Convention Bureau
convention@edinburgh.org
www.edinburgh.org/conference

13th International Symposium on Pancreatic & Biliary Endoscopy
20-23 January 2006
Los Angeles - CA
laner@cshs.org

2006 Gastrointestinal Cancers Symposium
26-28 January 2006
San Francisco - CA
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Society of American Gastrointestinal Endoscopic Surgeons
26-29 April 2006
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Digestive Disease Week 2006
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Annual Postgraduate Course
25-26 May 2006
Los Angeles, CA
American Society of Gastrointestinal Endoscopy
www.asge.org/education

American Society of Colon and Rectal Surgeons
3-7 June 2006
Seatlle - Washington
www.fascrs.org

71st ACG Annual Scientific and Postgraduate Course
20-25 October 2006
Venetian Hotel, Las Vegas, Neveda
The American College of Gastroenterology

AASLD 57th Annual - The Liver Meeting™
27-31 October 2006
Boston, MA
AASLD

New York Society for Gastrointestinal Endoscopy
13-16 December 2006
New York
www.nysge.org

## EVENTS AND MEETINGS IN 2007

9th World Congress on Gastrointestinal Cancer
20-23 June 2007
Barcelona
Imedex
meetings@imedex.com

***Gastro 2009, World Congress of Gastroenterology and Endoscopy London, United Kingdom 2009***

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### *Acknowledgments*

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

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

*In press*

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

*Organization as author*

4 **Diabetes Prevention Program Research Group**. Hypertension, insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension* 2002; **40**: 679-686 [ PMID: 12411462 ]

*Both personal authors and an organization as author*

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

*No author given*

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

*Volume with supplement*

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

*Issue with no volume*

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

*No volume or issue*

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

**Books**

*Personal author(s)*

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

*Chapter in a book (list all authors)*

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

*Author(s) and editor(s)*

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

*Conference proceedings*

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

*Conference paper*

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

**Electronic journal** (list all authors)

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

**Patent** (list all authors)

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

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Present 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 $\chi^2$ (in Greek), related coefficient as $r$ (in italics), degree of freedom as $\gamma$ (in Greek), sample number as $n$ (in italics), and probability as $P$ (in italics).

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

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***Italics***

Quantities: *t* time or temperature, *c* concentration, *A* area, *l* length, *m* mass, *V* volume.
Genotypes: *gyr*A, *arg* 1, *c myc*, *c fos*, *etc.*
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*EDITORIAL*

# Bacteria, inflammation, and colon cancer

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## Abstract

Our relationship with the colonic bacterial flora has long been viewed as benign, but recent studies suggest that this symbiosis has risks as well as benefits. This relationship requires that the host not only provide a supportive environment for the symbiotic bacteria, but also actively maintain intact mechanisms for properly managing the physiologic stresses that are closely associated with the symbiont's essential survival functions. Failure to do so breaches the host-symbiont contract, and can result in serious effects on the health of the host. Recent investigations that employ several knockout mouse models reveal the consequences of genetic deficiency in the host regarding these mechanisms, and the latent, pro-inflammatory, tumorigenic nature of normal bacterial flora. Further study of the interactions between normal bacterial flora and hosts could shed light on the etiologies and pathogenesis of inflammatory diseases and related cancers, with implications for human health.



**Key words**: Commensal bacteria; Chronic inflammation; Colon cancer; Germfree mice; Gene knockout

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## INTRODUCTION

Our relationship with the colonic bacterial flora has long been viewed as a symbiotic one. We provide a nutrient-rich habitat, while the bacteria play important roles in the development of the mucosal immune system, the maintenance of a physiological environment, the provision of essential nutrients, and the prevention of colonization by pathogenic bacteria[1,2]. Recently, the concept that normal bacterial flora are essential for the development of inflammation-induced carcinoma has emerged from studies of well-known colonic bacterial floras. This hypothesis is reviewed here because the lessons learned from investigations of the colonic flora could serve as a general guide to studies of the etiology and pathogenesis of chronic inflammatory diseases and related cancers in the gastrointestinal tract. These include conditions such as reflux esophagitis and esophageal adenocarcinoma, and *Helicobacter* gastritis and gastric adenocarcinoma and lymphoma, as well as inflammatory bowel diseases (IBD) and colorectal cancer.

Human colorectal carcinomas can be classified by etiology as inherited (e.g., hereditary nonpolyposis colorectal cancer due to genetic instability and familial adenomatous polyposis coli due to a mutation in the adenomatous polyposis coli gene, APC), inflammatory (e.g., Crohn's disease and ulcerative colitis), or sporadic (accounting for greater than 80% of colorectal cancers, but poorly defined etiologically). The availability of genetically-engineered rodent models has greatly facilitated the etiologic study of colorectal cancers.

### *The normal bacterial flora is a prerequisite for the development of inflammation-related colorectal tumors*

The role of bacteria in the development of colorectal tumors is best exemplified by the experimental dysplasia and cancer developed in the TCRβ/p53 double knockout mouse colitis model that mimics the development of adenocarcinoma in ulcerative colitis[3]. In conventional mice at four months of age, adenocarcinomas of the ileocecum developed in 70% of animals. However, there was no development of colonic adenocarcinoma in mice raised under germ-free housing conditions. A similar observation was also made in IL-10 knockout[4] and Gpx1/Gpx2[5] double knockout mice (Table 1). There is a growing list of genetically-engineered mouse models for gastrointestinal cancers, for which the effect of the normal bacterial flora on tumorigenesis has not yet been evaluated (Table 1)[6].

These include knockouts of the G protein subunit alpha i2 (Gαi2)[7], Smad3[8], Muc2[9], and double knockouts of TGF-β1/Rag2[10,11] and IL-2/β2m[12].

Comparison of the results for conventional and germ-free mice indicates that hosting a beneficial bacterial flora is not cost-free. The symbiotic contract that has evolved between the bacterial flora and its host includes obligations-it requires that the host actively maintain intact mechanisms for properly managing the physiologic stresses that are closely associated with essential survival functions of the symbiotic bacteria. These stresses include the generation of oxygen free radicals in basic metabolic pathways and the production of proinflammatory bacterial structures, among others. The health of the host thus depends on preserving functional genes for handling peroxidative stress, bacterial antigens, and inflammation, as well as on the maintenance of an intact mucosal barrier.

The serious consequences of this contract between host and symbiont become evident in its breach. Heretofore considered quite benign, the normal bacterial flora has been shown in recent studies with knockout mouse models to harbor latent tumorigenic potential. An imbalance in the host-symbiont relationship can trigger this hidden potential, to the detriment of the host. It is reasonable to suppose that similar pathways operate in the human body.

### The normal bacterial flora is a prerequisite for the development of inflammation in the colon

Though most genetically-engineered mouse colon cancer models have not yet been tested under germ-free conditions, they present the common picture of a close association between inflammation and cancer development. There are several studies on the development of inflammation in these models under germ-free conditions (Table 2).

One of the best-characterized models in this respect is the IL-10-deficient mouse. Interleukin-10 (IL-10) affects the growth and differentiation of many hemopoietic cells *in vitro*; in particular, it is a potent suppressor of macrophage and T cell functions. IL-10-deficient mice showed increased morbidity and mortality when exposed to normal bacterial flora. All male IL-10-deficient mice housed under conventional conditions died by 4 mo, while 50% of females remained alive. In contrast, both male and female IL-10-KO mice were healthy when housed under germ-free conditions (up to 8 mo). Marked inflammation was observed in the conventional IL-10-deficient mice. Abnormal changes included: (1) thickening of the mucosa, (2) disorganization and hyperplasia of crypts, (3) epithelial erosion/ulceration, (4) accumulation of bacteria, (5) marked infiltration of leukocytes, and (6) crypt abscess formation. None of these inflammatory changes were found in the germ-free IL-10-deficient animals[13,14]. Neutrophil chemokine KC was produced by epithelial cells in response to the inflammatory mediators TNF-alpha and IFN-gamma that were expressed following exposure to normal flora in animals lacking IL-10[13]. Inducible nitric oxide synthase (iNOS) had no impact on the development or severity of spontaneous chronic inflammation in IL-10-deficient mice[15]. In contrast to other genetically-engineered rodents, germ-free IL-2-deficient mice were not free of inflammation. They developed mild inflammation, but remained clinically healthy during observation periods of up to 46 wk with no mortality[16]. In contrast, specific pathogen-free IL-2-deficient mice developed massive mononuclear infiltration with frequent crypt abscesses, and usually died between 28 and 32 wk of age. The enteric bacterial flora was also required for the development of inflammation in HLA-$B_{27}$/β2m transgenic rats and TCRα-deficient mice[17,18]. Mice deficient in the multiple drug resistance (mdr) gene (*mdr1a*) were susceptible to developing a severe, spontaneous intestinal inflammation when maintained under specific pathogen-free animal facility conditions. This model has not been tested under nonspecific germ-free conditions, but treating *mdr1a*-deficient mice with oral antibiotics both prevented the development of disease and resolved active inflammation, supporting the hypothesis that the enteric bacterial flora is required for colonic inflammation[19]. Similar to *mdr1*a-deficient mice, colitis in keratin 8-deficient mice was amenable to antibiotic therapy[20]. These studies indicate an essential role for the normal bacterial flora in the pathogenesis of inflammation.

Colonic inflammation could also be induced with Tgfβ1/Rag2, Smad3, Gαi2, or cytokeratin 8 gene knockouts[7,8,11,21], but the effect of the normal enteric bacterial flora on the development of inflammation has not been examined under germ-free conditions in these models (Table 2).

**Table 1 Effect of colonic bacterial flora on the development of gastrointestinal cancers in genetically-engineered mice**

| Gene | Function | Incidence of carcinoma (%) | |
|---|---|---|---|
| | | Conventional | Germ-free |
| TCRβ/p53[3] | Cellular immunity/ growth and division | 70 | 0 |
| IL-10[4] | Anti-inflammation | 7 | 0 |
| Gpx1/Gpx2[5] | Peroxidative stress | 25 | 0 |
| Gαi2[7] | Cellular signaling | 31 | ND |
| Smad3[8] | Tgfβ signaling | 100 | ND |
| Muc2[9] | Major component of the mucus | 69 | ND |
| Tgfβ1/Rag2[10,11] | Anti-inflammation/antigen receptor rearrangement | 100 | ND |
| IL-2/β2m[12] | Proinflammation | 32 | ND |

ND: Not determined.

### The bacterial flora as a whole is important in colonic inflammation and tumorigenesis

Abundant data have implicated intestinal bacteria in the initiation and amplification stages of inflammatory bowel diseases. However, the precise role of intestinal bacteria remains elusive. One theory is that both "protective" species and "harmful" species exist within the normal enteric bacterial flora. A healthy balance between these two populations in a normal host might be detrimental for an inflammation-prone host. Alternatively, a breakdown in the balance between the two populations, termed "dysbiosis", could by itself promote inflammation in a normal host.

**Table 2 Effect of colonic bacterial flora on the development of intestinal inflammation in genetically-engineered rodents**

| Gene | Function | Type of inflammation | |
|---|---|---|---|
| | | Conventional | Germ-free |
| IL-10[13,14] | Anti-inflammation | Neutrophilic, severe, death | Negative |
| IL-2[16] | Proinflammation | Lymphocytic, severe, death | Mild, focal |
| HLA-B27/β2m[17] | Human leukocyte antigen/MHC β chain | Lymphocytic, severe | Negative |
| TCRα[18] | T cell receptor α chain | Lymphocytic | Negative |
| Gαi2[7] | Cellular signaling | Lymphocytic, plasmacytic | ND |
| Tgfβ1/Rag2[11] | Anti-inflammation/antigen receptor rearrangement | Granulocytic | ND |
| mdr1a[19] | ABC transporter | Lymphocytic, granulocytic | ND |
| K8[20] | Major intermediate filament protein | Lymphocytic | ND |
| Smad3[21] | Tgfβ signaling | Lymphocytic | ND |

ND: Not determined.

The pathogenic role of the normal enteric bacterial flora in the development of enterocolitis and colon cancer has been implicated in C57BL/6 IL-10-knockout mice. Probiotic Lactobacilli modify the enteric flora and are thought to have a beneficial effect on enterocolitis. Treatment of IL-10-deficient mice with the probiotic *Lactobacillus salivarius* ssp. *salivarius* UCC118 reduced the intensity of mucosal inflammation and the incidence of colon cancer from 50% to 10%. These effects were accompanied by significant reductions in fecal coliform, enterococci, and *Clostridium perfringens* levels[22]. This study exemplifies the effect of changes at the flora level on the development of inflammation, and supports the hypothesis that there are "protective" species and "harmful" species in the normal bacterial flora.

Human studies using culture techniques have linked populations of *Bacteroides vulgatus*, *Eubacterium rectale*, *Ruminococcus torques, Streptococcus hansenii, Bifidobacterium longum, Ruminococcus albus, Peptostreptococcus productus, Bacteroides stercoris, Bifidobacterium angulatum, Eubacterium eligens, Ruminococcus gnavus, Fusobacterium prausnitzii, Eubacterium cylindroids* to a high risk of colon cancer. The fact that *Bifidobacterium longum* and *Bifidobacterium angulatum* and the total concentrations of bifidobacteria also are significantly associated with a high risk of colon cancer is contrary to suggestions that the ingestion of *bifidobacterium* cultures acts to increase the numbers of intestinal floral bacteria that might offer increased protection against colon cancer[23-25]. *Lactobacillus* S06 and *Eubacterium aerofaciens* and total lactobacillus concentrations were significantly associated with a low risk of colon cancer[26].

Further studies of the population differences between normal and disease states might illuminate the contribution of specific flora changes to disease development.

### *The bacterial flora potentiates tumor formation independent of inflammation*

Mutation of the APC gene leads to the development of multiple intestinal neoplasia (Min), especially in the small intestine, with little inflammation[6,27]. Germ-free Min mice developed 50% fewer adenomas in the small intestine, although there were no significant differences in the remainder of the intestinal tract[28]. This model suggests that commensal bacteria are not required, but can potentiate tumor formation independent of inflammation.

### *Specific bacterial infection promotes colonic tumor formation in genetically susceptible mice*

Although resident enteric bacteria are necessary for the development of spontaneous colitis in many rodent models, not all bacteria have an equivalent capability to induce inflammation. Germ-free IL-10-deficient mice populated with bacterial strains, including *Bacteroides vulgatus*, *Clostridium sordellii*, *Streptococcus viridans*, *Escherichia coli, Lactobacillus casei, Lactobacillus reuteri, Lactobacillus acidophilus*, *Lactobacillus lactis,* a *Bifidobacterium* sp., and a *Bacillus* sp., did not exhibit significant colitis[4,14,29].

Conventional IL-10-deficient mice suffered from chronic enterocolitis[30]. In contrast, mutants kept under specific pathogen-free conditions developed only a focal inflammation limited to the proximal colon. These results suggest that the bowel inflammation in the mutants was stimulated by single or multiple specific bacterial species. *Citrobacter rodentium*, *Helicobacter hepaticus*, *Enterococcus faecalis* are examples of conditional cancer-causing bacteria that alone do not cause cancer, but are carcinogenic in certain genetically-engineered mice.

*Citrobacter rodentium* is a gram-negative bacterium that colonizes predominantly the distal colon of mice, causing a disease termed transmissible murine colonic hyperplasia (TMCH). This condition induces colitis and crypt cell proliferation, similar to that seen in human idiopathic inflammatory bowel diseases, including Crohn's disease and ulcerative colitis[31]. Infection with *C. rodentium* has not yet been associated with tumorigenesis, but TMCH promoted colon tumor development in mice administered the carcinogen DMH[32]. $Apc^{Min/+}$ mice infected with *C. rodentium* at 1 mo of age had a 4-fold increase in the number of colonic adenomas at 6 mo of age, as compared with uninfected Min mice[33].

*H. hepaticus* is a newly recognized bacterium associated with chronic active hepatitis, hepatic carcinoma, and inflammatory bowel disease in mice. *H. hepaticus* infection did not cause colon cancer in mice in the absence of an inflammatory trigger[34]. Germ-free and specific pathogen-free TGFβ1/Rag2-deficient mice were free of inflammation, hyperplasia, and cancer, but when reintroduced into a *H. hepaticus*-containing specific

pathogen-free room, developed colonic adenoma/carcinoma[11,35]. *H. hepaticus* in pure culture did not seem to induce IBD in IL-10 KO mice[36], suggestive of an essential collaboration between *H. hepaticus* and normal bacterial flora in the observed etiology.

*Enterococcus faecalis*, previously known as group D S*treptococcus* or *Streptococcus faecalis*, is an opportunistic pathogen that is found in the alimentary tract of both humans and animals. Its notorious capacity to acquire virulence factors and antibiotic resistance genes have made this opportunistic bacterium a major problem for patients and clinicians[37]. Inflammation, dysplasia, and rectal carcinoma developed in IL-10 KO mice colonized with *E. faecalis*, but not in germ-free mice[4].

### *Unusual bacterial infections associated with colorectal cancer in humans*

Although there is no established bacterial pathogen for human colorectal cancer, unusual infections might precede the clinical diagnosis of cancer in some instances.

*Streptococcus infantarius*, formally known as *Streptococcus bovis* or Non-enterococcal Group D S*treptococcus*, is a constituent of the human enteric flora. It is the causative agent in 5%-14% of endocarditis cases. Individuals with colon cancer had higher fecal carriage of *S. bovis* as compared to patients with nonmalignant enteric disease and healthy controls[38]. Panwalker reviewed 467 cases of adult *Streptococcus bovis* bacteremia[39]. Malignant colonic tumors were present in 62 of the 467 patients (13%). In some cases, the bacteremia will occur months or years after this species becomes established. It is not known whether the *S. bovis* carriage has any direct cause-and-effect relation to colon cancer. In azoxymethane-treated rats, administration of either *S. bovis* or its wall-extracted antigens promoted the progression of preneoplastic lesions through an increased formation of hyperproliferative aberrant colonic crypts, enhanced the expression of proliferation markers, and increased the production of IL-8 in the colonic mucosa, suggesting that *S. bovis* acts as a promoter of early preneoplastic lesions in the rat colon. It is interesting that bacterial cell wall proteins were more potent inducers of neoplastic transformation than were the intact bacteria[40]. When a cell wall protein fraction composed of 12 different proteins was applied to human epithelial colonic Caco-2 cells or the rat colonic mucosa, it was able to trigger the release of CXC chemokines (human IL-8 or rat CINC/GRO) and prostaglandin E2, which are correlated with an *in vitro* over-expression of COX-2. Moreover, these proteins were highly effective in the promotion of preneoplastic lesions in azoxymethane-treated rats. In the presence of these proteins, Caco-2 cells exhibited enhanced phosphorylation of MAP kinases. These data suggest that bacterial components possess both proinflammatory properties and procarcinogenic potentials, and support the hypothesis that colonic bacteria can contribute to cancer development, particularly in association with chronic infection/inflammation diseases where these bacterial components might interfere with cell function[41].

*Clostridium septicum* infections are rare, but are often associated with malignancy. This association has been discussed in a number of case reports[42]. In a review of 162 published cases of *C. septicum* infection in 1989, 81% had an associated malignancy, 34% had an associated colon carcinoma, while 40% had an associated hematologic malignancy[43]. A consequence of these close associations is that in patients for whom a *C. septicum* infection is diagnosed, a rigorous search for occult malignancy should be mounted. It is yet to be determined whether these associations represent a cause-and-effect relationship or a secondary phenomenon.

For both *S. bovis* and *C. septicus*, a question remains whether the bacteria induce bowel cancer or if the growth of the organisms is promoted by a preexisting carcinoma. Testing their carcinogenicity in genetically-engineered immunodeficient mouse models might shed light on our understanding of the role of bacteria in the development of human cancers.

## SUMMARY AND PERSPECTIVE

Traditional bacteriology is built upon concepts developed from studies of infectious diseases in which a pathogen can often be identified and pathogenesis explained by toxins or virulent factors. These concepts have clearly demonstrated their usefulness in the identification of etiologic agents of anthrax in the nineteenth century and *Helicobacter* gastritis more recently, among many others. Recent studies of tumorigenesis in rodent models have opened a new chapter in bacteriology, with the observation that the normal bacterial flora actively participates in the development of cancers. Searching for the responsible agents among normal flora bacteria, albeit not classic pathogens, is still a reasonable approach in the assessment of bacterial roles in chronic inflammatory disorders and related cancers. Support for this approach is found in evidence that certain bacterial species are required for tumorigenesis in genetically deficient rodent models, and in the association of specific bacterial species with human cancers.

The capacity for causing inflammation or cancers that is exhibited by a complex bacterial flora might depend on the aggregate activity of multiple constituents of the flora, rather than on a single species. This capacity might be enhanced or reduced as a function of significant changes in the species diversity and abundance within the flora. Thus, more detailed knowledge of how the bacterial flora is altered in association with disease conditions could significantly broaden our understanding of the etiology and pathogenesis of these diseases. In the past, the connection between a heterogeneous bacterial flora and a disease was often regarded as too complex to interpret. However, recent advances in 16S rDNA technology, DNA sequencing, and data analysis tools have made it possible to determine the differences between two diverse bacterial flora by defining and comparing the majority of bacterial species and their prevalence in each flora[2,44,45].

Alternatively, the host might also play a decisive role here. Colon cancers might develop in a genetically deficient host in the presence of a normal bacterial flora, without any requirement for specific bacterial species or alteration of the flora. No human counterparts of the critical gene

defects artificially created in rodents have been identified, but it is plausible that weak defects or polymorphisms of human genes that might not be aberrant enough to confer an overt clinical disease could, in collaboration with a normal bacterial flora, lead to cancers in a patient over a period of decades. Future investigations could seek to discover any relevant weak gene defects in human patients, and correlate them with long-term pathogenesis.

The present studies in rodent models of the role of the bacterial flora in tumorigenesis obviously raise some interesting possibilities in relation to human cancers. Can the concepts derived from bacterial flora in colon cancers be extrapolated to cancers in other anatomic sites where cancer development is related to chronic inflammation, such as gastric adenocarcinoma in *H pylori* gastritis, esophageal adenocarcinoma in reflux esophagitis, or oral squamous cell carcinoma in periodontitis? Can the natural history of cancer development be altered toward the benefit of hosts by manipulation of bacterial flora using probiotic and antibiotic therapies or vaccination? The potential contributions to improving human health make finding the answers to these questions a clear priority for future research.

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*EDITORIAL*

# Colorectal cancer screening

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## Abstract

Colorectal cancer is a major public health burden worldwide. There is clear-cut evidence that screening will reduce colorectal cancer mortality and the only contentious issue is which screening tool to use. Most evidence points towards screening with fecal occult blood testing. The immunochemical fecal occult blood tests have a higher sensitivity than the guaiac-based tests. In addition, their automation and haemoglobin quantification allows a threshold for colonoscopy to be selected that can be accommodated within individual health care systems.



**Key words:** Screening; Colorectal cancer; Fecal occult blood testing; Colonoscopy

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## INTRODUCTION

Colorectal cancer is a major public health burden. It is the fourth most common form of cancer worldwide and the most frequent in North America, Australia, New Zealand, Argentina, and parts of Europe[1]. When colorectal cancer is detected at an early stage, the prognosis is excellent with a five year survival in excess of 97%[2]. The risk of nodal metastases and thus poorer prognosis increases as colorectal cancer develops with a risk of 2%-3% when confined to the submucosa compared with 8%-12% when confined to the muscularis propria[3,4]. The evidence that screening for colorectal cancer leads to earlier detection and improved survival is now incontrovertible and has led to a consensus that colorectal cancer screening will reduce mortality; however there is no consensus as to the best screening tool[5-11]. The four possible screening options currently cited are barium enema, fecal occult blood testing or fecal occult blood testing (FOBT), sigmoidoscopy, colonoscopy, or a combination thereof. Cost-effective analyses do not help distinguish between the strategies with the median incremental cost-effectiveness ratios being €9950/life-year saved, €13 200/life-year saved, and €10 000/life-year saved, for FOBT, sigmoidoscopy, and colonoscopy respectively compared with no screening[12].

Inadequate screening capacity is an important barrier to consider in colorectal cancer screening. Colonoscopy is the final pathway of all colorectal cancer screening. Depending on which fecal test is used, 2%-15% of participants will need a colonoscopy[13]. Between 5%-10% of participants undergoing screening flexible sigmoidoscopy screening will need a colonoscopy[14]. If colonoscopy is used as the screening option then potential annual demand for screening colonoscopy can be calculated at 45%-50% of those aged 50-70 years[15]. The endoscopy capacity of a health care system carries significant weight when deciding the best screening tool.

## BARIUM ENEMA

There are drawbacks to each of the screening options. Although double contrast barium enema is recommended for screening no published studies of its efficacy in this role exist. The sensitivity of barium enema for polyps > 1 cm is 48% and for colorectal cancer 82.9%[16,17]. Despite the presence of barium enema in screening guidelines, its place lies for those individuals who have had a failed colonoscopy rather than as a screening tool.

## SIGMOIDOSCOPY

Sigmoidoscopy is an attractive option as it takes only five minutes, requires no sedation and only a self-administered enema to clear the bowel. In a pivotal retrospective case controlled study those with colorectal cancer were less likely to have had a prior sigmoidoscopy than the control group with an odds ratio of 0.3[8]. Four flexible sigmoidoscopy trials were carried out on the strength of retrospective studies[10,18-20]. Long-term follow up was available from only one of these studies with a reduction in colorectal cancer incidence and mortality of 80% and 50% respectively over 13 years[10]. Much controversy has surrounded the question of right-sided colonic lesions missed by sigmoidoscopy. In men sigmoidoscopy failed to identify 29.7% of advanced colonic lesions and in women 65.3%[21,22]. As a consequence, a very persuasive argument against sigmoidoscopy has been the claim that screening flexible sigmoidoscopy is as illogical as screen-

ing only one breast during mammography[23]. In reality, just 2%-5% of asymptomatic individuals are believed to have isolated advanced proximal neoplasia[24]. The real concern surrounding sigmoidoscopy is its acceptance rate and its invasiveness as a screening test in asymptomatic individuals. In studies, which represent the ideal clinical situation, the acceptance rate varies from 33%-80%[10,18,19]. Screening sigmoidoscopy is an invasive procedure and is associated with risks which may not be acceptable in asymptomatic individuals: a perforation rate of 1 in 25 000, bleeding in 3.2%, and pain in 14%[25,26].

## COLONOSCOPY

Evidence-based guidelines place greatest weight on large-scale randomized trials, but the corroboration for colonoscopy comes from indirect evidence or small case-control studies. The US National Polyp Study and the Italian multi-center study showed a reduction in colorectal cancer incidence of 75%-90% over a follow-up period of 5.9 and 10.5 years respectively[9,27]. However, the primary aim of both studies was to determine the effects of resecting colorectal polyps on colorectal cancer incidence. Case-control studies have evaluated the feasibility of colonoscopy in colorectal cancer screening with advanced lesions detected in 10.5%-12.5% of asymptomatic individuals[28,29]. It should be born in mind that both studies enrolled individuals who wished to participate in colorectal cancer screening, and population-based studies reveal that the acceptance rate for colonoscopy is less than 20%[30,31]. For any screening test to be effective the acceptance rate must be over 60% and the acceptability of colonoscopy to the population may limit its usefulness as a screening tool. In addition, in clinical practice the yield from screening colonoscopy in average-risk individuals has been lower than case control studies with only 5.1% having a polyp > 9 mm[32]. The sensitivity of colonoscopy for colorectal cancer and polyps may have been over-estimated. In retrospective studies and prospective FOBT screening trials the sensitivity of colonoscopy for colorectal cancer is over 95%[17,33]. Back-to-back colonoscopy studies reveal that the miss rates for polyps > 1 cm is 0%-6%[34,35]. Virtual colonoscopy followed by colonoscopy reveals a miss rate of 12% for polyps > 1 cm[36]. Colonoscopy technique is of crucial importance in detection rates. In particular, the quality of bowel preparation and colonoscopy withdrawal times have been identified as factors impacting on colonoscopy detection rates[37-39]. Indeed, improving the quality of colonoscopy techniques is estimated to reduce interval cancers by up to 50%[40]. Finally as with sigmoidoscopy, colonoscopy is an invasive procedure and may not be acceptable as a screening tool in asymptomatic individuals. Although the risk associated with a single colonoscopy is small with a 0.2%-0.3% risk of serious complication and a 0.1% risk of death, the cumulative risk of repeat screening colonoscopy may outweigh the benefit obtained from screening colonoscopy[41].

## FECAL OCCULT BLOOD TESTING

The most robust evidence for the effectiveness of colorectal cancer screening lies with FOBT. Long-term follow up available in four randomized controlled trials of over 330 000 individuals and three non-randomized trials of just over 200 000 individuals reveals a reduction in colorectal cancer mortality of 12%-33%. Adjusting for compliance gives a 23% reduction in colorectal mortality with guaiac-based FOBT[5-7,42-44]. It is sometimes claimed that the mortality reduction in the American study is attributable to the high colonoscopy rate of 28%-38%[6]. However only 4% of individuals in the European studies, and just over 6% of individuals in the Chinese study underwent endoscopy[5,7,43].

The sensitivity of FOBT is often seen as a liability, and it is true that the once-off sensitivity of FOBT for colorectal cancer ranges from 26%-69%, and for adenomas ranges from 9%-36%[13,21,45]. Yet FOBT screening involves repeated testing at 1-2 yearly intervals and within this context the programme sensitivity of FOBT for detecting cancer is as high as 90%[6]. From an acceptability point of view FOBT is non-invasive, can be carried out at home, and does not necessitate any bowel preparation or taking time off work.

Guaiac-based FOBT depends on fecal blood to catalyze the phenolic oxidation of guaiac in the presence of hydrogen peroxide to produce a blue chromogen. Immunochemical FOBTs use antibodies specific for human haemoglobin and have a higher sensitivity of 66%-90% and a specificity > 90%[46,47]. Immunochemical FOBTs have evolved further with the development of quantitative results, thus allowing the positivity threshold to be varied and achieve an increased sensitivity with lowered specificity or a decreased sensitivity with an increased specificity[48,49].

## ALTERNATIVE SCREENING TOOLS

Virtual colonoscopy, despite improvements in technology, continues to show widely varying results with sensitivities for detecting polyps ⩾ 10 mm ranging from 55% to 92%[36,50]. As such it is still not ready for population screening. Fecal calprotectin failed to live up to its initial promise in subsequent studies[51]. Fecal DNA tests are affected by the relatively low frequency of single marker alterations in colorectal cancer. Even the use of a broad panel of markers in fecal DNA still fails to be convincing for population screening[52].

## CONCLUSION

Screening will reduce the mortality from colorectal cancer and should be implemented now. For those at average risk of developing colorectal cancer most evidence points towards screening with FOBT. Depending on which FOBT is used, between 2%-15% of individuals screened will need colonoscopy, a figure that can be accommodated without too much difficulty.

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*REVIEW*

# Crohn's disease: Innate immunodeficiency?

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## Abstract

In the past, Crohn's disease (CD) has been understood primarily as an immunologic disorder characterized by an abnormal T-cell response. Recent *in vitro* and *in vivo* data suggests that CD may instead be precipitated by innate immune dysfunction resulting from a combination of genetic and environmental factors. Some reports have demonstrated a defective immune response in a variety of other cellular components, including neutrophils, monocytes and dendritic cells. Recent studies of granulocyte-macrophage colony-stimulating factor (GM-CSF) in CD, aiming to stimulate the innate immune system with the conception that an innate immune defect underlies the development of the disease, have been demonstrated a clinical benefit and reinforce this evolving understanding of the disease.



**Key words:** Crohn's disease; Innate immunity; Immunodeficiency; NOD2

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## INTRODUCTION

Over a period of many years Crohn's Disease (CD) has been thought to result predominantly from excessive activation of type 1 helper T cells (TH1) with a characteristic cytokine profile including elevated interferon-γ and IL-2. However the pathways by which T cells became activated have remained an unsolved dilemma. Collectively recent studies using cell and animal models as well as studies of individuals with CD suggest that an aberrant innate immune response to luminal bacteria may be a critical initiating step in the development of the disease. These studies suggest that in at least some individuals with CD, innate immune responses are paradoxically impaired compared to normal controls.

## NOD2/CARD15 FUNCTION AND EFFECT OF ITS MUTATIONS

Improved, albeit still incomplete, understanding of the function of NOD2/CARD15 have been particularly key to an appreciation of the importance of innate immune dysfunction in CD. NOD2 is expressed constitutively in macrophages, neutrophils and dendritic cells[1], as well as in Paneth and epithelial cells[2]. NOD2 is a cytoplasmic protein that serves as a microbial sensor, and its leucine-rich repeat (LRR) domain is required for recognition of muramyl dipeptide (MDP), a fragment of peptidoglycan present in bacterial cell walls. The ligand MDP ultimately leads to activation of the transcription nuclear factor (NF-κB), and induction of proinflammatory cytokines[3,4]. Membrane recruitment of NOD2 is essential for NF-κB activation after the recognition of MDP in intestinal epithelial cells and is mediated by a motif comprising two leucine residues and a tryptophan in the COOH-terminal domain of NOD2[5]. Evidence that NOD2 may function as an antibacterial factor in intestinal epithelial cells was demonstrated in Caco-2 cells stably expressing wild type NOD2 when infected with *Salmonella typhimurium*. This protective effect was lost in cells expressing a most common mutant NOD2 associated with CD (3020insC)[6].

Specific mutations of the NOD2 gene have been definitively associated with increased susceptibility to ileal Crohn's disease in Western (but not Asian) populations: Arg702Trp, Gly908Arg, and leu1007fsinsC (a frameshift mutation that truncates the carboxy terminal 33 aminoacids)[7,8]. Heterozygous carriage of the risk alleles confers a 2-4 fold increased risk, and homozygotes or compound heterozygotes have a 20-40 fold increased risk [9]. More than 90% of all CD associated mutations are located in the LRR domain, suggesting that these may affect the function of NOD2 with respect to bacterial recognition and signaling. Transient transfection experiments indicate that CD-associated NOD2 mutants no longer activate NF-κB in response to MDP[3,10], which suggests that defective NF-κB activation facilitates infection of the lamina propia by enteric bacteria.

Abbott DW *et al*[11] demonstrated that NOD2 activation leads to ubiquitinylation of NEMO, a key component of the NF-κB signaling complex. They showed that NOD2-

dependent ubiquitinylation of NEMO is dependent on the scaffolding protein kinase RIP2. Crohn's disease-associated mutants of NOD2 exhibited a decreased ability to bind RIP2, and this decreased ability to bind RIP2 correlates with a decreased ability to ubiquitinylate NEMO.

NOD2 mutants produce selective functional defects in leukocytes of patients with CD as shown by van Heel *et al*[12] who analyzed cytokine expression of peripheral blood mononuclear cells after exposure to MDP. In PBMC from CD patients the NOD2 ligand induced little TNFα and IL-1β, but strong IL-8 secretion. Futhermore, monocytes isolated from CD patients carrying the 1007fs (3020insC) mutation were reported to exhibit defects in the production of the proinflammatory cytokines, TNFα, IL-6 and IL-8, as well as the anti-inflammatory cytokine IL-10[13]. Dendritic cells derived from CD patients homozygous for leu1007fsinsC also fail to up-regulate the costimulatory molecules CD80 and CD86 in response to MDP and lack production of cytokines such as TNF-α, IL-12 and IL-10[14].

## RELATION OF NOD2 AND TLR PATHWAYS

Intersection between TLR and NOD2 pathways is suggested by reports of synergistic induction of proinflammatory cytokines such as TNFα and IL-1β upon costimulation with MDP and specific TLR ligands[15,16]. MDP also substantially upregulated secretion of TNFα and IL-1β induced by ligands to five different TLR ligands, TLRs 2, 4, 5, 7 and 9: ($Pam_3CysSerLys_4$, LPS, Flagellin, MALP-2 and R-848, respectively). Of note, these effects were observed in the presence of the most common NOD2 mutants associated with CD. In studies using mice lacking NOD2, Watanabe *et al*[17] observed reduced responses to MDP, but enhanced responses to the TLR2 ligand, peptidoglycan e.g. increases in IL-12. They interpreted these findings to suggest that the NOD2 signaling pathways normally downregulate the TLR2 pathways. In their model, loss of function mutation of NOD2 together with TLR2 signals delivered by other bacterial products could result in enhanced cytokine responses to commensal bacteria by macrophages. These findings suggest that interaction between NOD2 and specific TLR pathways may represent an important modulatory mechanism of innate immune responses which is altered in some patients with CD.

However, controversy remains about the role of NOD2 and the interactions between NOD and TLR signaling pathways. While these results suggest that this NOD2 mutation may have enhanced responsiveness to bacterial peptidoglycan, interpretation of the significance of these findings remains controversial, because transient transfection assays using the CD-associated NOD2 mutants, as described above, have consistently shown defective cytokine responses to MDP and decreased activation of NF-κB[18]. In contrast to Watanabe *et al* Kobayashi and colleagues[19] did not find upregulation of TLR responses following disruption of NOD2. Kobayashi *et al* did find that mutations in NOD2 in mice affect the expression of small antibacterial proteins called cryptidins (α-defensins in humans) by intestinal epithelial cells. This data is consistent with the notion that NOD2 regulates the production of antibacterial peptides in Paneth cells in the intestinal crypts, and that this may contribute to local control of pathogenic bacteria (Figure 1).

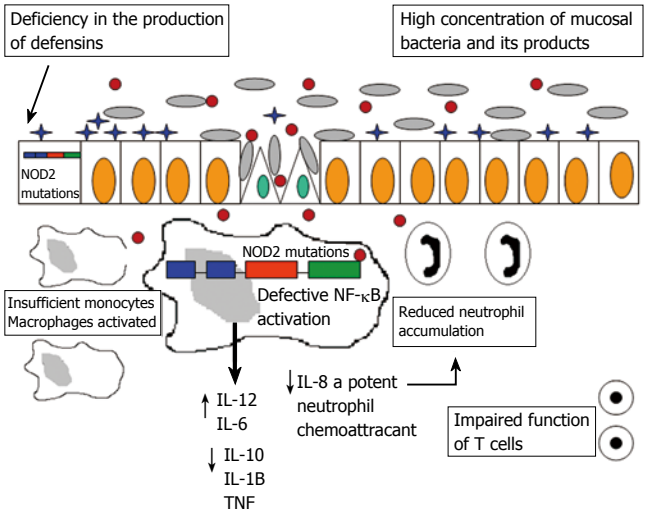


**Figure 1** Impairment of different innate immune mechanisms. These mechanisms play a central role in the homeostasis of intestinal barrier function. Identified environmental risks and genetic susceptibility may contribute to the innate immune dysfunction.

## OTHER POTENTIAL DEFECTS IN INNATE MUCOSAL DEFENSE MECHANISMS IN CD PATIENTS

### *Altered defensin expression in CD*

In general, α-defensins (1-3, 5 and 6) are induced in the colonic mucosa of CD and UC patients. However, NOD2 mutations in CD patients are associated with diminished mucosal α-defensin expression[20]. Decreased β-defensin 1 and the lack of induction of both inducible antimicrobial peptides β-defensins 2 and 3 in CD could result in enhanced bacterial survival and perhaps invasion[21].

### *Neutrophil function in CD*

Neutrophils act as a first line defense at the mucosal-microbial interface by killing and digesting bacteria within phagocytic vacuoles. Neutrophil-mediated clearance of mucosal microbes would prevent activation and recruitment of monocytes/macrophages. Several defects have been described in patients with CD, including impairment in migration of neutrophils[22]; complement dysfunction that produces impaired neutrophil recruitment[23]; decrease of phagocytic and bactericidal neutrophil function[24] and deficient superoxide generation in neutrophils[25].

A number of genetic syndromes with well described defects of the innate immune system may also provide insights into pathophysiology relevant to CD. Patients with glycogen storage disease (GSD) Ib[26,27], chronic granulomatous disease (CGD)[28], Chediak Higashi syndrome[29], Hermansky-Pudlak syndrome[30] leukocyte adhesion deficiency[31], Turner's syndrome[32], and

congenital[33], cyclic[34] and autoimmune[35] neutropenias can all manifest features of CD or CD-like phenotype[36]. Each of these syndromes comprises a quantitative or qualitative deficiency in the function of neutrophils, monocytes, or macrophages, suggesting that varied functional cellular deficiencies can result in a common intestinal phenotype of CD.

The finding that two murine lines with disruption of either CCAAT/enhancer binding protein (CEBP)-ε[37] and a cell-type specific disruption of the Stat3 gene in neutrophils and macrophages[38] develops enterocolitis, support the role of the neutrophil and the macrophage in the development of CD. Stat-3 disrupted mice show an immune response skewed toward Th-1 activity, demonstrating that neutrophil and macrophage dysfunction may eventuate in a Th-1 phenotype.

Thus a variety of studies and clinical insights have suggested that CD may result from an impaired mucosal innate immune response. In the context of these earlier findings, a recent study by Marks *et al*[39] support the hypothesis that the mucosal innate immune system plays a central role early in the development of CD. Marks *et al*[39] found a significantly decreased production of IL-8 and IL-1β (45% and 50% reduction respectively) from macrophages of patients with CD. This defect in the production of these cytokines was independent of the presence of NOD2 mutations and helps to clarify the controversy generated by Li *et al*[40] who reported that the presence of NOD2 mutations showed no induction (Leu1007finsC) or modest induction (Gly908Arg and Arg702Trp) of IL-8 and IL-1β). Macrophages from CD patients produce less IL-8 in response to pro-inflammatory agonists, suggesting that these cells may influence the acute inflammatory response. Impaired secretion of IL-8 appears to result in a failure of neutrophil migration. This finding was confirmed by normalizing neutrophil efflux after augmentation of endogenous IL-8 secretion by topical MDP. The authors suggest that in CD, reduced or delayed recruitment of neutrophils to sites where bacteria penetrate the intestinal wall may result in the persistence of bacteria and other debris in the tissue and lead to the chronic inflammation typical of this disease.

The data of Marks *et al*[39] suggests that CD patients possess a generalized impaired innate immune response as reflected by diminished response to intradermal injection of killed bacteria as well as trauma of the skin or the intestine. When killed bacteria were injected into the forearms of CD patients, there was less blood flow to the injection site than non-CD patients. They also found that CD patients had reduced neutrophil accumulation and interleukin-8 (IL-8) production at sites of tissue trauma in the intestine and skin. This study supports the idea that CD may in some way be associated with relative inability to mount an acute inflammatory response compared to normal individuals.

## THERAPEUTIC APPROACHES

Marks *et al*[39] suggest the provocative notion that IL-8 either by direct enteral administration or through synthesis by genetically modified gut organisms might have therapeutic value. Similarly, they propose that if diminished recruitment of neutrophils is a backdrop to CD, agents that increase blood flow such as long-acting phosphodiesterase-5 inhibitors might be useful in healing or preventing lesions in CD. In support of this possibility, they report that oral administration of sildenafil markedly increased blood flow to sites of bacterial injection in CD patients.

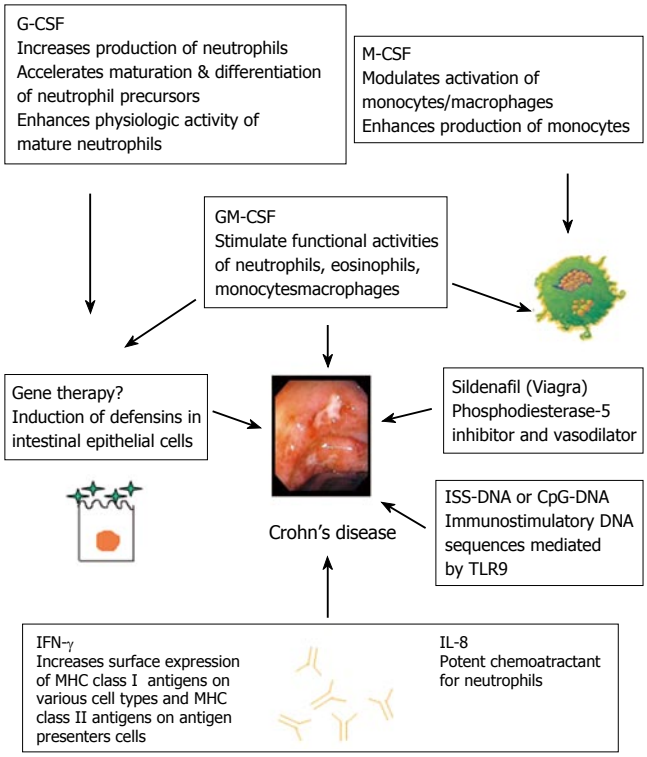


**Figure 2** Targets of immunostimulation therapy in Crohn's disease. Potential therapies encompass interventions focused on augmenting the intestinal innate immune function in different mechanisms of action.

Recent reports suggest that agents which act to enhance innate immune defenses can indeed confer therapeutic benefit. The endogenous growth factor granulocyte-macrophage colony-stimulating factor (GM-CSF) performs important functions in both the phagocytic and epithelial components of intestinal early innate immune defense. GM-CSF is expressed by both CD4+ T cells and Paneth cells in the intestine, and its receptors have been recently demonstrated to be present on intestinal epithelial cells which proliferate in response to GM-CSF *in vitro*[41,42]. Within the immune system, GM-CSF increases phagocytic cell function through its effects on oxidative burst, phagocytosis, and intracellular bacterial killing[43].

In the context of the evolving concepts of CD pathophysiology, these observations provided the rationale for clinical trials of granulocyte colony stimulating factor (G-CSF, specifically filgrastim) and GM-CSF (sargramostim). Both pilot studies suggested a benefit[44,45], though GM-CSF appeared more effective. A recent randomized controlled trial of 124 patients found a significant benefit in response at 100-point decrease in CDAI and in remission. The response was sustained for a mean of 8-10 wk after discontinuation of therapy[46].

While mechanistic studies were not included in the trial, recent translational studies[47] have demonstrated that GM-CSF can reverse several neutrophil impairments in cells obtained from individuals with CD (Figure 2).

In summary, mucosal innate immunodeficiency characterized by impaired dysfunction of neutrophils, monocytes and dendritic cells as well as intestinal epithelium play a critical early role in the development of CD. These defects may arise from a variety of genetic defects which are presumably worsened by environmental factors to culminate in decreased cytokine production and insufficient bacterial killing. Persistence of microbial derived stimuli subsequently leads to T cell activation, accounting for the T cell driven nature of the established disease. This concept would provide insight into why immunosuppression may be effective in some individuals by limiting the secondary, chronic T-cell response, while immune stimulation, possibly with GM-CSF, may also prove to be effective as a general strategy particularly in acute and early phases of the disease. A better understanding of the early initiating events in CD may result in even better therapeutic approaches that enhance the innate immune system.

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*REVIEW*

# Mechanisms and significance of liver steatosis in hepatitis C virus infection

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## Abstract

The pathogenesis of liver damage associated with chronic hepatitis C virus (HCV) infection is thought to be largely immunomediated. However, some frequent histopathological features, such as steatosis, suggest a direct cytopathic effect of HCV. The direct responsibility of HCV in the pathogenesis of steatosis is shown by: (1) the association with HCV genotype 3 infection, suggesting that some viral sequences are involved in the intracellular accumulation of lipids; (2) the correlation between severity of steatosis and HCV replication levels; (3) association between response to treatment and disappearance of steatosis. Experimental studies have shown that the nucleocapsid protein of HCV (core protein) is capable and sufficient to induce lipid accumulation in hepatocytes. Moreover, the observation that chronic hepatitis C patients have reduced serum levels of ApoB suggests an interference with the very-low density lipoprotein (VLDL) assembly, although other mechanisms are possible. In patients with sustained virological response induced by antiviral therapy, such levels are normalized. Other observations suggest that the pathogenesis of steatosis in chronic hepatitis C is not solely due to HCV. The origin of the mild steatosis observed in most patients may be metabolic, since its severity correlates with body mass index and insulin resistance. Most studies have shown a correlation between presence and/or severity of steatosis and fibrosis stage, but it is unclear whether this effect is direct or mediated by the associated insulin resistance, increased susceptibility to apoptosis, or by inflammatory cytokines. Finally, steatosis negatively influences the rate of response to antiviral treatment, as confirmed by large clinical trials. Management of steatosis in chronic hepatitis C requires knowledge of its pathogenesis and may involve both life-style changes and pharmacological interventions, although the latter remain largely experimental.

## INTRODUCTION

The hepatitis C virus (HCV) is a major cause of chronic liver disease with an estimated 170 million people infected worldwide. The spectrum of severity of the liver disease associated with HCV varies widely from non-specific, minimal inflammatory changes to cirrhosis and hepatocellular carcinoma[1]. The rate of progression of chronic hepatitis C is also variable, depending on many cofactors, mostly host-related, such as age, gender, alcohol consumption, overweightness and coinfections[2,3]. Steatosis, defined as an increased fat content of the liver, essentially accounted for by triglycerides, has been recognized as one of these factors capable of influencing both liver fibrosis progression and the rate of response to interferon-alpha-based therapy. Steatosis is a common lesion associated with many conditions affecting the liver, some of which, like overweightness and alcohol consumption, being very frequent. Interestingly, however, the proportion of chronic hepatitis C patients with steatosis is higher than one would predict by simple chance association, suggesting a direct role of HCV, at least in some cases, in the intrahepatic accumulation of triglycerides. This proportion is indeed so high that, in the pre-serology era, steatosis has been used as a diagnostic tool to identify patients with non-A, non-B hepatitis[4,5].

The proper appreciation of fatty liver associated with chronic hepatitis C is important due to its clinically significant consequences. The scope of this review is to discuss the pathogenetic, clinical and therapeutic aspects related to steatosis in chronic hepatitis C patients.

## VIRAL STEATOSIS IN CHRONIC HEPATITIS C

The reported prevalence of steatosis in patients with chronic hepatitis C varies between 40% and 80%,

depending on the features of the population studied in terms of alcohol consumption, prevalence of overweightness/obesity, diabetes and other risk factors of fatty liver[6]. If all known factors of fatty liver are excluded, the prevalence of steatosis in chronic hepatitis C is still about 40%. This figure represents an approximately 2-fold increase compared to the prevalence of steatosis in another common chronic liver disease like hepatitis B (20%)[7,8]. This evidence alone suggests that HCV may directly cause steatosis, at least in some patients.

An early observation, later confirmed by nearly all reports that have addressed this issue, showed that steatosis was more frequent and more severe in patients infected with HCV genotype 3[9-13]. This added to the evidence linking HCV and fatty liver, because it hinted at the presence of "steatogenic" sequences across the genome of genotype 3. In other words, this viral genotype, more often and more significantly than other genotypes (albeit not exclusively, see below), would be directly involved in the accumulation of triglycerides in hepatocytes (so-called "viral" steatosis). This interpretation is supported by two additional observations. The first is that, especially in patients with genotype 3, the severity of steatosis correlates with the level of HCV replication, expressed as HCV RNA level in liver[10] or in serum[11]. Similar results have been reported measuring the level of HCV core protein expressed in the liver of chronic hepatitis C patients by immunoblot[14]. The second important observation is that the fatty liver is significantly reduced or disappears when patients are successfully treated with antivirals. This effect, again, is more evident in patients with genotype 3, while those with genotypes non-3 may remain steatotic even in case of sustained virological response[15,16]. A relapse after the end of therapy may result in the reappearance of steatosis in patients in whom it had disappeared while on-treatment[17].

The mechanism of triglyceride accumulation by HCV remains largely speculative. From a general standpoint, and based on currently available evidence, HCV may interfere with lipid metabolism at three levels: impaired secretion, increased neosynthesis, and impaired degradation (Figure 1).

Historically, impaired secretion of lipids from the infected hepatocyte has been the first proposed mechanism of HCV-induced steatosis. In fact, serum levels of apolipoprotein B (ApoB) and cholesterol are reduced in chronic hepatitis C patients in whom steatosis responds to antiviral therapy[18,19], suggesting that HCV may interfere with the very-low density lipoprotein (VLDL) assembly and/or secretion. The disappearance of fatty liver in sustained virological responders to antiviral therapy correlates with normalization of ApoB and cholesterol levels[16,18]. Hypocholesterolemia in patients with chronic hepatitis C has been reported to be specifically associated with genotype 3[19]. Thus, clinical data suggest that HCV may interfere with VLDL secretion, a defect corrected by antiviral treatment.

*In vitro* studies and the transgenic mouse model have both suggested that the HCV core protein is sufficient to induce a lipid accumulation in hepatocytes[20-23]. This viral protein is localized on the surface of lipid droplets, and its over-expression seems to further stimulate the formation of lipid droplets. These models have predominantly used genotype 1-derived constructs, but recent work has reported similar results using other viral genotypes, including type 3a, which seems to be the most efficient in terms of fat accumulation[24]. In fact, although some degree of intra-hepatocyte fat deposition occurs with all viral genotypes, the genotype 3 core protein expression results in about 3-fold fat accumulation with respect to genotype 1[24], in agreement with the clinical evidence. Based on the experimental model of the transgenic mouse, the HCV core protein seems to inhibit the microsomal triglyceride transfer protein (MTP) activity[23]. Since this enzyme plays a key, rate-limiting role in VLDL assembly, the consequence of its inhibition is the accumulation of triglycerides, i.e. steatosis. A direct interaction between core protein and MTP is unlikely, as it would require the secretion of the viral protein into the endoplasmic reticulum lumen, which has not been reported. However, the MTP inhibition may still be indirect. Strangely enough, transgenic mice for the HCV core protein have normal ApoB levels in serum[23]: Were it not for this discrepancy, this mouse model may be an ideal candidate to study the HCV-related steatosis seen in chronic hepatitis C patients. Recent data in human liver are, however, in agreement with this proposed mechanism, since the intrahepatic levels of MTP mRNA were reduced in patients with chronic hepatitis C, especially those with steatosis and/or genotype 3[25]. According to another experimental model, the HCV core protein may accumulate in the mitochondria and

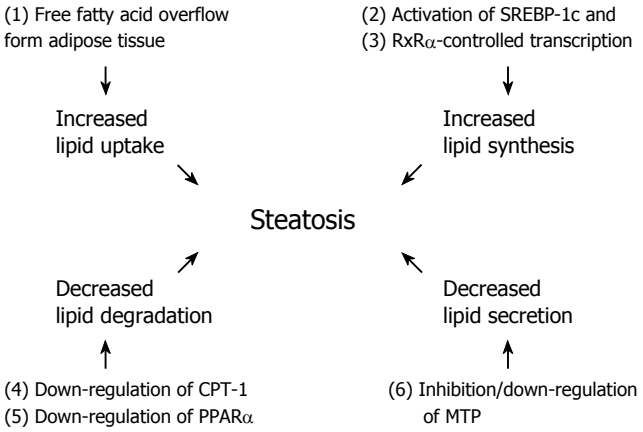


**Figure 1** Some of the major mechanisms leading to steatosis in patients with chronic hepatitis C. HCV, especially (but not only) genotype 3a, has been shown to be directly involved in triglyceride accumulation by several mechanisms: activation of fatty acid neosynthesis *via* SREPBP-1c (2) and RxRα (3), impaired degradation through down-regulation of CPT-1 (4) and PPARα (5), and inhibition and/or down-regulation of MTP (6). Another potential mechanism (not shown) is the cell damage inflicted by reactive oxygen species consequent to localization of the HCV core protein in the mitochondria. This may also trigger lipid peroxidation of microsomal membranes, leading to impaired VLDL secretion. In patients who are insulin resistant, the major mechanism leading to steatosis is the free fatty acid overflow from adipose tissue (1). Hyperglycemic/hyperinsulinemic states are also associated with activation of fatty acid neosynthesis *via* SREPBP-1c (2) and impaired degradation *via* down-regulation of CPT-1 (4). It has to be added that HCV may contribute to insulin resistance by impairing the IRS-1 signaling in hepatocytes by multiple mechanisms. Not shown in the figure are also the interference with ApoB synthesis and the activation of MTP reported in insulin resistant states: the latter phenomenon may be partially counterbalanced by the strong inhibition of MTP observed in patients with predominantly viral steatosis .

induce liver damage *via* the production of reactive oxygen species. These effects were prevented by a mitochondrial electron transport inhibitor[26]. In HCV transgenic mice, increased intrahepatic lipid peroxidation products occurred in response to carbon tetrachloride[27]. The subsequent production of ROS may result, among other effects, in the peroxidation of membrane lipids and structural proteins, such as those involved in the trafficking and secretion apparatuses. This would then block VLDL secretion, leading to steatosis. Furthermore, the intracellular accumulation of triglycerides would further contribute to the pathogenesis of steatosis by providing the fuel for continuing lipid peroxidation, in a typical free radical-driven amplification reaction.

HCV may also induce steatosis *via ex novo* synthesis of fatty acids. For example, HCV has been reported to upregulate the sterol regulatory element binding protein-1c (SREBP-1c) signaling pathway[28]. SREBP-1c is a transcription factor leading to the up-regulation of enzymes involved in *de novo* lipogenesis, an event that can favor intracellular accumulation of triglycerides. Chimpanzees experimentally infected with HCV show an increased intrahepatic activity of enzymes involved in lipogenesis, such as ATP citrate lyase, which are regulated by SREBP-1c[28]. The HCV core protein may additionally bind to and activate the DNA-binding domain of the retinoid receptor α (RxRα), a transcriptional regulator that controls many cellular functions, including cellular lipid synthesis[29-31]. On the other hand, accumulation of fat in hepatocytes transiently expressing the HCV core protein seems to depend on the presence of exogenous lipids, which indirectly decreases the likelihood of a significant fatty acids neosynthesis activated by this viral protein[24]. However, one cannot rule out that other viral proteins may activate the neosynthesis of fatty acids.

HCV may finally cause steatosis by impairing fatty acid oxidation. Transfection of hepatoma cells with the HCV core protein is followed by a reduced expression of peroxisome proliferators-activated receptor α (PPARα), a nuclear receptor regulating several genes responsible for fatty acid degradation[29]. These same authors have also reported a down-regulation of mitochondrial carnitine palmitoyl transferase-1 (CPT-1), the rate-limiting enzyme of mitochondrial β-oxidation, which is the main catabolic pathway of fatty acids, and of the acyl CoA oxidase (AOX)[29]. A reduced expression of CPT-1 in the liver of chronic hepatitis C patients has been reported recently[30]. However, the down-regulation of several genes, such as CPT-1 and AOX, is transcriptionally controlled by PPARα. Thus, the reported effects may be secondary to down-regulation of PPARα. PPARα mRNA is significantly reduced in the liver of patients infected with genotype 3 compared to genotype 1[32], and down-regulation of PPARα mRNA in chronic hepatitis C has been reported at least by another group[33]. Overall, the data support the hypothesis that HCV core protein may modulate the expression of various lipid degradation-associated genes, possibly *via* the down-regulation of PPARα.

The search for the viral sequences responsible for the genotype-specific effects on triglyceride accumulation has been so far elusive. No single mutation has been identified as being responsible for steatosis, suggesting that more complex mutation clusters may be involved in virally-driven steatosis[24]. The C-terminal signal sequence of the core protein appears particularly interesting. In this domain, the core protein of genotype 3a contains several unique mutations that, alone, or more likely in combination, may confer the steatogenic phenotype.

## STEATOSIS OF OTHER CAUSES OCCURRING IN HEPATITIS C

The HCV-induced steatosis may co-exist with a fatty liver due to other causes. In chronic hepatitis C patients who do not drink alcohol and are infected with non-3a genotypes, the most frequent correlate of fatty liver is an increased body mass index. Not only being obese, but also being merely overweight (BMI higher than 25 but less than 30) is an independent risk factor for hepatic steatosis in patients infected with HCV[8,11,16]. In genotype 1 infection, as observed in patients with non-alcoholic fatty liver disease (NAFLD), the fat distribution seems more important than total fat in determining steatosis. Visceral obesity, rather than merely increased BMI, seems to play a major role in the development of HCV-related steatosis[11]. In initial studies, when genotype was taken into account, it soon became clear that there was no association between BMI and the prevalence and the severity of steatosis in genotype 3 infected patients whereas, among patients with non-3 genotype, steatosis correlated with BMI[11,34]. These findings have been repeatedly confirmed and when patients with risk factors for NAFLD are excluded genotype 3 infection remains the single most important, independent predictor of steatosis[10,34].

This BMI-associated (or "metabolic") steatosis is not or very little modified by successful antiviral therapy[15,16]. However, a partial amelioration of steatosis is sometimes observed also in genotype non-3 infection following HCV eradication[16]. This implies that the assignment of either of the two types of steatosis (viral and metabolic) to a specific viral genotype should not be so clear-cut. Careful analysis of experimental and clinical data indicates that also HCV genotypes non-3 may induce some degree of viral steatosis, whereas metabolic abnormalities may be associated with a fatty liver also in genotype 3-infected persons. Thus, it is likely that the two types of steatosis can coexist in at least some chronic hepatitis C patients, although, in genotype 3 infection steatosis will be primarily of viral origin and in genotype non-3 primarily metabolic.

The most likely cause of metabolic steatosis in chronic hepatitis C, therefore preceding its appearance, is the insulin resistance that accompanies overweightness[35]. The mechanisms that underlie liver steatosis in the insulin resistant state are multiple (Figure 1). On the one hand, free fatty acid overflow from adipose tissue to the liver is a direct consequence of the failure to block lipoprotein lipase, resulting in increased uptake by peripheral tissues, including liver[36]. Second, the deregulated hyperglycemic/hyperinsulinemic state stimulates the expression of a variety of enzymes involved in fatty acid neosynthesis, while at the same time inhibit the

mitochondrial β-oxidation[36]. This imbalance between uptake, *ex novo* synthesis and degradation results in excess triglyceride accumulation within hepatocytes. In case of HCV infection, some degree of synergism may occur at the level of the above metabolic pathways, such as CPT-1 inhibition[29]. Moreover, insulin is a known down-regulator of ApoB synthesis[37]. However, recent data support the notion that chronic insulin resistant state may stimulate MTP activity in order to increase hepatocyte VLDL output[38]. If this is confirmed, two conflicting events would occur in insulin resistant chronic hepatitis C patients: MTP inhibition, directly-or indirectly-mediated by HCV, and MTP stimulation, mediated by insulin. Presumably, these two mechanisms may not operate in the same subgroups of patients. In those with genotype 3, who have the lowest levels of insulin resistance (see below), the viral inhibition of MTP would predominate, with hypotriglyceridemia, low ApoB serum levels and "viral" steatosis as a final consequence. In patients with genotype non-3 infection, hypertriglyceridemia may occur, but the inability to counterbalance the stimulation of fatty acid neosynthesis and the inhibition of mitochondrial β-oxidation would still result in "metabolic" steatosis. Clearly, further work is warranted to dissect the role of each of these metabolic changes in the pathogenesis of steatosis in hepatitis C.

Insulin resistance is a hallmark of the metabolic syndrome and, as such, proceeds independently of HCV. However, there is some good evidence that HCV may play a significant role in influencing the level of insulin resistance, and this irrespectively of the stage of liver disease. The fact that HCV-infected cirrhotic patients may present with type 2 diabetes more frequently than patients with cirrhosis of other origin was first reported in 1994[39], and has been later confirmed by several cohort and case-control studies, also in special populations like patients having received transplantation[40-48]. In particular, a retrospective analysis of 1117 patients with chronic viral hepatitis[40] reported that diabetes was diagnosed in 21% of HCV but only in 12% of HBV-infected patients. By multivariate analysis, HCV infection and age resulted in independent predictors of diabetes. In the same study, when 594 diabetics were compared with 377 patients evaluated for thyroid disturbances, 4.2% of diabetic patients were found to be infected with HCV as compared with 1.6% of controls[40]. In another study, conducted within the Third National Health and Nutrition Examination Survey (NANHES-III), a significant association with age was observed, i.e. chronic hepatitis C patients 40 years of age or older were more than three times more likely to have type 2 diabetes than those without HCV infection[46]. This raised the issue that diabetes may be due to the stage of liver disease rather than to the viral infection. However, Hui *et al*[49] have subsequently reported that 121 HCV-infected patients with stage 0 or 1 hepatic fibrosis had higher levels of HOMA scores compared with 137 healthy volunteers matched by sex, body mass index, and waist-to-hip ratio. This work also provided some evidence that the level of insulin resistance may be genotype-specific, since patients with genotype 3 had lower levels of HOMA scores than patients with genotype 1. Further evidence that HCV may provoke an insulin resistant state comes from some limited data on the correlation between the severity of insulin resistance and the HCV replication levels[50], and from the observation that insulin sensitivity may improve in patients who achieve HCV RNA clearance following antiviral therapy, while remaining unchanged in non-responders, despite a decrease in BMI[51]. However, further, independent confirmation of these data is warranted.

Experimental data suggest a direct interference of HCV with the insulin signaling pathway *via* proteasomal degradation of the insulin receptor substrate-1 and -2[52]. In addition, functional impairment of this signaling pathway may occur *via* increased levels of pro-inflammatory cytokines such as TNF-α[53] or other post-receptor defects[54]. It has been proposed that chronic hepatitis C patients with more severe liver disease may have an exaggerated intrahepatic TNF-α response, resulting in insulin resistance and a higher risk of developing diabetes[55]. In patients with genotype 3a, HCV may alter the intrahepatic insulin signaling through a down-regulation of PPARγ[32]. Interestingly, although interference with the insulin signaling shows some HCV genotype-specificity, as discussed above, insulin resistance has been reported to occur in all HCV genotypes[56].

Thus, HCV may induce liver steatosis by interfering with lipid metabolism in hepatocytes, and, indirectly, by influencing the level of insulin resistance. As a consequence, the metabolic steatosis, although largely due to disorders independent of the viral infection, may at least partially be ameliorated by antiviral therapy. It has to be noted, however, that as many as 30% of patients with fatty liver who do not drink alcohol and are infected with genotypes other than 3a have normal BMI and HOMA score[56], suggesting that other causes of fatty liver exist in hepatitis C.

A relatively new field is represented by the metabolic impact of cytokines secreted by adipose tissue, i.e. the so-called adipokines. As an example, an association has been reported for serum levels of adiponectin and HCV-related steatosis[57]. Adiponectin is a cytokine secreted by adipocytes with antilipogenic effects that may protect non-adipocyte tissues, such as liver, from fat accumulation. Chronic hepatitis C patients infected with genotype 3 have the lowest levels of adiponectin. Low circulating levels of adiponectin lead to increased serum free fatty acids, which are then taken up by hepatocytes. Further work is needed to fully appreciate the significance of adiponectin in steatosis associated with hepatitis C and more in general in the metabolic syndrome.

Apart from viral and metabolic factors, some specific host genetic polymorphisms may also play a role in the pathogenesis of steatosis. Hyperhomocysteinemia, by inducing endoplasmic reticulum (ER) stress, causes deregulation of the endogenous sterol response pathway *via* SREBP, leading to increased hepatic biosynthesis and uptake of cholesterol and triglycerides. This in turn leads to steatosis. It has also been suggested that hyperhomocysteinemia may increase oxidative stress by inhibiting the expression of several antioxidant enzymes, thus sensitizing hepatocytes to the cytotoxic effect of

pro-oxidant agents. Interestingly, a close association has been reported between the severity of steatosis and homocysteine serum levels in chronic hepatitis C patients[58]. The hyperhomocysteinemia-induced steatosis model may explain why only some, but not all, HCV-infected patients develop steatosis, and why only a minority of patients, e.g. those with higher homocysteine levels, accumulate a greater amount of fat in the liver. Hyperhomocysteinemia may result from a methylenetetrahydrofolate reductase (MTHFR) C677T polymorphism. In that study, it was shown that the polymorphism of the MTHFR gene at position 677, which has a prevalence of 12%-15% for the TT genotype in the general population, was associated with both hyperhomocysteinemia and a greater degree of steatosis in chronic hepatitis C patients. It was estimated that the relative risk of developing more severe steatosis was six-fold higher for patients with the CT genotype and 20-fold higher for those with the TT genotype[58].

## IS THERE A ROLE FOR STEATOSIS IN HCV REPLICATION?

HCV replication occurs entirely in the hepatocyte cytoplasm and, like many other plus-strand RNA viruses, proceeds in association with the endoplasmic reticulum membranes. Perturbation of host cell lipid and cholesterol metabolism can disrupt replication complexes by altering membranous structures where replication occurs. Thus, it is very likely that alterations brought about by HCV on lipid metabolism may affect its own replication.

The question that has been repeatedly raised is whether steatosis may play a role in the HCV life cycle, e.g. by stimulating HCV replication or facilitating cell-to-cell spread. Most models are currently highly speculative. For example, HCV is known for circulating bound to lipoproteins under the form of lipo-viro-particles (LPVs)[59]. Interestingly, binding LPVs to hepatocyte cell lines can be out competed by VLDL and LDL from noninfected controls and is blocked by anti-ApoB antibodies. Conversely, up-regulation of the LDL receptor increases their internalization[59]. Thus, low levels of circulating VLDL, as induced by HCV genotype 3a and, in general, in all patients with viral steatosis, may facilitate cell-to-cell spread of LPVs. This fascinating hypothesis needs, however further confirmation.

Another interesting aspect of the interaction between HCV replication and lipids comes from the observation that HCV RNA replication in hepatoma cells can be disrupted by treatment with lovastatin, a drug that decreases the production of mevalonate by inhibiting 3-hydroxy-3-methylglutaryl CoA reductase[60]. Mevalonate is a precursor of hydrophobic prenyl prosthetic groups, like the geranylgeranyl group, which are necessary to anchor various proteins to cell membranes. The inhibition of HCV RNA replication by lovastatin was overcome by the addition of geranylgeraniol, suggesting that HCV RNA replication requires one or more geranylgeranylated protein[60,61]. This protein has been recently identified as the FBL2 protein (F-box and leucine-rich repeat-containing protein[62]), which would form a stable complex with the non-structural protein 5A of HCV[63]. Knockdown of FBL2 mRNA was paralleled by inhibition of HCV replication *in vitro*[63]. It is interesting to add that several statins were recently shown to possess anti-HCV activity *in vitro*[64]. Fluvastatin exhibited the strongest anti-HCV activity, atorvastatin and simvastatin showed moderate inhibitory effects, whereas lovastatin had weakest anti-HCV activity. Moreover, the anti-HCV activities of statins were reversed by the addition of both mevalonate and geranylgeraniol[64]. If confirmed, these observations may have important consequences on the management of chronic hepatitis C.

Thus, the fatty acid content of the infected hepatocyte may be crucial to modulate the rate of viral replication. However, some caution is mandatory before hasty conclusions are drawn, based on these data. Fatty acids accumulated in the form of triglycerides as in the case of steatosis may not be available to replication complexes involving HCV, and their degree of saturation may also vary. It is known that, in patients with viral steatosis, the severity of the fatty liver correlates with HCV replication level: however, in these patients, replication precedes fatty accumulation, and not vice-versa, as shown by antiviral treatment data. Conversely, in patients with metabolic steatosis, in whom steatosis precedes viral infection and proceeds independently of it, the level of viral replication is not increased in parallel with the severity of fatty liver. Thus, further work is warranted to understand the relationship between steatosis and HCV life cycle, if any exists.

## STEATOSIS AS COFACTOR OF LIVER FIBROSIS PROGRESSION

The vast majority of cross-sectional[10,34,65,66-71] and longitudinal[11,66-68] studies have repeatedly shown an association between steatosis and progressing liver fibrosis in HCV-infected patients. At present, it is unclear whether the two types of HCV-associated steatosis (viral and metabolic) have a similar or different impact on fibrosis progression, because the reported data are controversial. It is likely that the two forms of steatosis act in an additive way. However, data are not unequivocal, as some studies suggest that steatosis may accelerate fibrosis only in genotype 3-infected persons[67-69], whereas others support an association in patients infected with genotype 1[70,71]. At least one study denies any association between steatosis and fibrosis[72]. Interestingly, as the disease progresses to advanced cirrhosis, steatosis tends to disappear[69], a phenomenon already observed in NAFLD[73]. Longitudinal studies are particularly important in emphasizing the role of steatosis in fibrosis progression. In a recent study on paired liver biopsies performed across a median interval of 61 mo in 135 untreated patients with chronic hepatitis C[66], steatosis was the only independent factor predictive of progression of fibrosis, and the probability of progression of fibrosis was significantly related to the percentage of hepatocytes with steatosis. The authors concluded by supporting the attitude of treating patients with mild

hepatitis and steatosis, regardless of HCV genotype.

The mechanisms by which HCV-related steatosis promotes liver fibrosis progression are far from being elucidated. Available data suggest that oxidative stress, pro-inflammatory cytokines, insulin resistance, and increased susceptibility to apoptosis may mediate the fibrogenic effect of steatosis. However, since virtually all studies are correlative, it is difficult to ascertain whether steatosis directly participates in the fibrogenic process, or whether it should be considered as an innocent by-product of another, directly fibrogenic mechanism.

The association between HCV and intrahepatic oxidative stress has been mentioned above[26,27]. It has been reported that in the presence of hepatic steatosis, oxidative stress is enhanced in HCV infection and may promote fibrogenesis, similarly to the second "hit" proposed in NAFLD. In a recent study, Kitase *et al*[74] detected by immunohistochemistry some protein adducts with lipid peroxidation end-products in the liver of chronic hepatitis C patients. Interestingly, areas positive for 4-hydroxy-2-hexenal-protein adducts, a good marker for oxidative stress, were larger in steatotic livers compared to non-steatotic. Thus, the authors concluded that steatosis in chronic hepatitis C may amplify the oxidative stress-driven lipid peroxidation by providing the necessary fuel[74]. There is also some limited evidence that antioxidant therapies may ameliorate the necro-inflammatory activity in chronic hepatitis C[75], although long-term effects on the fibrosis progression rate have not been reported so far.

Pro-inflammatory cytokines may mediate fibrogenesis in the steatotic liver, although it is unclear whether steatosis would facilitate this process. A recent meta-analysis (the HCV MAID study)[70], which included individual patients data of 3068 patients with chronic hepatitis C from 10 centers in five countries, demonstrated that liver steatosis is associated with increased liver inflammatory activity and accelerates the progression of liver fibrosis. This observation has been reported by others[34,71].

Insulin resistance is fibrogenic in the liver. However, in most studies, the relative contribution of steatosis and insulin resistance to fibrosis has not been determined. The HCV MAID Study has suggested that steatosis and diabetes are both independent factors of fibrogenesis in patients with genotype 1 infection[70]. However, when insulin resistance, an earlier and more sensitive parameter of glucose metabolism dysfunction, is added to a logistic regression analysis, the association between steatosis and fibrosis disappears[49]. A body of epidemiologic work suggests that the presence of diabetes and insulin resistance *per se* are risk factors of severe fibrosis and more rapid fibrosis progression in chronic hepatitis C[49,76,77]. The connective tissue growth factor (CTGF) is over-expressed in the liver of patients with non-alcoholic steatohepatitis as well as of diabetic Zucker rats[78]. In addition, both CTGF mRNA and protein are significantly increased when hepatic stellate cells are incubated with either glucose or insulin[78]. Similar mechanisms may operate in chronic hepatitis C[79], where the level of intrahepatic expression of CTGF was correlated with serum levels of leptin and the scores of steatosis and fibrosis. Since hepatic stellate cells possess leptin receptors[80], it is intriguing to speculate that leptin may represent the link between steatosis, insulin resistance and fibrosis in chronic hepatitis C.

Finally, hepatocyte apoptosis is a well recognized condition associated with both necroinflammatory activity and fibrosis in NAFLD[81]. In chronic hepatitis C, in the presence of steatosis, increased apoptosis is associated with activation of stellate cells and increased stage of fibrosis[82]. In addition, steatosis is associated with decreased Bcl-2 mRNA levels and an increase in the proapoptotic Bax/Bcl-2 ratio. It has also been reported that caspase activity, which controls apoptosis, is increased in both liver biopsy and sera from HCV patients[83] and is strictly correlated to the extension of steatosis. All these data seem to indicate a relationship between steatosis, apoptosis and development of fibrosis, although the fine pathogenetic mechanisms await clarification.

## HCV-RELATED STEATOSIS AND HEPATOCELLULAR CARCINOMA

Experimental data from the transgenic mice model have shown that HCV may play a causative role in the development of steatosis and hepatocellular carcinoma (HCC)[27,84]. It has been speculated that, among the possible mechanisms, reactive oxygen species may play a major role in mutagenesis[27,85]. In addition, the HCV core protein may interact with RxR$\alpha$[31], a transcriptional regulator that controls many aspects of cell proliferation and differentiation. A recent study has reported steatosis to be an independent risk factor for the development of HCC in chronic hepatitis C patients[85]. The authors prospectively followed 161 patients with chronic HCV infection for up to 15 years and found that the presence of steatosis was significantly associated with the incidence of HCC by multivariate analysis. Recently, however, a retrospective study including a smaller number of chronic hepatitis C patients did not confirm that steatosis may be a risk factor for HCC development[86]. Thus, further prospective studies are needed to assess the role of HCV-associated steatosis in liver carcinogenesis.

## HCV-RELATED STEATOSIS AND RESPONSE TO ANTIVIRAL TREATMENT

Steatosis has been recognized as a negative factor response to antiviral therapy for many years[87]. This observation has been repeatedly confirmed by data coming from large clinical trials[16,88,89]. The effect is significant for the steatosis seen among patients with non-3a genotype, hinting at the insulin resistance as the pathogenetic factor affecting responsiveness to interferon-alpha. This was confirmed by a recent study[51], where the sustained virological response rate was inversely correlated with the baseline HOMA score. Indirect evidence in favor of this negative association comes also from the reduced response to antivirals seen in African Americans, reportedly due to their high rate of visceral obesity and insulin resistance[90], and from the correlation between high levels of circulating TNF-$\alpha$, as seen in insulin resistance, and poor response to interferon therapy[90].

The molecular reasons for the correlation existing between insulin resistance and interferon-alpha resistance are still unclear. Chronic hepatitis C patients who do not respond to interferon-alpha may have increased levels of suppressor of cytokine signalling 3 (SOCS-3) in the liver, a factor promoting the proteasomal degradation of IRS-1[91]. Interestingly, members of the SOCS family are negative regulators of STAT-1, a factor involved in the transduction of the interferon-alpha signaling[92]. Thus, we cannot exclude that HCV activates some members of the SOCS family as a mechanism to inhibit the interferon-alpha signaling, with the simultaneous impairment of the insulin signaling just being a collateral effect.

## CONCLUSION

Steatosis is an established risk factor for disease progression in chronic hepatitis C. Furthermore, it has also been shown to impact significantly on the response to antiviral therapy. Thus, appropriate therapeutic strategies for HCV-related steatosis are required with the aim to improve both the natural history of chronic hepatitis C and its drug management. Ideally, it would be important to assess whether steatosis is mainly virally-induced (like in many cases of genotype 3 infection) or whether metabolic host factors play a predominant role, like in many patients infected with non-3 genotypes. In chronic hepatitis C patients with genotype 3, successful treatment is associated with disappearance or significant amelioration of fatty liver. Whether the efficacy of antivirals may be improved by specific interventions aimed at reducing the degree of steatosis remains to be proven. In patients with non-3 genotype infection, in whom insulin resistance may play a role as cofactor of disease and fatty infiltration of the liver, the first-line intervention is represented by those life-style modifications that may reduce body weight. In a recent study, a weight-reduction program followed by chronic hepatitis C patients was effective in reducing steatosis and improving liver biochemistry and fibrosis in the absence of effects on virological parameters and irrespectively of the infecting genotype[93]. One should be reminded that the diagnosis of viral and/or metabolic steatosis in any given patient should not be based solely on the infecting genotype, but should be corroborated by a whole set of clinical and laboratory parameters, including the assessment of the insulin resistance score. Finally, because alcohol, even at low doses, synergistically interacts with steatosis to promote fibrosis progression, it is mandatory to advise the patients to avoid its use. The additional role of drugs like metformin and thiazolidinediones, which improve insulin sensitivity, remains to be evaluated in carefully planned clinical trials. Of additional interest is the use of drugs that may inhibit the geranylgeranylation, a cellular enzymatic activity apparently essential for HCV replication. All these pharmacological strategies, however, remain experimental, for the time being, and should not be attempted in routine clinical practice until new data from controlled trials will be available.

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*GASTRIC CANCER*

# Inhibition of human gastric carcinoma cell growth by atofluding derivative N3-*o*-toluyl-fluorouracil

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## Abstract

**AIM:** To evaluate the growth inhibition efficacy of atofluding derivative N3-*o*-toluyl-fluorouracil (TFU) on human gastric carcinoma cell lines SGC-7901 and MKN-45.

**METHODS:** Cell growth inhibition by TFU was measured by MTT and clonogenic assays without or with liver microsomal enzymes. Xenografts of cancer cells in nude mice were employed to study the anti-proliferative effects of TFU *in vivo*.

**RESULTS:** TFU inhibited the growth of SGC-7901 and MKN-45 cells. However, the inhibitory effects of TFU on cell growth were not significant. The inhibition rates were enhanced in the presence of liver microsomal enzymes, ranging 4.73%-48.57% in SGC-7901 cells and 9.0%-62.02% in MKN-45 cells. *In vivo*, TFU delayed the growth of SGC-7901 and MKN-45 cells in nude mice. The inhibition rates were 40.49%, 63.24%, and 75.98% in SGC-7901 cells and 40.76%, 61.41%, and 82.07% in MKN-45 cells when the oral doses were 25, 50, and 100 mg/kg, respectively. TFU treatment was generally well tolerated by mice with less than 20% reduction in body weight.

**CONCLUSION:** TFU inhibits the growth of human gastric carcinoma cells. The inhibition rates are increased in the presence of liver microsomal enzymes. The efficacy of TFU may be associated with the sustaining release of 5-fluorouracil (5-FU) mediated by the enzymes.



**Key words:** N3-*o*-toluyl-fluorouracil; Gastric carcinoma cells; Pro-drug; Growth inhibition

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## INTRODUCTION

5-fluorouracil (5-FU), a pyrimidine analog, has become a backbone in the therapy of gastric and colon cancer since its introduction in 1957. However, its clinically effective dosage is very close to its toxic dosage when given intravenously, resulting in strong toxicities to gastric and intestinal mucosa and bone marrow[1-3]. In addition, its plasma half-life is very short (15-20 min) and the drug is administered only as a continuous iv infusion[4-7]. In order to overcome these disadvantages, attempts have been made to design and synthesize new 5-FU derivatives. In the 1980s, a new oral 5-FU derivative, N1-acetyl-N3-*o*-toluyl-fluorouracil (atofluding), was developed[8]. Studies have shown that atofluding can effectively treat many types of tumor with few side effects. Nevertheless, the acetyl group on the N1 position of atofluding is not stable and prone to decompose, impairing quality control for the preparation. The pharmacokinetics showed that atofluding, N3-*o*-toluyl-fluorouracil (TFU), rather than atofluding itself could be detected in the serum after oral administration. TFU is the decomposition products of atofluding that hydrolyze the acetyl group by fluorine on C5 position (Figure 1). TFU is extracted and dissolved in acetone and water before its

determination by high-pressure liquid chromatography[8,9]. The results also showed that steady-state concentrations of TFU can remain in the body for 30 h[8]. These results have led us to the conclusion that TFU is stable in the preparation. Encouraged by these observations, the anti-tumor activities of TFU were studied and the inhibition efficacy of TFU on the growth of human gastric carcinoma cells was evaluated in the present study in order to replace the unstable atofluding.

## MATERIALS AND METHODS

### Cell lines and cell culture

Human gastric carcinoma cell lines, SGC-7901 and MKN-45 were obtained from the Division of Cancer Treatment, National Cancer Institute of China (Beijing, China). Cells were maintained in RPMI-1640 supplemented with 10% (v/v) heat-inactivated fetal bovine serum, penicillin-streptomycin (100 IU/mL-100 μg/mL), 2 mmol/L glutamine, and 10 mM Hepes buffer at 37℃ in a humid atmosphere containing 50 mL/L $CO_2$. Cells were fed every three to four days, and harvested by brief incubation in PBS containing 0.02% trypsin-EDTA.

### Chemical

TFU was synthesized by acylation of 5-FU with 2-methylbenzoyl chloride in pyridine at room temperature as previously described[8,9] and dissolved in dimethylsulfoxide (DMSO) for *in vitro* assay and in 5% amylum for *in vivo* study.

### MTT assay

Cells (2-5 × $10^4$ per well) seeded in 96-well plates (Corning Costar Corporation, Cambridge, MA, USA) for 12 h were treated with different doses of TFU for the required time period. The medium was then removed and the wells were washed with PBS. Cell viability was assessed by adding 20 μL of MTT [3-(4, 5-dimethylthiazol-2-yl)-2], 5 mg/mL 5-diphenyltetrazolium bromide (Sigma, USA) for 4 h[10]. Light absorbance of the solution was measured at 540 nm on the plate reader (TECAN, Grodig, Salzburg, Austria). The growth of experimental and control cells was compared. Experiments were performed in triplicate.

### Liver microsomal enzymes and preparation

SD male rats (150 ± 10 g) from the animal breeding house were treated with intra-peritoneal injections of phenobarbital (80 mg/kg body weight) daily for three consecutive days in 0.5 mL of peanut oil. The animals were sacrificed 24 h after the last injection. Livers were removed, washed with chilled 0.1 mol/L phosphate buffer (pH 7.4) and homogenized. Liver microsomal enzymes were prepared as described by Rastogi S *et al*[11]. The microsomal enzymes were resuspended in 0.1 mol/L phosphate buffer (pH 7.4) containing 10 mmol/L dithiothrietol, 10 mmol/L EDTA and 20% glycerol. Ten mL of the mixture of enzymes containing 1 mL of microsomes and 31.4 mg of nicotinamide adenine dinucleotide phosphate (NADPH; Merck, Darmstadt, Germany) was used[12]. The enzyme mixture (2 μL) was applied to each well of the 96-well plates after cells were seeded and TFU was added. Cell growth inhibition was measured as above.

### Clonogenic assay

Cells (250 to 300 per well) grown in 6-well plates (Falcon, Becton Dickinson, Franklin lakes, New Jersey, USA) for 12 h were treated with different doses of TFU at 37℃. After two weeks, colonies (greater than 50 cells) were stained with crystal violet and counted as previously described[10,13].

### In vivo inhibition of tumor growth

The *in vivo* efficacy of TFU was assessed in nude mice bearing tumors. Balb/c athymic (nu+/nu+) female mice, 4-6 wk of age, were purchased from the Experimental Animal Laboratory, Chinese Academy of Medical Sciences (Beijing, China). The research protocol was approved in accordance with the institutional guidelines of the Animal Care and Use Committee at Shandong University. Animals were housed under pathogen-free conditions. Cells (1 × $10^7$) were suspended in 100 μL of Matrigel (Collaborative Biomedical, Bedford, MA, USA) and injected subcutaneously into the right anterior flank of nude mice. After seven days, when tumor volume reached approximately 0.1-0.2 $cm^3$, the mice were divided into different groups ($n$ = 8) and orally administered 0, 25, 50, 100 mg/kg of TFU in 0.5 mL of 5% amylum[14]. Administrations were performed six days per week for three consecutive weeks. Tumor growth inhibition rates were defined as a ratio to the control tumor weight.

### Statistical analysis

Statistical significance was determined by the Student' s two-tailed $t$-test. The Kruskal-Wallis test was used to detect the percent of cell growth inhibition. $P < 0.05$ was considered statistically significant. Statistical analysis was performed with SPSS/Win11.0 software (SPSS, Chicago, IL, USA).

## RESULTS

### Growth inhibitory effects of TFU

To select optimal times for analysis of the relationship between treatment with TFU and cell growth inhibition, untreated cells were tested over six days. SGC-7901 and MKN-45 cells reached their confluence by d 6. We then selected d 5 within the linear growth period for concentration analysis and a 5-d period for the time course of treated cells (data not shown).

Gastric carcinoma cells were treated with TFU (0.1, 1, 10, 100, 1000 μg/mL) for up to 120 h and the viable cells were evaluated as described in MATERIALS and METHODS. As shown in Figure 2A, TFU showed its anti-proliferative effects on SGC-7901 cells in a dose- and time-dependent manner. Significant differences were seen across the doses tested (Kruskal-wallis H = 18.870, $P$ = 0.042). The growth inhibitory effects of TFU were significantly increased from 24 to 120 h of incubation (Kruskal-wallis H = 18.870, $P$ = 0.042). However, the inhibitory effects of TFU on cell growth were not high. Cells cultured with 10 μg/mL of TFU achieved only 11.09% of growth inhibition

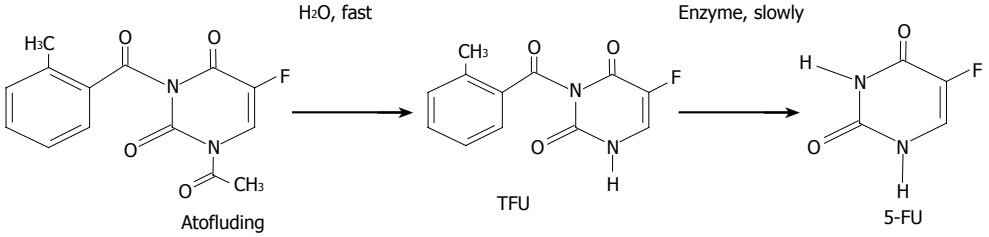


**Figure 1** Metabolism of atofluding to into TFU and 5-FU. The acetyl group on the N1 position of atofluding was hydrolyzed in the presence of $H_2O$ into TFU rapidly due to the influence of fluorine on the C5 position. 5-FU was transformed from TFU slowly and continually by the liver microsomal enzymes.

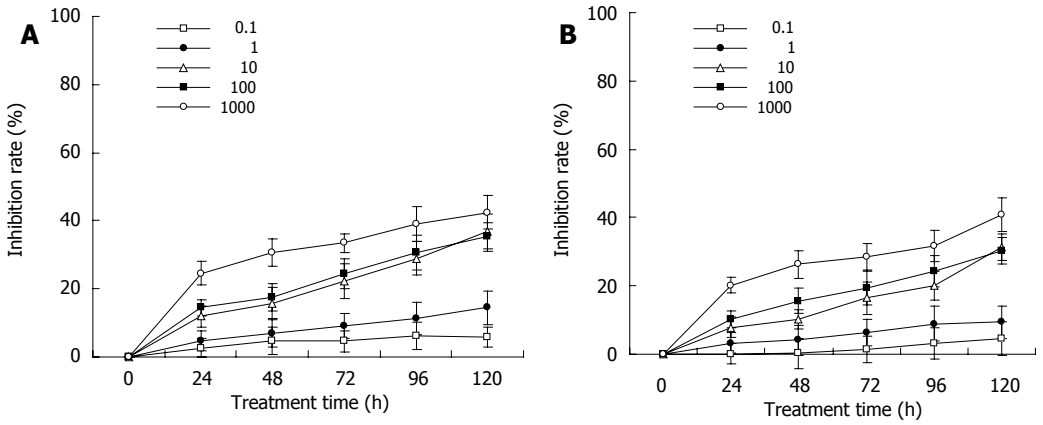


**Figure 2** TFU inhibits MKN-45 (**A**) and SGC-7901 (**B**) cell growth *in vitro*. Cells were treated with TFU (0.1, 1, 10, 100, 1000 μg/mL) for up to 120 h. Viable cell numbers were evaluated by the MTT assay and expressed as percentage of untreated controls at the concurrent time point. The bars indicate means ± SD ($n$ = 3).

**Table 1 Enhancement of TFU induces growth inhibition of SGC-7901 and NKM-45 cells in the presence of liver microsomal enzymes**

| Cell line | Time (h) | Inhibition (%) | | | | |
|---|---|---|---|---|---|---|
| | | 0.1 μg/mL | 1 μg/mL | 10 μg/mL | 100 μg/mL | 1000 μg/mL |
| SGC-7901 | | | | | | |
| | 24 | 4.73 | 8.21 | 11.92 | 14.84 | 24.08 |
| | 48 | 9.56 | 15.10 | 22.31 | 36.02 | 39.89 |
| | 72 | 17.29 | 24.35 | 30.75 | 40.03 | 43.72 |
| | 96 | 22.03 | 31.76 | 32.97 | 42.43 | 45.51 |
| | 120 | 37.67 | 36.67 | 35.24 | 45.86 | 48.57 |
| MKN-45 | | | | | | |
| | 24 | 9.00 | 18.38 | 20.00 | 36.99 | 42.02 |
| | 48 | 20.00 | 34.00 | 40.00 | 51.40 | 55.00 |
| | 72 | 45.09 | 52.63 | 57.11 | 58.18 | 60.16 |
| | 96 | 52.50 | 61.64 | 57.87 | 55.53 | 61.41 |
| | 120 | 59.19 | 62.02 | 59.12 | 55.84 | 53.98 |

Enhancement was calculated using the inhibition rate in the presence of microsomal enzymes minus the inhibition rate in the absence of enzymes at the same concentration.

at 24 h, 15.69% at 48 h, 22.24% at 72 h, 28.93% at 96 h and 36.86% at 120 h. The maximum inhibition rate was 42.51% at the concentration of 1000 μg/mL after 120 h treatment. MKN-45 cells expressed similar results (Figure 2B).

### *Enhancement of growth inhibition on human gastric carcinoma cells by liver microsomal enzymes*

The inhibitory effects of TFU on human gastric carcinoma cells were then examined in the presence of liver microsomal enzymes. The inhibition rates were significantly increased at all concentrations (0.1, 1, 10, 100, 1000 μg/mL) and treatment time points (24 to 120 h), being 4.73%-48.57% in SGC-7901 cells and 9.0%-62.02% in MKN-45 cells (Table 1). The growth inhibition was increased in a dose- and time-dependent manner (data not shown).

We also used a clonogenic assay to further test the sensitivity of SGC-7901 and MKN-45 cells to TFU over a relatively long culture period (two weeks). The anti-proliferative effect of TFU on clone formation was significantly increased in the presence of liver microsomal enzymes across the concentrations ($P < 0.01$, data not shown).

### *Inhibitory effects of TFU in vivo*

The effects of TFU on gastric carcinoma cell xenografts in nude mice were then examined. As shown in Table 2, TFU delayed the growth of SGC-7901 and MKN-45 cells after three weeks of treatment with 25, 50, and 100 mg/kg ($P < 0.01$). TFU treatment inhibited tumor growth in a dose-dependent manner. This inhibitory effect on the xenograft was more pronounced in MKN-45 cells than in SGC-7901 cells. Except for the 100 mg/kg group, TFU treatment was generally well tolerated by mice with less than 20% reduction in body weight ($P > 0.05$).

## DISCUSSION

In this report, we evaluated the efficacy of TFU, the pro-drug of 5-FU, on growth inhibition of human gastric carcinoma cells. The results showed that TFU had anti-proliferative effects on SGC-7901 and MKN-45 cell growth. The inhibition rates were increased significantly in the presence of liver microsomal enzymes.

Structurally, the difference between atofluding and TFU is that the former has an acetyl group on its $N_1$ position. Nevertheless, the acetyl group is prone to be hydrolyzed into TFU rapidly by fluorine on the C5 position, impairing quality control for the preparation. On the other hand, TFU is very stable *in vitro* and *in vivo*[8,9,15].

**Table 2 Growth inhibitory effects of TFU on SGC-7901 and MKN-45 cells in nude mice ($n = 8$)**

| Cell line | Dosage (mg/kg) | Body weight (mean ± SD, g) | Tumor weight (mean ± SD, g) | Tumor growth inhibition (%) |
|---|---|---|---|---|
| SGC-7901 | | | | |
| | 0 | 22.12 ± 2.31 | 2.30 ± 0.67 | |
| | 25 | 21.62 ± 1.96 | 1.21 ± 0.61 | 30.49 |
| | 50 | 20.28 ± 2.95 | 0.75 ± 0.17 | 53.24 |
| | 100 | 18.05 ± 2.40 | 1.23 ± 0.43 | 65.98 |
| MKN-45 | | | | |
| | 0 | 22.72 ± 1.75 | 1.84 ± 0.72 | |
| | 25 | 21.62 ± 1.96 | 1.09 ± 0.81 | 40.76 |
| | 50 | 20.28 ± 2.95 | 0.71 ± 0.40 | 61.41 |
| | 100 | 18.75 ± 1.44 | 0.33 ± 0.49 | 82.07 |

Differences were analyzed using the Student's two-tailed test.

Consistent results have been seen in our previous studies. The pharmacokinetic studies showed that TFU is detectable some minutes after oral administration of atofluding[8]. In this study, however, atofluding was not detected in the required time period, the steady-state concentrations of TFU in blood (9.97 ± 0.7 mg/mL) were achieved 30 h after multiple administrations. TFU was subsequently slowly metabolized to release 5-FU. Though the subsequent steady-state concentrations of 5-FU were lower than those of TFU, the concentrations could remain for up to 50-52 h[15], suggesting that TFU has good anti-tumor activities and low side effects. TFU would replace the unstable atofluding as the preparation. Sustained release of 5-FU from TFU is a catalytic procedure and enzymes are needed to mediate the bio-transformation[8]. In this study, liver microsomal enzymes promoted the activities of TFU, suggesting that the enzymes can mediate the metabolism.

However, the mechanism of the pathways is not known. Liver microsomal enzymes in a reaction mixture contain many forms of reductase and oxygenase[11,12,16,17]. It is presumed that TFU might transform into 5-FU by hydrolyzing in the presence of NADPH-dependent reductase[18]. The activity of reductase is initiated by donating electrons from NADPH consumption[11,12]. The bio-transformation slowly releases 5-FU into the blood to keep the relatively long steady-state concentrations. 5-FU is then converted to deoxynucleotide (5-FdUMP) mediated by phosphoribosyl transferase in tumor cells and competes with deoxyuridine monophosphate (dUMP) for thymidylate synthetase[19-23]. DNA synthesis decreases, thus leading to cell death. Liver microsomal enzyme assay is currently underway to analyze the metabolism of sustained release of 5-FU.

In conclusion, TFU inhibits the growth of gastric carcinoma cells by sustaining the release of 5-FU. Liver microsomal enzymes mediate this bio-transformation. Experiments on a broad-spectrum of cancer cell lines are in progress.

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# Inhibition of hepatic tumor cell proliferation *in vitro* and tumor growth *in vivo* by taltobulin, a synthetic analogue of the tripeptide hemiasterlin

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## Abstract

**AIM:** To investigate the inhibitory effects of taltobulin (HTI-286), a synthetic analogue of natural hemiasterlin derived from marine sponges, on hepatic tumor growth *in vitro* and *in vivo*.

**METHODS:** The potential anti-proliferative effects of HTI-286 on different hepatic tumor cell lines *in vitro* and *in vivo* were examined.

**RESULTS:** HTI-286 significantly inhibited proliferation of all three hepatic tumor cell lines (mean $IC_{50}$ = 2 nmol/L ± 1 nmol/L) *in vitro*. Interestingly, no decrease in viable primary human hepatocytes (PHH) was detected under HTI-286 exposure. Moreover, intravenous administration of HTI-286 significantly inhibited tumor growth *in vivo* (rat allograft model).

**CONCLUSION:** HTI-286 might be considered a potent promising drug in treatment of liver malignancies. HTI-286 is currently undergoing clinical evaluation in cancer patients.



**Key words**: Hepatocellular carcinoma; Antimicrotubule agent; HTI-286

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## INTRODUCTION

Hepatocellular carcinoma (HCC) is the fifth most common tumor entity causing over one million deaths annually worldwide[1,2]. Most patients with HCC suffer two different diseases, namely a chronic and a malignant liver disease. More than 80% of all HCCs are developed in cirrhotic livers. Currently, only curative treatment options are surgical resection or liver transplantation. However, only a very few patients are eligible for these treatment options. Almost 80% of the newly diagnosed patients with HCC do not qualify for resection because of insufficient functional parenchyma or transplantation due to multi-centricity and/or other co-morbidities. Consequently, most patients unfortunately only qualify for a palliative treatment[3,4]. For these patients, a number of therapeutic interventions have been developed, termed as ablative procedures (transarterial chemoembolization, radio-frequency- or laser-induced thermo-ablation and percutaneous ethanol injection)[5]. Furthermore, several systemic chemotherapy regimes have been evaluated[6-8]. For all conservative therapies, randomised clinical trials are missing to date. Currently, chemotherapy in HCC is only applied in framework of clinical trails or arterial chemoembolization. To date, only one group has proven a survival benefit of chemoembolization in a specific subpopulation of HCC patients[9]. European Association for the Study of the Liver concluded in a consensus meeting for clinical management of HCC in 2001 that chemotherapy for HCC currently is negligible[5].

Microtubule (MT) function is essential for cell growth and interference of the dynamics of microtubule formation is considered a promising therapeutic approach in cancer treatment[10]. Unfortunately, inherent resistance

to anti-microtubule agents has been found in many tumor types and acquired resistance occurs during multiple cycles of therapy. Thus, great interest lies in identifying new anti-microtubule drugs that overcome various modes of resistance. Anti-microtubule agents have not been commonly tested against HCC since initial studies had shown inherent or acquired resistance to anti-microtubule agents because of high intrinsic activity of multidrug resistance gene (MDR) in HCC[11-13].

HTI-286 is a synthetic tubulin inhibitor and a hemiasterlin analogue. Hemiasterlin was originally identified as natural product from marine sponges. It is a potent inhibitor of cell growth, depolymerizes MTs and arrests cells in the $G_2$-M phase of cell cycle. Loganzo *et al*[14] have shown that HTI-286 retains potency in cellular models resistant to several chemotherapeutics like taxanes and vinca alkaloids. HTI-286 has been fount to inhibit growth of various human tumor xenograft models, such as colon, skin, prostate, brain and breast cancer, that are resistant to currently approved anti-microtubule drugs. Additionally, HTI-286 circumvents the P-glycoprotein-mediated resistance, which hampers efficacy of several anti-microtubule agents[14,15].

Accordingly, we hypothesised that HTI-286 has a promising potential in the treatment of HCC. We investigated the proliferation inhibiting effect of HTI-286 on hepatic tumor cell lines, such as like Morris hepatoma (MH), HepG2 and Hep3B, *in vitro*. Primary human hepatocytes (PHH) in culture were also exposed to HTI-286 to evaluate a possible cytotoxic effect. Furthermore, in a rat allograft model, *in vivo* effect of intravenous HTI-286 administration on tumor growth was studied. We found that HTI-286 markedly inhibited cell proliferation of hepatocarcinoma cells *in vitro* and tumor growth *in vivo*. Interestingly, no significant cell death appeared in PHH cell culture under HTI-286 exposure.

## MATERIALS AND METHODS

### Compound

HTI-286 (N, β, β- trimethyl- L- phenylalanyl- N-(1S, 2E)-3-carboxy-1-isopropylbut-2-enyl N, 3 dimethyl-L-valinamide), also known as taltobulin or SPA-110, was generously provided by Dr. Frank Loganzo (Wyeth, Pearl River, NY, USA). HTI-286 was dissolved in Dulbecco's modified Eagle's medium (DMEM) to make two stock solutions of 5 and 0.05 mmol/L, which were then diluted in culture medium to obtain the desired concentrations.

### Cell lines

Rat liver tumor Morris hepatoma 3924A (MH) cells were grown in RPMI 1640 with Glutamax I (Invitrogen) supplemented with 200 mL/L heat-inactivated fetal bovine serum (FBS) and 1% penicillin/streptomycin, gentamycin 50 μg/mL (Invitrogen).

Human hepatocarcinoma HepG2 cells were cultured in Minimum Essential Medium Eagle with glutamine (Sigma) supplemented with 0.11 g/L sodium pyruvate and 1.5 g/L sodium bicarbonate (pH 7.2), 100 mL/L heat-inactivated FBS and 1% penicillin/streptomycin, gentamycin 50 μg/mL.

Human hepatocarcinoma Hep3B cells were cultured in DMEM (4.5 g/L glucose), Glutamax I, NaPyr (Invitrogen), 100 mL/L FBS, 1% penicillin/streptomycin and gentamycin. Isolation of primary human hepatocytes was done by a co-research group and kindly provided to us. Primary human hepatocytes were cultured in William's medium E (Sigma) containing supplemented factors.

### Evaluation of cell doubling time and determination of 50% cell growth inhibition ($IC_{50}$)

Anti-proliferative activity of HTI-286 was determined *in vitro* using 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl-tetrazolium bromide colorimetric method, also known as the MTT reduction assay. This assay is based on the ability of viable cells to reduce MTT-tetrazolium salt into MTT-formazan by the mitochondrial enzyme succinate-dehydrogenase[16].

Before initiating proliferation assay, cell doubling time of each tested cell line was evaluated with the MTT assay. Afterwards MH, Hep3B and HepG2 tumor cells were cultured in 96-well microtiter plates and seeded at densities predetermined. Ensuring cell confluence rates of > 50%, fresh medium with or without HTI-286 was added at different concentrations for 1, 2 or 3 doubling times. Then, 12.5 μL of an MTT solution in medium (5 mg/mL MTT; Sigma Chemical Co., St Louis, USA) was added for 3 h. The medium was removed and the MTT-formazan crystals were solubilized by adding DMSO (100 μL/well). Absorbance ($A_{562}$) was determined at 562 nm. Drug concentration inhibiting 50% ($IC_{50}$) growth compared to untreated cells was calculated.

### Proliferation assay

MH, Hep3B and HepG2 tumor cells were cultured in 96-well microtiter plates and seeded at densities predetermined after they were starved in growth factor-depleted medium for one doubling time. Ensuring cell confluence rates of > 50%, fresh medium with or without HTI-286 was added at different concentrations for two doubling times. MTT assay was processed as described above. Results were expressed as percentage of the control. The absorbance of the control (cell culture without any treatment) corresponds to 100% MTT reduction. Three independent experiments were performed for each cell line and data were presented as mean ± SD.

### DNA synthesis measurement using 5-bromodeoxyuridine (BrdU) incorporation

To evaluate the cell cycle arrest, MH cells were $A_{405}$ seeded at the same densities as used for proliferation assay, but BrdU, a labelled DNA precursor (5-bromo-2'-deoxyuridine), was applied for three and half hours. After removing the medium and according to the manufacturer's recommendations, absorbance was determined at 405 nm. The labelled precursor is incorporated into genomic DNA during the S-phase (synthesis) of the cell cycle and the detected amount is directly proportional to the rate of cell division occurring in the sample.

### Immunofluorescence assay

Morris hepatoma, Hep3B and HepG2 cells were plated on

cover slips placed in 6-well dishes. Two days later, different concentrations of fresh medium with or without HTI-286 were added to the wells for two cell doubling times. Cells were washed with phosphate-buffered solution (PBS), fixed for 15 min in methanol at 4℃ and incubated for 1 h at room temperature with 1:500 dilution of anti-α-tubulin antibody (clone DM 1A; Sigma) in PBS. After washing with PBS, cells were incubated with 1:100 dilution of FITC-conjugated F (ab') 2 fragment of goat anti-mouse IgG (Jackson Immunoresearch, West Grove, PA) for 30 min at room temperature in PBS. After washing, cells were mounted in one drop of Dako Faramount (DAKO Corporation, USA). Cells were examined with an Olympus B × 61 microscope and × 40 magnification objective.

### *In vivo study*

A total of 17 female American Cancer Institute (ACI) rats, weighing 120-145 g, were used. The official Animal Care Committee of the University of Bern approved all experimental procedures. The animals had free access to water and standard pelleted diet and were housed in a temperature-controlled room under constant 12 h light/dark cycles. Operations were carried out under anaesthesia using clean but not sterile technique. Rats were pre-anaesthetized with ether and then put under narcosis with a mixture of dormitor, climasol and fentanyl. As antidote a mixture of antisedan, sarmasol and narcan was used. The liver was exposed *via* a midline incision and a single cube (1-2 $mm^3$) of subcutaneously grown Morris hepatoma tumor was implanted between the right and left lobe. Tumors were allowed to grow for 10 d. Tumor growth was traced with magnetic resonance tomography (MRT). MRT was performed in a 1.5 Teele unit (Sonata, Siemens) using a phased array wrist coil. T2-weighted images (TSE; 3D; TRC200; TE 43 ms) in axial plane were acquired with continuous 1 mm slices (isotrope resolution).

The tumor volume was calculated according to a web-based volumetric calculator (S.D. Filip; Mississippi State University). After confirmation of successful tumor implantation, the animals were randomly divided into control and HTI-286-treated arms, each consisted of six animals, by a third person who was not involved in the study. HTI-286 was administered *via* tail-injection in a 0.12 mg/kg rat-weight concentration on d 1, 5, 9 and 15. The tumor growth was followed with MRI on d 7, 21, and 28 after tumor implantation. After the final MRI, animals were sacrificed under a deep narcosis.

### *Statistical analysis*

QuickCals calculator for scientists (GraphPad Software 2002) was used for statistical analysis. Student's *t* test was chosen to compare the means. Differences were considered statistically significant with two-tailed *P* values less than 0.05.

## RESULTS

### *Cell doubling time and $IC_{50}$*

To determine a test confluence in which active cell proliferation is permitted during the experiments and also a sufficient confluence for adding the drug, the cell doubling time of each tumor cell line was first evaluated using different initial seeding densities and by following the cell growth for 7 d (data not shown). The doubling times for MH, Hep3B and HepG2 tumor cells were found to be 22 h, 43 h and 59 h, respectively. For our experiments, we chose to add the drug for two cell doubling times.

We evaluated the required $IC_{50}$ concentration for each cell line with the MTT assay. All three hepatocarcinoma cell lines were exposed for 1, 2 and 3 cell doubling times to the drug. Sensitivity to HTI-286 was different in all cell lines. Mean $IC_{50}$ for all tested hepatic tumor cell lines was 2 nmol/L ± 1 nmol. At 1 nmol/L, HTI-286 inhibited MH cell growth by 50%. The $IC_{50}$ for Hep3B and HepG2 was 2 nmol/L and 3 nmol/L, respectively. HepG2 was the most sensitive and Hep3B the least sensitive cell line among the tested hepatocarcinoma cell lines.

### *Proliferation assay*

Ability of HTI-286 to inhibit cell proliferation was examined. Morris hepatoma, Hep3B and HepG2 cells were exposed at $IC_{50}$ concentration to HTI-286 for two cell doubling times. All three tested hepatic tumor cell lines were significantly inhibited compared to the controls with *P* values of 0.005, 0.001, and 0.002 for MH, HepG2, and Hep3B, respectively (Figure 1A-C).

In contrast, PHH did not show an obvious decrease in viable cells in the MTT assay after continuous exposure to HTI-286 for 24 or 48 h at concentrations between 1 nmol/L and 1 mmol/L (Figure 2).

These data suggest that hepatic neoplastic cells are more sensitive to HTI-286 than primary human hepatocytes. Furthermore, our data are consistent with the effects of HTI-286 on 18 different human tumor cell lines reported by Loganzo *et al*[14].

### *Effect of HTI-286 on cell cycle distribution*

Hemiasterlin arrests cells in mitosis and induces apoptosis. HTI-286-treated MH cells were analysed by 5-bromodeoxyuridine (BrdU) incorporation assay. Cells showed significant decrease in the S phase when being exposed to HTI-286 at 1 nmol/L for two cell doubling times. DNA synthesis was significantly reduced in the treated cells ($P = 0.02$) indicated by reduced BrdU incorporation compared to the untreated cells (Figure 3). With increasing concentration, more cells were found to be arrested into this phase (data not shown). Loganzo *et al*[14], using fluorescence-activated cell sorting analysis (FACS), identified the cell cycle arrest in the $G_2$-M phase. However, we did not have the possibility to perform a FACS analysis. These data are consistent with reported effect of hemiasterlin and HTI-286 on cell cycle and cell proliferation.

### *Effect of HTI-286 on microtubule structure and cell morphology*

The effect of HTI-286 on microtubule structure was examined by immunofluorescence microscopy using an antibody specific for α-tubulin. Untreated MH, Hep3B and HepG2 cells contained a dense and complex network

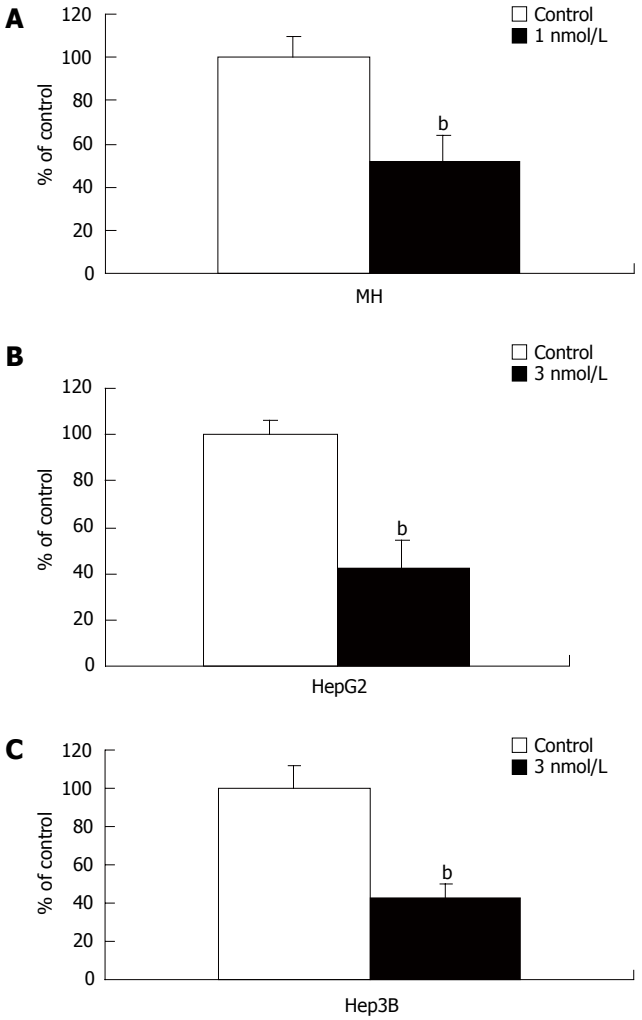


**Figure 1** Inhibitory effect of HTI-286 on proliferation of MH, HepG2, and Hep3B cells. Inhibitory effect upon HTI-286 treatment was highly significant in all three hepatic carcinoma cell lines. MH (**A**), HepG2 (**B**) and Hep3B (**C**) cells were exposed to 1, 3, and 3 nmol/L of HTI-286, respectively, for two cell doubling times. Student's *t* test analysis showed two-sided *P* values of 0.005, 0.002 and 0.001 for MH, HepG2, and Hep3B, respectively.

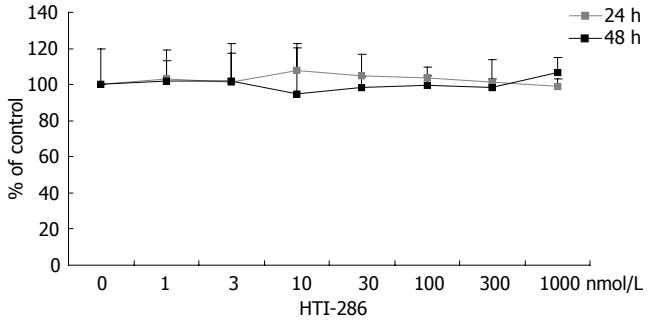


**Figure 2** Effect of HTI-286 on primary human hepatocytes. Human hepatocytes were exposed to HTI-286 for 24 and 48 h at concentrations between 1 nmol/L and 1 mmol/L. No significant decrease in viable cells was detected in MTT assay at any concentration.

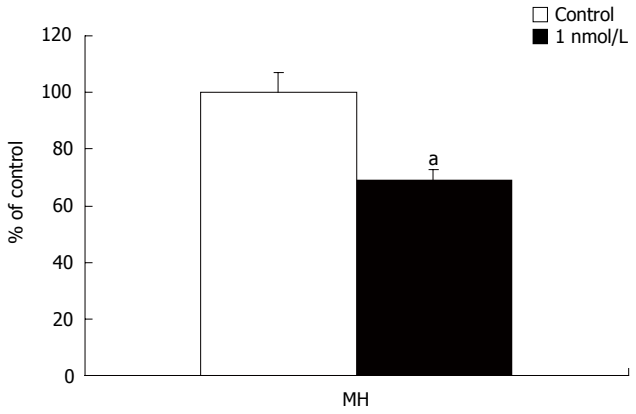


**Figure 3** BrdU incorporation in MH cells treated with 1 nmol/L HTI-286. Decrease in S phase was observed as BrdU incorporation decreased significantly ($P = 0.024$) in MH cells compared to the untreated cells when exposed to 1 nmol/L HTI-286 for two cell doubling times.

of MTs in general extending from the perinuclear region to the cell periphery. MH, Hep3B, and HepG2 cells were exposed to different concentrations (1 nmol/L-30 nmol/L) of HTI-286. Microtubule structure was disrupted by HTI-286 at low concentrations (Figure 4A and B). Furthermore, cell morphology changed during drug exposure. Cell rounding and swelling phenomena were the most significant attributes accompanied by a decrease in cell number. Accumulation of cells in metaphase was seen (data not shown). With increasing concentration of HTI-286, diffuse tubulin staining was evident with loss of cell adhesion, indicating a general disruption of cellular integrity. Additionally, multipolar spindles appeared, indicating disturbed spindle function in cells undergoing mitosis (Figure 4C). These data confirm that HTI-286 permeates hepatocarcinoma cells and modify MT structure and are consistent with the previous reported effect of HTI-286 on tubulin.

### *In vivo efficacy of HTI-286*

Morris hepatoma tumors were implanted into the liver of rats as described in methods. The perioperative mortality (tumor implantation) was zero percent. Successful tumor growth was seen in almost 70% of the animals on $10^{th}$ post-implantation day. Of those animals with good tumor development, 12 were randomized into two groups, each consisting of six animals. One group was given 0.12 mg/kg of HTI-286 intravenously on d 1, 5, 9 and 15. The dose was chosen according to advice by Wyeth. Rats tolerated intravenous HTI-286 treatment well. No clinical signs of hepatic or renal insufficiency were seen in the animals. The control group was not given any specific treatment but received the vehicle and the natural tumor progression was followed. The tumor volume at randomization did not differ between both groups ($P = 0.14$). Two animals from the control group died during the second MRI examination due to anaesthesia complications and were excluded from the analysis. By d 7, after only two doses of HTI-286, tumor growth was significantly inhibited in the treated group compared to the control group ($P = 0.04$). The last dose of HTI-286 was applied on d 15. The growth difference on d 21 reached high significance after completion of treatment (4 doses in total) in the HTI-286-treated group compared to the control group ($P = 0.0001$). Tumor growth was followed for one week more without application of HTI-286. On d 28, the MRI still revealed

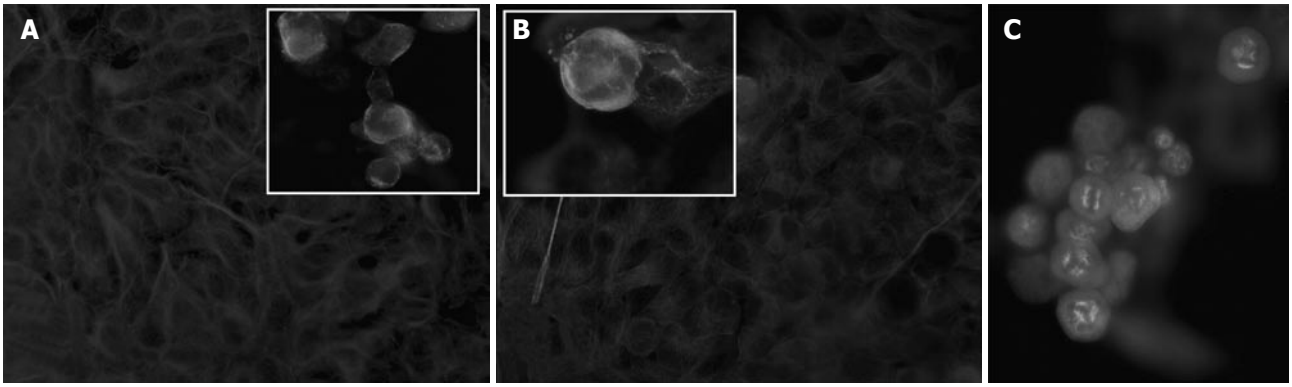


**Figure 4** Microtubule (MT) structure assessed by immunofluorescene microscopy with a specific α-tubulin antibody. **A** and **B**: Untreated Hep3B and HepG2 cells showed dense and complex MT network in the cytoplasm. Insets A and B represent cells after HTI-286 treatment at 3 nmol/L for two cell doubling times. Decreased cytoplasmic microtubule density and diffuse staining were observed. Magnification x 40 except for insets, which are magnified further to outline the morphological changes. **C**: MH cells treated with HTI-286 at 1 nmol/L for two cell doubling times showed multipolar spindles, indicating disruption of the microtubule network in mitotic cells.

smaller tumor in the treated group, indicating lasting inhibitory effect; however, the difference did not reach statistical significance ($P = 0.056$) (Figure 5).

## DISCUSSION

HCC is the most common cause of death due to cancer in some areas of Asia and with steadily rising incidence in Europe and USA as well. In Europe, it is already the primary cause of death in patients with cirrhosis[17]. Late presentation and lack of systemic therapy result in median survival of less than 12 mo. Less than 25% of HCC patients qualify for resection or transplantation[18]. To date, no chemotherapy regimen has been established for patients with unresectable HCC. Ultimately, the majority of HCC patients can only be treated symptomatically and they have dismal prognosis[19].

The few studies of anti-microtubule agents used for the treatment of HCC have shown high intrinsic multidrug resistance (MDR) gene activity, resulting in ATP-dependent export of chemotherapeutic drugs across the plasma membrane mediated by transporters of the MDR P-glycoprotein family (P-gp). Tubulin binding agents are typically good substrates for P-gp and may be partially responsible for the poor response rates observed in HCC patients[20-22].

Hemiasterlins are a family of potent cytotoxic peptides initially isolated from marine sponges. Anderson *et al*[15] have reported *in vitro* and *in vivo* anticancer activity of hemiasterlins and clarified underlying mechanisms of cytotoxicity. HTI-286 is a synthetic analogue of naturally occurring hemiasterlins and a potent antimitotic agent. In 18 different human tumor cell lines, HTI-286 has been proven as a potent inhibitor of cell proliferation and having substantially less interaction with P-gp than currently used taxanes and vinca alkaloids. HTI-286 has been shown significantly effective in tumors resistant to paclitaxel and vincristine. Furthermore, in human tumor xenografts, HTI-286 inhibited growth of tumors derived from colon, brain, skin, prostate, and breast[14].

It has been shown that neoplastic cells are either inherently refractory to tubulin binding agents (taxanes and vinca alkaloids) or acquire resistance during repetitive therapy[23-25]. Such resistance, whether inherent or acquired, is associated with induction of signal transduction pathways, expression of MDR, differential tubulin isotypes, tubulin mutations, interaction of tubulin with the cytoskeleton, and alteration of apoptosis-regulating proteins, such as p53, bcl family members, or survivin[10,26,27].

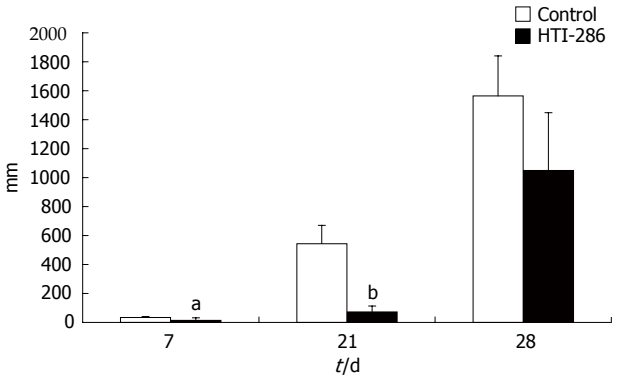


**Figure 5** *In vivo* inhibition of tumor growth under HTI-286 treatment. HTI-286 was injected on d 1, 5, 9 and 15. By d 7, tumor growth significantly differed between the control and HTI-286 group ($^aP = 0.042$; $^bP = 0.0001$). Two weeks after the last HTI-286 injection, the inhibitory effect was diminished between both groups ($P = 0.056$).

Our data demonstrate that HTI-286 is an effective inhibitor of hepatic tumor cell proliferation *in vitro*. The mean $IC_{50}$ of HTI-286 in our experiments (2 nmol/L ± 1 nmol/L) is consistent with the reported $IC_{50}$ of 2.5 nmol/L reported by Loganzo *et al*[14]. Anderson *et al*[15] reported $IC_{50}$ for hemiasterlin A and B of 0.5 and 2 nmol/L, respectively. With intravenous HTI-286 treatment, growth of Morris hepatoma tumors was also significantly inhibited. However, two weeks after the last intravenous dose, the inhibitory effect diminished. These findings are also consistent with the *in vivo* efficacy reported by Loganzo *et al*[14].

The data shown here demonstrate sensitivity of hepatic tumor cells to HTI-286. The observed differences in sensitivity and viability between the tested tumor cell

lines and PHH may be related to different tubulin isotype expression in malignant *versus* normal parenchymal cells. Although human hepatocytes do not proliferate in culture and lose their hepatic-specific metabolic activity and differentiation irreversibly after being maintained in culture for 5 to 7 d, our data suggest a less cytotoxic effect of HTI-286 on PHH compared with hepatic tumor cells[28,29]. Interestingly, no significant decrease in viable cells was observed at concentrations from 1 nmol/L to 1 mmol/L in PHH culture. In contrast, 50% growth inhibition was observed in MH, Hep3B, and HepG2 cells exposed to only 1-3 nmol/L HTI-286. However, the cell metabolism of human hepatocytes under HTI-286 has not been evaluated.

Six different alpha- and beta-tubulins have been described to date[30,31]. Furthermore, the six isotypes are sub-classified according to their C-terminal composition, which is highly divergent. In addition, post-translational modifications enhance the divergence[32,33]. Several studies have shown a positive correlation between expression of beta-tubulin isotype and resistance to paclitaxel and docetaxel. Accordingly, alterations of alpha-tubulin have been described resulting in resistance to tubulin binding agents as well[34-38]. Currently, only one study is available on expression of tubulin isotypes in HCC, response rate and resistance to vinca alkaloids and taxanes[39].

Microtubules are in dynamic equilibrium with the pool of soluble tubulin dimers in the cell, with constant incorporation and release of the dimers into the polymerized structures and back into the soluble tubulin pool[40]. Microtubule dynamic (MD) is the phenomenon of switching between growing and shortening states of MT ends. In proliferating and neoplastic cells, MD is increased[41,42]. Microtubule dynamic is suppressed by HTI-286[43]. Drugs targeting tubulin induce apoptosis. In the case of tubulin-binding agents, although the mechanisms have not yet been completely clarified, it seems that inhibition of MD is closely associated with changes in the expression profile and functional status of apoptotic/anti-apoptotic-regulatory molecules and MT-associated proteins[34,44,45]. Additionally, drug-resistance to tubulin-binding agents is also linked to cytoprotective and anti-apoptotic factors, such as p53, bcl-2, or bcl-xL. Current molecular understanding of the mechanisms of cell death by tubulin-targeting agents suggests that these agents inhibit MD and cell cycle $G_2$/M-phase transition, which triggers molecular signalling that induces mitochondrial permeability and the release of pro-death molecules into the cytosol inducing caspase-dependent apoptosis[46,47]. Giannakakou *et al*[48] showed suppression of MD with low concentrations of MT-targeting compounds enhancing microtubule-dependent trafficking and accumulation of p53 into the nucleus and activation of p53-downstream target genes as well as induction of p53-upregulated modulators of apoptosis. Evidence is strong that MDR1 expression is negatively correlated with p53, and MDR1 has been shown to be responsible for drug resistance to tubulin-binding agents[49]. HTI-286 is a very poor substrate for MDR1 encoded drug-efflux pump. p53 directly affects the MD by transcriptional regulation of proteins involved in modulation of microtubules, known as MT-associated proteins (MAPs). Bcl family members have been identified as transcriptional targets of p53. Anti-apoptotic function of bcl2 and pro-apoptotic function of bax depend on their dimerization status. Furthermore, anti-microtubule agents induce bcl2 inactivation through phosphorylation, which parallels their inability to form heterodimers with bax. Consequently, bax-bax homodimer levels rise and evoke apoptosis[50]. These findings are supported by the fact that bcl2 phosphorylation appears at $G_2$-M phase[51]. Microtubule-targeting drugs cause cell cycle arrest in $G_2$-M phase. Although the alteration of the molecular environment during HTI-286 exposure has not yet been evaluated, it is likely that the aforementioned molecular mechanisms leading to apoptosis also apply to HTI-286-induced cell death. HTI-286 inhibits proliferation at very low concentrations, suggesting that molecular events are impacted at low drug concentrations. Notably, suppression of MD is the most decisive factor in initiation of apoptosis and that all types of MT-targeting drugs, independent of their class (depolymerizing or polymerizing agents), modulate MD.

In addition to mitosis, tubulin plays an important role in the interphase cell in maintaining the celluar subcompartiments and distribution of organelles (mitochondria, Golgi, endoplasmis reticulum, lysosomes, endosomes and nucleus)[52-57]. This finding suggests a complex role for tubulin in signal transduction and transcription. Our $\alpha$-tubulin staining data implicate a complex network of MT in the interphase cell and a role in cellular organisation and metabolism.

In conclusion, our data suggest that HTI-286 is highly effective in inhibiting hepatic tumor cell proliferation *in vitro* and tumor growth *in vivo*. Primary human hepatocytes appear to be unaffected by high doses of HTI-286. Further investigation of HTI-286 in patients with advanced HCC or with primary unresectable HCC is warranted. Currently, HTI-286 is in clinical development.

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# A single dose of caffeic acid phenethyl ester prevents initiation in a medium-term rat hepatocarcinogenesis model

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## Abstract

**AIM:** To study of the effect of caffeic acid phenethyl ester (CAPE) on the initiation period in a medium-term assay of hepatocarcinogenesis.

**METHODS:** Male Wistar rats were subjected to a carcinogenic treatment (CT) and sacrificed at 25$^{th}$ d; altered hepatic foci (AHF) were generated efficiently. To a second group of rats a single 20 mg/kg doses of CAPE was given 12 h before initiation with CT and were sacrificed at 25$^{th}$ d. We evaluated the expression of preneoplastic markers as γ-glutamyltranspeptidase (GGT) and glutathione S-transferase type pi protein (GSTp) by histochemistry, RT-PCR and Western blot analyses, respectively. We measured thiobarbituric acid reactive substances (TBARS) in homogenates of liver and used Unscheduled DNA Synthesis (UDS) assay by incorporation of [$^3$H] thymidine ($^3$HdT) in primary hepatocyte cultures (PHC).

**RESULTS:** At 25$^{th}$ d after CT CAPE reduced the observed increase of GGT$^+$AHF by 84% and liver expression of *ggt* mRNA by 100%. In case of the GSTp protein, the level was reduced by 90%. As indicative of oxidative stress generated by diethylnitrosamine (DEN) 12 h after its administration, we detected a 68% increase of TBARS. When CAPE was administered before DEN, it completely protected from liver TBARS induction. To have an indication of the sole effect of CAPE on initiation, two carcinogens were tested in a UDS assay in PHC, we used methyl-n-nitrosoguanidine as a direct carcinogen and DEN, as indirect carcinogen. In this assay, genotoxic damage caused by carcinogens was abolished at 5μM CAPE concentration.

**CONCLUSION:** Our results demonstrated that CAPE possesses anti-genotoxic and antineoplastic capabilities, by an anti-oxidative and free-radical scavenging mechanism.



**Key words:** Caffeic acid phenethyl ester; Antioxidant; Hepatocarcinogenesis; Initiation

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## INTRODUCTION

CAPE (2-phenylethyl 3(3,4-dihdroxyphenyl)-2-propenoate), exhibits a broad spectrum of biological activities antibacterial[1], anti-inflammatory[2], antiviral[3], antiatherosclerotic[4], antiproliferative[5], neuroprotective[6-8] and antitumoral actions[9]. In *in vivo* models, CAPE has been identified as an experimental antineoplastic agent by inhibition of colon Aberrant Crypt Foci formation[10], mice skin tumor induction[11,12], intestinal tumor in Min/+ mice[13]; and, recently, we demonstrated that CAPE inhibits induction of AHF at a promotion stage of a chemical hepatocarcinogenesis model in rats[14].

The mechanism by which CAPE exerts its anti-carcinogenic effect is not completely known. In our previous work of the inhibition of promotion by this substance, we hypothesized that the activation of NF-kappaB induced by reactive oxygen species (ROS) generation produced by the metabolism of 2-acetamidofluorene (2-AAF) is inhibited by CAPE through its antioxidant capacity. This capacity has been interpreted as the mechanism which prevents oxidative

stress caused by different stimuli[12,15,16]. The inhibition of oxidative stress generated in ischemic-reperfusion conditions in different experimental models including rat and rabbit has been explained similarly[17-19]. The *in vitro* antioxidant activity of CAPE has also been shown, since it reduced the levels of intracellular $H_2O_2$ and oxidized bases in DNA, probably by selective scavenging activity[20,21].

At present, evidence has accumulated that electrophiles are generated by direct and indirect carcinogens. In our hepatocarcinogenesis system, DEN metabolism produces free-radical metabolites. Additionally, cytochrome isoform 2E1 (CYP2E1) is the main cytochrome responsible of DEN metabolism, and capable of generating a prolonged burst of ROS near the site of substrate oxidation[22]. The products of these events are involved as cellular oxidant participants in neoplastic development[23]. Since oxidative stress during carcinogen metabolism participates in the initiation of hepatocarcinogenesis in the rat and CAPE counteracts oxidative stress, the present study was designed to examine the effect of CAPE on the initiation stage, evaluating its possible antioxidative mechanism of action *in vivo* in a medium-term hepatocarcinogenesis model and *in vitro* using a PHC.

## MATERIALS AND METHODS

### Animals

Male Wistar rats (180-200 g) obtained from the Production Unit of Experimental Laboratory Animals (UPEAL-Cinvestav, México D.F., México), had access to food (PMI Feeds Inc., Laboratories Diet) and water at all times; food cups were replenished with diet three times weekly. During treatment, the rats were transferred to the holding room, under controlled conditions of 12-h light/12-h dark cycle, 50% humidity, and 21℃ temperature. All animals received human care according to the institutional guidelines for use of laboratory animals.

### Experimental hepatocarcinogenic protocol

Animals were treated as described by Carrasco-Legleu *et al*[14]. Briefly, animals were administered 200 mg/kg DEN intraperitoneally; one week later, 2-AAF was orally administered with a stainless steel feeding tube at doses of 20 mg/kg per day on 3 consecutive days before partial hepatectomy (PH). Animals were sacrificed 25 d after DEN administration[24] and the livers excised, quickly frozen in liquid nitrogen and stored at -80℃ until analysis. CAPE was obtained by esterification of caffeic acid with phenethyl alcohol as detailed previously[25], and dissolved in dimethylsulfoxide (DMSO) to evaluate its effect on initiation; it was administered IP at a dose of 20 mg/kg weight 12 h before DEN application (Figure 1).

### Histochemistry of GGT

Histological liver sections of 15 μm thickness were obtained with a cryostat (Slee cryostat MTC, Germany) and stained for GGT activity according to Rutenburg *et al*[26]. Images of the GGT positive ($GGT^+$) foci were captured with a digital camera (Color View 12, Soft Imaging System GmbH, Germany) and quantified with analysis software (AnalySIS Soft Imaging System GmbH, Germany). $GGT^+$ areas larger than 0.01 $mm^2$ were registered to avoid basal detection. Rat livers were sectioned in 5 mm slices, and 3 slices were randomly selected from each liver. Twenty histological sections were prepared from each liver slice and, randomly again, 4 out of the 20 were selected and prepared for computer analysis.

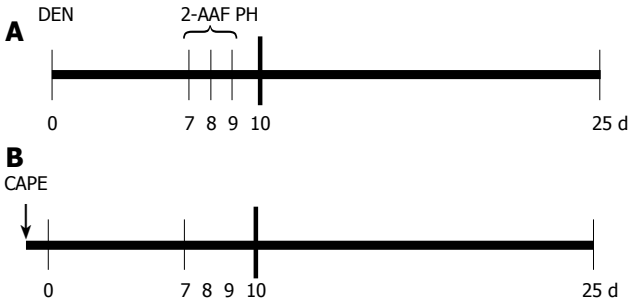


**Figure 1** CAPE administration protocol. **A**: Carcinogenic treatment (CT): at time 0 the rats were treated with 200 mg/kg of DEN; on d 7, 8 and 9, 2-AAF was administered by gavage at doses of 20 mg/kg per day, a PH was performed at d 10 and rats were sacrificed at d 25; **B**: CAPE (↓) was administered 12 h before DEN.

### RT-PCR analysis

Total RNA of liver tissue was isolated with the method developed by Chomczynski and Sacchi[27]. It was treated with RNase-free DNase 1 (Boehringer-Mannheim, Germany) and one microgram of RNA was reverse transcribed into cDNA using the commercial kit (Gibco BRL/Life Technology, Inc., Gaithersburg, MD) at a final volume of 12.5 μL. α-Actin was used as an internal reference gene. The 2 primer sequences of rat GGT and αactin used were: 5'-CTCTGCATCTGGCTACCCAC-3' 5' -GGATGCTGGGTTGGAAGAGG-3' and 5'-CCAAGG CCAACCGCGAGAAGATGAC-3' 5'-GGTACATGGTG GTGCCGCCAGAC-3' (sense and antisense), respectively. The mixture was heated at 45℃ for 30 min in a GeneAmp PCR System 2400 (Perkin-Elmer, Corp., CT). GGT and α-actin were amplified in 40 and 30 sequential cycles, the program was denaturation (94℃, 30 s), annealing (59℃, 45 s), and extension (70℃, 45 s). The PCR products were 418 bp and 586 bp, respectively. The amplified samples were visualized on 2% agarose gels, stained with ethidium bromide and captured with a digital camera (Kodak electrophoretic documentation and Analysis system 120).

### Western blot analysis

For detection of GSTp in the cytosolic fraction, liver homogenates were prepared with lysis buffer (Tris-HCl 10 mmol/L pH 7.4, NaCl 150 mmol/L and phenylmethylsulfonyl fluoride (PMSF) 1 mmol/L) and were spun at 9000 r/min in a microcentrifuge at 4℃ for 15 min. The cytoplasm protein concentration was measured with the bicinchoninic acid method[28]. SDS/PAGE was performed under reducing conditions on 12% polyacrylamide gels. Resolved proteins were transferred onto nitrocellulose sheets (Bio-Rad Lab., Hercules, CA), probed with a rabbit polyclonal anti-GSTp (Dako Corporation, Carpinteria, CA), treated with horseradish peroxidase-conjugated secondary antibody (Zymed, San Francisco, CA), detected by Enhanced Chemiluminescense

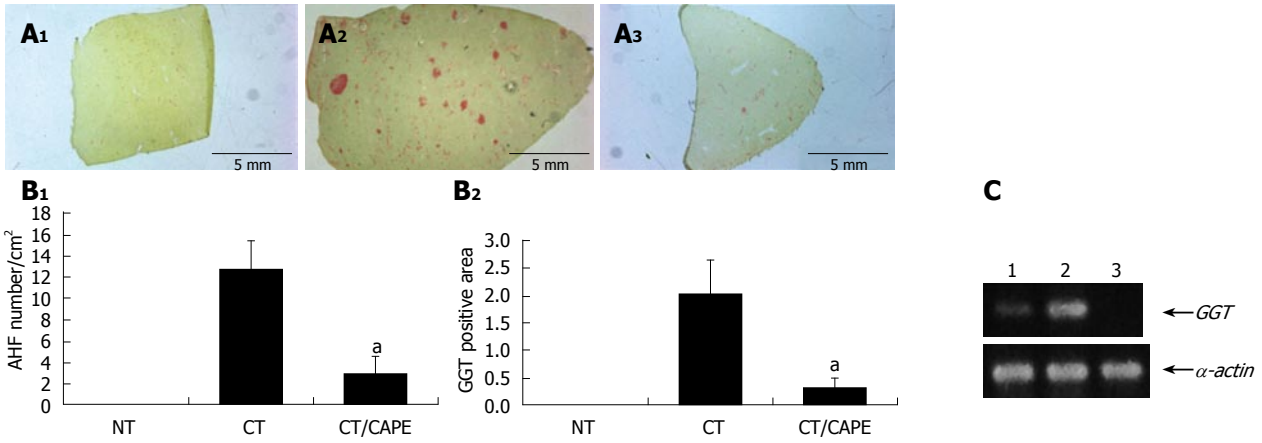


**Figure 2** Effect of CAPE on GGT expression. **A**: GGT histochemically stained sections (1) non-treated group (NT), (2) carcinogenic treatment group (CT), (3) CT plus CAPE before DEN; **B**: CAPE effect on number/cm$^2$ of AHF and percentage of GGT$^+$ area/tissue. The differences of AHF number and area after the effect of CAPE in these groups were compared with the respective control group (CT + vehicle) and obtained values were significant by *t* with $^aP < 0.05$. Twelve histological liver sections per rat from each treatment were randomly selected. NT ($n = 3$), CT ($n = 4$), CT plus CAPE ($n = 7$); **C**: The RT-PCR assay was used to determine *GGT* mRNA expression. L1: Control NT; L1: CT group; L1: CT plus CAPE. The series of the extreme right represents α-actin transcripts as control. This experiment was performed with a pool of total RNA isolated from 3 animals.

detection reagent (Santa Cruz Biotechnology Inc., Santa Cruz, CA) and developed with Konica Film (Tokyo, Japan.). These membranes were then re-probed with anti-α-actin monoclonal antibody (Cinvestav, Mexico City) used as loading control.

### *Thiobarbituric acid reactive substances*

Samples of frozen liver were homogenized in a buffer containing 10 mmol/L Tris, PMSF and NaCl, and protein was determined. Lipid peroxidation (LPX) was measured as TBARS, a widely used method, according to Buege and Aust[29]. Briefly, 600 mg of protein liver homogenates plus 300 μL of 0.4% thiobarbituric acid in 20% acetic acid, pH 3.0, were mixed and heated at 100℃ for 45 min. Next, the samples were cooled, added with 200 μL of 1.2% KCl, 0.5 ml of 1:15 pyridine/butanol and centrifuged at 7500 r/min for 10 min. The resulting solution was determined at *A*532 nm, and using the extinction coefficient, $E = 1.56 \times 10^5$, TBARS were expressed as nmoles of malonyldialdehyde as mg of proteins.

### *Unscheduled DNA synthesis assay in primary hepatocyte cultures*

PHC were prepared from male Wistar rats (180-200 g) by the collagenase perfusion method as previously described[30], and three replicates of $8 \times 10^5$ cells were seeded onto 35 mm culture dishes. PHC were seeded in DMEM supplemented with 10% bovine fetal serum (HyClone, Laboratories Inc., Logan Utah) and 5 μg insulin/mL (Eli Lilly, México, DF) and placed in a 37℃ humidified incubator in an atmosphere of 90% air/10% $CO_2$.

After 2 h of incubation, the medium was removed and replaced by one ml of serum-free medium containing 10 μmol/L HU, 5 μCi of $^3$HdT from Amersham Life Science (Cleveland, OH) and 4 μL of DMSO as control. DEN, CAPE or MNNG were dispensed from 0.625 to a final concentration of 10 μmol/L per dish. Control dishes received the corresponding vehicle of CAPE and carcinogens. PHC were incubated for 4 h with their respective treatment. To account for variation in the number of cells between dishes, results were normalized as a function of DNA concentration. For this purpose DNA hydrolysate was obtained with the method by Leyva and Kelly[31] as described by Pérez-Carreón[32]. Incorporation of $^3$HdT in dpm per μg DNA was determined and the results were expressed, as percent of control incorporation.

### *Statistical analysis*

Number and area of GGT$^+$ AHF and GSTp protein expression of the group treated with CAPE were compared with the respective control group (CT plus vehicle). Results were analyzed by *t*. Differences were considered statistically significant at $P < 0.05$.

## RESULTS

### *In vivo assays*

**Protective effect of CAPE on hepatocarcinogenesis initiation:** In this *in vivo* medium-term hepatocarcinogenesis assay, CAPE was tested on initiation. Rats which received the CT (DEN, 2-AAF and PH) were sacrificed 25 d after initiation. At this time, copious liver GGT$^+$ AHF were histochemically detected, and their average-quantity was considered 100%. The CT animal group that received a single dose of CAPE before initiation clearly showed a decrease of GGT$^+$ AHF formation, resembling that of the non-treated (NT) group (Figure 2A). Quantification of this phenomenon showed a drastic decrease in number and area of GGT$^+$ AHF. CAPE had a protective effect of 84% and 91%, respectively (Figure 2B). Differences were considered significant when $P < 0.05$. We also evaluated the expression of transcripts of the GGT protein by the RT-PCR assay, and observed a decrease of transcripts due to the CAPE effect (Figure 2C)

**CAPE decreases the expression of the GSTp protein:** The protective effects of CAPE with respect to GGT were toward its activity and its mRNA expression. GSTp is another marker related to the hepatocarcinogenesis model. We analyzed if CAPE modulates this marker at protein

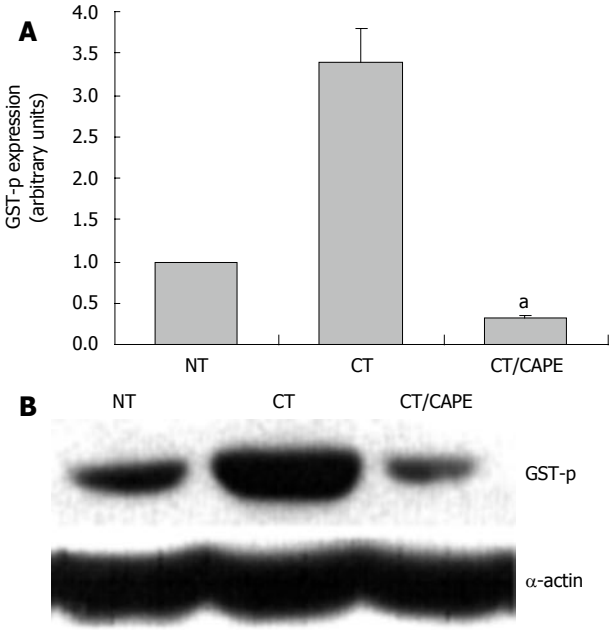


**Figure 3** The effect of CAPE on GSTp protein expression. Western blot was used to analyze the expression of the GSTP protein marker. **A:** Densitometric analysis of GSTP in: Control NT, CT and CT plus CAPE given before DEN. Results were statistically significant with $^{a}P < 0.05$; **B:** Representative Western blot.

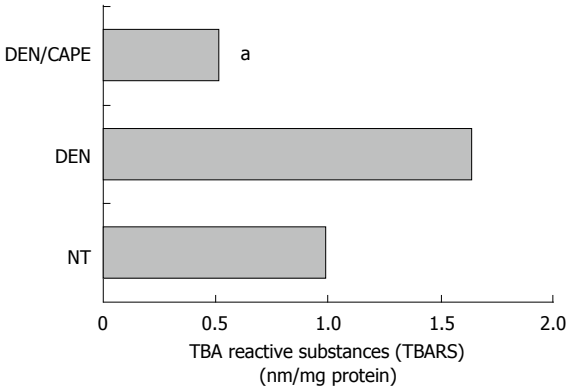


**Figure 4** Effect of CAPE on LPX levels induced by DEN. LPX was determined by detection of malonyldialdehyde concentration and expressed as TBARS. DEN increased TBARS levels detected at 12 h. CAPE administered 12 h before the initiator, clearly reduced the TBARS induced by DEN. Six hundred microgram of liver homogenates of each treated group were used, group NT, DEN (12 h after) and DEN plus CAPE (24 h after CAPE administration). Results were statistically significant with $^{a}P < 0.05$.

level. By Western blot we detected that CAPE drastically decreased the expression of the GSTp protein by 90% at d 25 (Figure 3). These results, in congruence with those related to GGT, showed the same protective profile of CAPE.

**DEN-generated lipid peroxidation was decreased by CAPE:** Since our laboratory previously has shown that LPX is an expression of oxidative stress in cells[33], we determined the TBARS as a global approach to measure LPX, and compared their levels obtained 24 h after administration of CAPE before initiation. Twelve hours after initiation with DEN, an increment of LPX of 68% above control level was observed (Figure 4). The administration of CAPE 12 h before administration of DEN importantly prevented full expression of LPX. CAPE decreased it to 52% with respect to the NT group of rats.

In sum, these *in vivo* results showed that a single dose of CAPE, in addition to the protective effect on induction of $GGT^{+}$ AHF, transcription of *GGT*, and expression of GSTp, produced a very important protective effect against oxidative stress.

### *In vitro assay*

**Protective effect of CAPE *in vitro*:** The genotoxic alteration produced by carcinogens, one of the first measurable effects involved in initiation, can be analyzed with the UDS assay. This assay was used to test if CAPE protects against the genotoxic effect induced by 2 carcinogens with different mechanisms of activation: DEN, an indirect carcinogen that is activated by CYP2E1 and generates electrophylic species as well as ROS, and MNNG, a direct carcinogen that produces electrophylic proximal carcinogens by hydrolysis. The effect of CAPE on DEN-treated PHC of Wistar rats was analyzed; the incorporation of $^{3}$HdT in presence of vehicle (water or DMSO alone) was defined as control. Figure 5A shows that DEN at 1.25 μmol/L induced an increment of $^{3}$HdT incorporation of 60% above control level. CAPE alone at 1.25 and 2.5 μmol/L did not induce incorporation above control level (data not shown). The CAPE concentrations (0.625 to 5 μmol/L) abolished increments in $^{3}$HdT incorporation caused by 1.25 μmol/L DEN in a concentration-dependent form. We also analyzed if the action of CAPE is due to its free-radical scavenging abilities, MNNG being a mutagenic electrophylic species generator by hydrolysis. CAPE protected from the genotoxic damage to DNA produced by MNNG. The increment of $^{3}$HdT incorporation induced by MNNG was more pronounced than that induced by DEN. The maximum evoked response was 182% above control level at 0.625 μmol/L MNNG concentration. CAPE was tested at 5 μmol/L for its protective effect, and this concentration abolished the carcinogenic effect of 0.625 to 10 μmol/L MNNG and decreased $^{3}$HdT incorporation between 20 and 40% below control level (Figure 5B). CAPE inhibited the genotoxicity induced by both carcinogens tested, DEN and MNNG, suggesting that its protective mechanism is probably its capacity as scavenger of electrophylic molecules and not as inhibitor of carcinogenic activation in our system.

## DISCUSSION

Present results clearly show a protective effect of CAPE when a single dose was given before initiation in the rat hepatocarcinogenesis model. CAPE decreased the induction of both, area and number of $GGT^{+}$ AHF, GGT transcripts and of the GSTp protein. Our results showed a protective effect against induction of preneoplastic lesions and are an addition to previously reported data[11-14]. These effects are in accordance with the effective chemoprotective antioxidant activity reported for CAPE[34,35] and are further supported by *in vitro* evaluation, in mouse epidermal cells, where CAPE was found to be

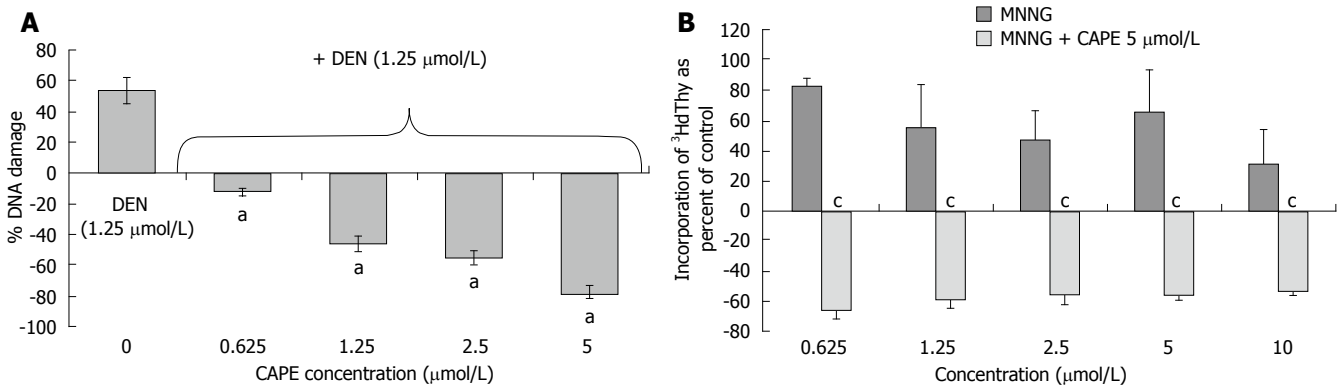


**Figure 5** CAPE protects from genotoxic damage caused by DEN and MNNG in PHC. **A**: DEN at 1.25 μmol/L concentration induced $^3$HdT incorporation and this dose was tested with several concentrations of CAPE (0.625, 1.25, 2.5, 5 and 10 μmol/L). CAPE decreased DEN genotoxic damage to levels even below the control; **B**: Curve concentration of MNNG from 0.625-10 μmol/L. One dose of 5 μmol/L of CAPE protects from genotoxic damage caused by MNNG in PHC. Data incorporation of $^3$HdT was estimated as dpm/mg of DNA and results are expressed as percent of control samples, the zero data represent $^3$HdT incorporation of basal level of control group. Results were obtained from 3 replicates of at least 3 animals. $^a P < 0.05$ *vs* DEN only; $^c P < 0.05$ *vs* MNNG only.

one of the four better chemo-protective agents among 25 well-known substances with this activity[36].

In this context, it has been postulated that overproduction of ROS and oxidative DNA damage play a major role in cancer development. Increased concentrations of active oxygen, organic peroxides and radicals can promote initiated cells to neoplastic growth, inducing alterations in DNA structure or producing epigenetic mechanisms[23,37]. This justified the investigation of the effect of CAPE during the early stages of liver carcinogenesis, where we had specifically demonstrated the obligated participation of oxidative stress in the production of preneoplastic lesions during initiation in our medium-term hepatocarcinogenesis model. We had previously shown that ROS importantly participate in our model of hepatocarcinogenesis, and quercetin, an antioxidant molecule, prevents the appearance of AHF and drastically decreases LPX induced by DEN[33]. These results are in accord with observations in another system where CAPE decreased LPX levels[38]. Our proposition is that many, if not all, protective effects of CAPE have a common mechanism, which is its anti-oxidative and free-radical scavenging abilities that involve several cellular pathways.

Present *in vitro* UDS assay results in PHC with two carcinogens, one indirect and the other a direct, support our hypothesis. The protective effect of CAPE against DEN involves at least two possible explanations: (1) CAPE intercepts electrophylic metabolites and ROS formed through the CYP2E1 activity and, in this way, blocks their interaction with DNA and prevents induction of DNA damage, or (2), CAPE inhibits CYP2E1, as is the case of the chemoprotector molecule, sulforophane[39], and prevents formation of DEN electrophylic metabolites, which prevents UDS. Prevention of MNNG-induced UDS could be explained only by the CAPE anti-oxidative and free-radical scavenging ability since MNNG electrophylic metabolites are generated without participation of cytochrome enzymes and ROS generation. Moreover, it has been shown that tea polyphenols prevent MNNG induction of gastric cancer[40]. A stronger evidence in this context has been obtained in Swiss mice treated with MNNG; garlic, tomato or neem leaf extracts prevent the induction of micronuclei and the increment of LPX in bone marrow, as well as enhanced GSH-dependent antioxidant activities[41,42]. Even though, in the case of DEN, we cannot discard the possibility that the mechanism by which CAPE prevents AHF is via CYP2E1 inhibition, the fact that the MNNG genotoxic effect is prevented by CAPE, supports the notion that the protective mechanism of CAPE is due to its anti-oxidative and free-radical scavenging activities. This proposition is also supported by results in a cell-free assay used to evaluate antioxidant and free-radical scavenging activities of several chemopreventive agents[43]. In these studies, CAPE revealed the third highest antioxidant activity after caffeic acid and α-tocopherol, and the second highest free-radical scavenging activity after rosmarinic acid. This evidence together with previous studies in models that show that the protective effect of CAPE against cancer in rodents is related to its anti-oxidant activity support our proposal[11-13,44].

The chemoprotective effect of CAPE in our medium-term model of hepatocarcinogenesis diminished *ggt* mRNA expression, drastically decreases the number and area of GGT$^+$ AHF, and the GSTP protein expression; it abolished LPX and, in addition, it prevented the UDS induced by DEN or by MNNG in PHC. In conclusion, we suggest that the protective effects of CAPE at initiation as shown *in vivo* and *in vitro* are due to its anti-genotoxic, anti-oxidative and free-radical scavenging abilities.

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*VIRAL HEPATITIS*

# Interferon augments the anti-fibrotic activity of an angiotensin-converting enzyme inhibitor in patients with refractory chronic hepatitis C

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## Abstract

**AIM:** To evaluate the effect of combination treatment with the interferon (IFN) and angiotensin-converting enzyme inhibitor (ACE-Ⅰ) on several fibrotic indices in patients with refractory chronic hepatitis C (CHC).

**METHODS:** Perindopril (an ACE-Ⅰ; 4 mg/d) and/or natural IFN (3 MU/L; 3 times a week) were administered for 12 mo to refractory CHC patients, and several indices of serum fibrosis markers were analyzed.

**RESULTS:** ACE-Ⅰ decreased the serum fibrosis markers, whereas single treatment with IFN did not exert these inhibitory effects. However, IFN significantly augmented the effects of ACE-Ⅰ, and the combination treatment exerted the most potent inhibitory effects. The serum levels of alanine transaminase and HCV-RNA were not significantly different between the groups, whereas the plasma level of transforming growth factor-β was significantly attenuated almost in parallel with suppression of the serum fibrosis markers.

**CONCLUSION:** The combination therapy of an ACE-Ⅰ and IFN may have a diverse effect on disease progression in patients with CHC refractory to IFN therapy through its anti-fibrotic effect.



**Key words**: Interferon; Angiotensin-converting enzyme inhibitor; Liver fibrosis; Chronic hepatitis C; Transforming growth factor-β

## INTRODUCTION

The goal of therapy for chronic hepatitis C (CHC) is the eradication of the hepatitis C virus (HCV)[1]. In the recent decade, anti-viral therapy against HCV has been markedly improved, especially treatment with ribavirin and peg-interferon (IFN)[2]. However, the overall sustained viral response (SVR), defined as undetectable HCV-RNA 6 mo after the end of treatment, even with the combination of peg-IFN and ribavirin is still unsatisfactory, since many patients have not achieved SVR yet. It is well known that continuous inflammation associated with HCV infection slowly results in liver fibrosis development, and eventually leads to hepatocellular carcinoma (HCC). Accordingly, one of the clinical goals in the treatment of refractory CHC is to prevent the progression of liver fibrosis development[3]. It has been reported that the prognosis and clinical management of chronic liver diseases depend on the extent of liver fibrosis, as complications mainly develop in the advanced stages, which is also applicable to HCV infection[1]. Also, it has been shown that the degree of fibrosis development correlates with the incidence of HCC[4]. Collectively, it is likely that the effective anti-fibrotic therapy will significantly improve the prognosis of patients, especially in non-SVR refractory CHC.

Recent studies have shown that long-term IFN could exert beneficial effects in patients with refractory CHC[5]. Apart from the anti-viral effect, several evidences suggested that IFN could improve liver fibrosis. However, the histological improvement of liver fibrosis could be observed only in the SVR patients[6]. It has been reported that IFN inhibited the collagen promoter activity in the activated hepatic stellate cells (HSC), which play a pivotal role in liver fibrosis development[7]. Furthermore, it is well

known that the combination treatment with different anti-fibrotic agents may provide synergistic rather than additive effects. Alternative strategies with other safe agents in the non-SVR patients should be used.

Recent studies have revealed that the renin-angiotensin system (RAS) plays an important role in the liver fibrosis development[8]. Angiotensin-Ⅱ (AT-Ⅱ) induces the proliferation of the activated HSC, and the local RAS components were significantly up-regulated during the liver fibrosis development[9]. It has been reported that inhibition of RAS by the clinically used angiotensin-converting enzyme inhibitor (ACE-Ⅰ) and AT-Ⅱ type-1 receptor blocker (ARB) significantly attenuated liver fibrosis development in several experimental models[10,11]. In the clinical practice, these RAS-inhibitory agents also exerted anti-fibrotic effects in several types of chronic liver diseases, including CHC[12,13]. We previously reported that the combination treatment with IFN and ACE-I exerted a more potent inhibitory effect on murine liver fibrosis development than either single agent[14]. In a preliminary study, we also demonstrated that the combination treatment with IFN and ACE-I suppressed the serum fibrosis markers in CHC[15].

In the current study, we investigated whether this combination treatment would exert a more potent anti-fibrotic effect than either single agent in patients of refractory CHC. To elucidate the possible mechanisms, we also examined the serum transforming growth factor-β (TGF-β) level, which is known as a key cytokine in the liver fibrogenesis.

## MATERIALS AND METHODS

### Patients and methods

In the current study, we examined a total 40 patients with CHC refractory with mild hypertension from July 2002 to December 2005 at Nara Medical University Hospital. The refractory CHC is defined as CHC that failed to respond to IFN mono-therapy. The patients were randomly divided into 4 groups of matching clinical background (e.g., age, sex), HCV status, and serum fibrosis markers. The treated groups were given either single or combination of oral ACE-I (perindopril: 4 mg/d) and an intramuscular injection of IFN (natural IFN: 3 MU/L; 3 times a week) for 12 mo, whereas the control group received neither agent. We recommended all patients to stop alcohol intake completely. This protocol was approved by the Institutional Review Board (IRB) of Nara Medical University, and the study was conducted in conformity with ethical and humane principles.

### Evaluation of laboratory data

The serum fibrosis markers; namely, hyaluronic acid, type IV collagen 7S (7S-collagen), and amino-terminal peptide of type-Ⅲ pro-collagen (P-Ⅲ-P) were measured before and after the treatment in all patients by latex agglutination, enzyme immunoassay (EIA), and radioimmunoassay (RIA) respectively, with routine laboratory methods. The serum level was measured by ELISA kit (R&D systems, Tokyo, Japan) according to the manufacturer's instructions as described previously[14]. The serum HCV status was measured by using the quantitative or qualitative reverse transcription-polymerase chain reaction (RT-PCR) analysis. Other serum biochemical markers, such as alanine transaminase (ALT), were measured by the routine laboratory methods.

**Table 1 Characteristics in patients of refractry chronic hepatitis C**

| | Control | IFN | ACE-Ⅰ | IFN+ACE-Ⅰ |
|---|---|---|---|---|
| No. of patients | 10 | 7 | 7 | 11 |
| Mean age ± SD (yr) | 60 ± 7 | 60 ± 9 | 58 ± 9 | 62 ± 8 |
| Sex (M/F) | 6/4 | 3/4 | 4/3 | 5/6 |
| Serum HCV-RNA[1] (M copies/L) | 632 ± 120 | 641 ± 131 | 620 ± 106 | 628 ± 108 |
| Genotype (1 b/others) | 9/1 | 6/1 | 7/0 | 10/1 |

[1]Data represent mean ± SD.

### Statistical analysis

To assess the statistical significance of the inter-group differences in the quantitative data, Bonferroni's multiple comparison test was performed after one-way analysis of variance (ANOVA). This was followed by Barlett's test to determine the homology of variance.

## RESULTS

### Patient characteristics

The characteristics of the refractory CHC patients in all groups are shown in Table 1. There were no significant differences in the patient's clinical background among the groups. Most patients in all groups had high titer of HCV of genotype 1b, which is known for its resistance to the IFN based anti-viral therapy[2].

### Serum fibrosis markers

All medications were well tolerated by all patients. To examine the therapeutic effects of IFN and/or ACE-I on the progression of liver fibrosis in patients with refractory CHC, we compared several serum fibrosis markers before and after the respective treatment. As shown in Figure 1A, there were differences in the serum hyaluronic acid among the groups. Without treatment, the mean serum hyaluronic acid level was increased (1.2% ± 1.1%) at the end of the study as compared with the starting level. Both IFN- and ACE-I treated-groups had decreased mean serum hyaluronic acid level at the end of study (-4% ± 2.2% and -9% ± 3.4%, respectively). However, only ACE-I exerted a significant difference as compared with the untreated control group ($P < 0.01$). The combination treatment of ACE-I and IFN (IFN + ACE-Ⅰ) exerted the most potent suppressive effect (-21% ± 5.9%) among all groups, and the inhibitory effect of this combination was more potent than that in the ACE-I -treated group ($P < 0.01$) (Figure 1B). Similar results were observed in the 7-S-collagen and P-Ⅲ-P levels (Figures 2 and 3, respectively).

### Biochemical markers and serum TGF-β level

We next examined the changes in several biochemical

**Table 2 Changes of seveal markrs in patients of refractry chronic hepatitis C (mean ± SD)**

| | Control | | IFN | | ACE- I | | IFN+ACE- I | |
|---|---|---|---|---|---|---|---|---|
| | Before | After | Before | After | Before | After | Before | After |
| Mean BP (mmHg) | 132 ± 14 | 130 ± 12 | 130 ± 14 | 128 ± 13 | 141 ± 16 | 128 ± 12[a] | 142 ± 15 | 130 ± 12[a] |
| HCV-RNA (M copies/L) | 632 ± 120 | 604 ± 117 | 641 ± 131 | 598 ± 95 | 620 ± 106 | 613 ± 110 | 628 ± 108 | 586 ± 90 |
| S-Cre (mg/L) | 9 ± 2 | 9 ± 2 | 9 ± 2 | 9 ± 1 | 9 ± 1 | 8 ± 1 | 8 ± 1 | 8 ± 1 |

[a]$P < 0.05$ *vs* before treatment.

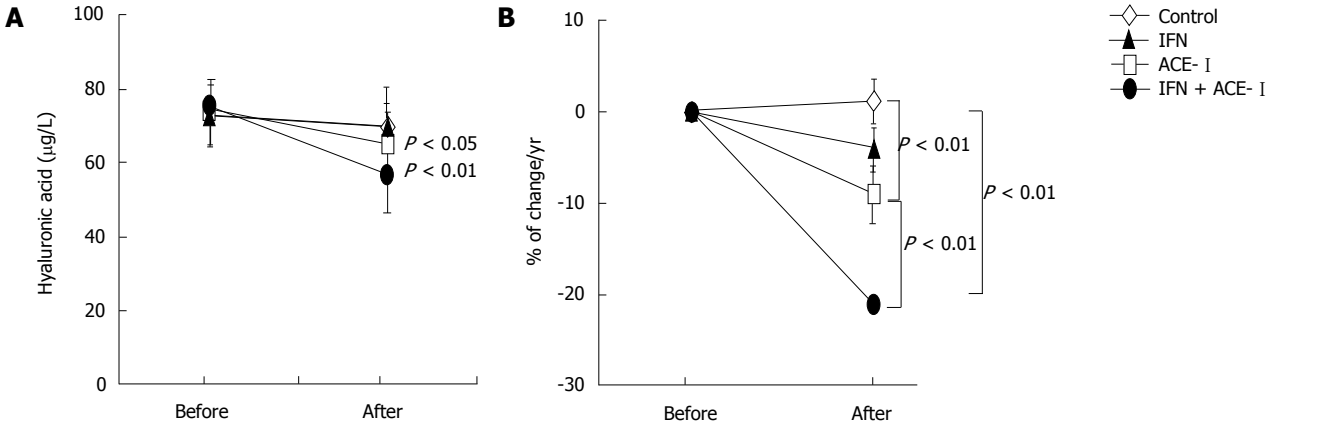


**Figure 1** The effects of IFN and ACE- I on the serum hyaluronic acid in the patients with refractory chronic hepatitis C. **A**: Hyaluronic acid level before and after the study; **B**: % of change in the hyaluronic acid level in each group. Control: untreated control group; ACE- I , IFN: ACE- I - and IFN-treated groups, respectively; IFN + ACE- I : combination treated group with ACE- I and IFN. The data represent the mean ± SD.

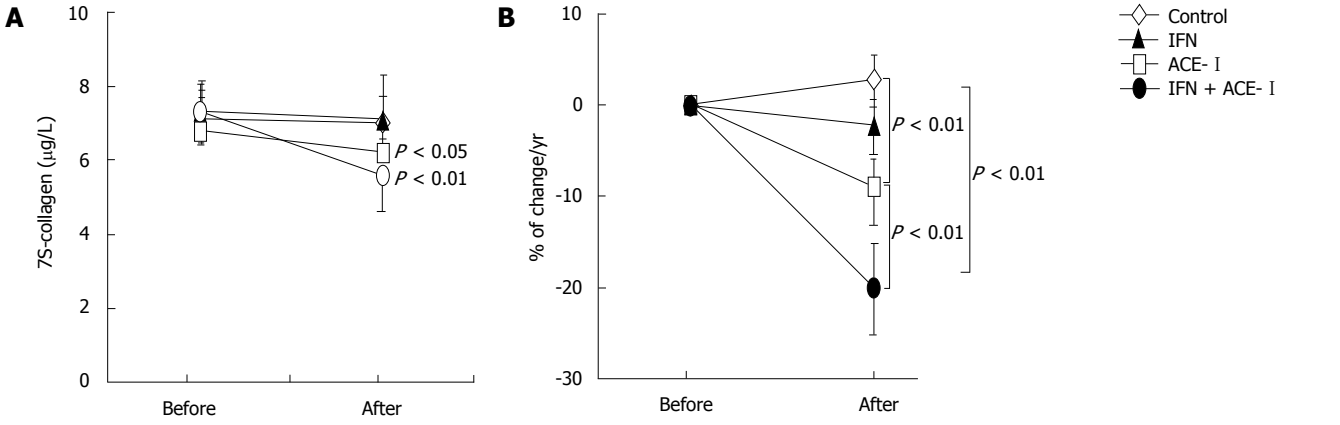


**Figure 2** The effect of IFN and ACE- I on the serum 7-S-collagen in the patients with refractory chronic hepatitis C. **A**: The 7-S-collagen level before and after the study; **B**: % of change in the 7-S-collagen level in each group. Control: untreated control group. ACE- I , IFN: ACE- I - and IFN-treated groups, respectively. IFN + ACE- I : combination treated group with ACE- I and IFN. The data represent the means ± SD.

markers. The ACE- I - and IFN + ACE- I -treated groups showed improvement in the serum ALT level at the end of the study (Figure 4A). However, in contrast to the serum fibrotic markers, there were no marked differences between both groups (Figure 4B). These results indicated that the improvement of fibrosis markers induced by ACE- I and/or IFN were not due to the non-specific cytoprotective effects. Also, the viral status of HCV and serum creatinine (Cre) did not significantly change before and after the study in all groups. The mean blood pressure decreased in the ACE- I - and IFN + ACE- I -treated groups, although, similar to ALT, there was no difference between both groups (Table 2). Since TGF-β is known as a key cytokine in the liver fibrosis development, we next measured the TGF-β level in all experimental groups. The alterations of the TGF-β level were almost parallel to those of the fibrosis markers. The IFN + ACE- I treatment significantly attenuated the TGF-β level at the end of the study (-20% ± 5.6%) (Figure 5).

## DISCUSSION

In the current study, we showed that the combination treatment with ACE- I and IFN significantly suppressed

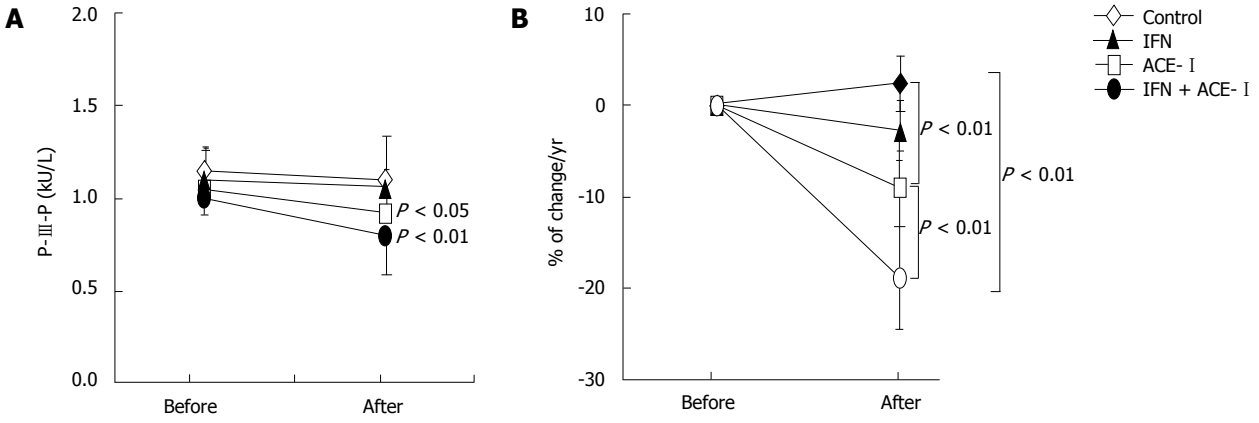


**Figure 3** The effects of IFN and ACE-Ⅰ on the serum P-Ⅲ-P in the patients with refractory chronic hepatitis C. **A**: P-Ⅲ-P level before and after the study; **B**: % of change in the P-Ⅲ-P level in each group. Control: untreated control group. ACE-Ⅰ, IFN: ACE-Ⅰ- and IFN-treated group, respectively. IFN + ACE-Ⅰ: combination treated group with ACE-Ⅰ and IFN. The data represent the means ± SD.

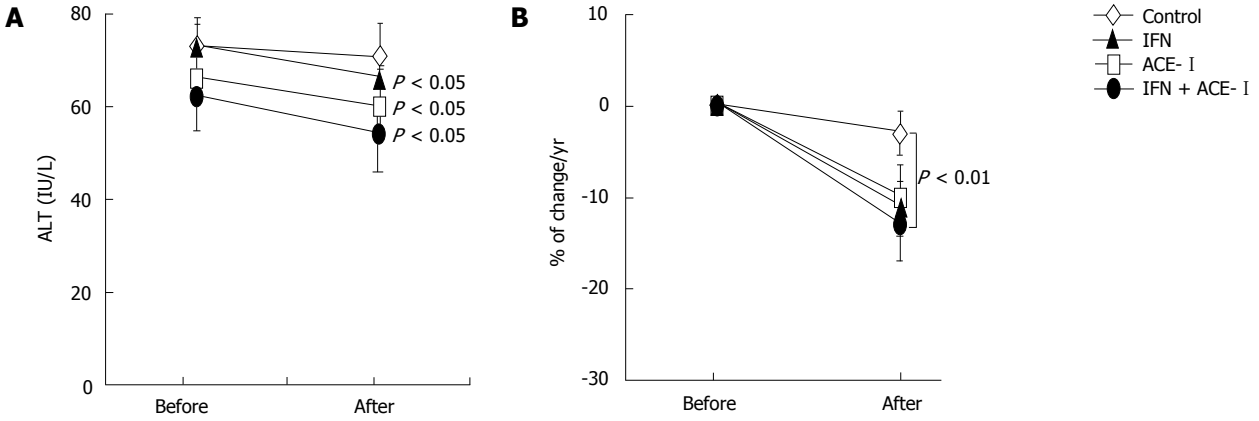


**Figure 4** The effects of IFN and ACE-Ⅰ on the serum ALT in the patients with refractory chronic hepatitis C. **A**: ALT level before and after the study; **B**: % of change in the ALT level in each group. Control: untreated control group. ACE-Ⅰ, IFN: ACE-Ⅰ- and IFN-treated groups, respectively. IFN + ACE-Ⅰ: combination treated group with ACE-Ⅰ and IFN. The data represent the means ± SD.

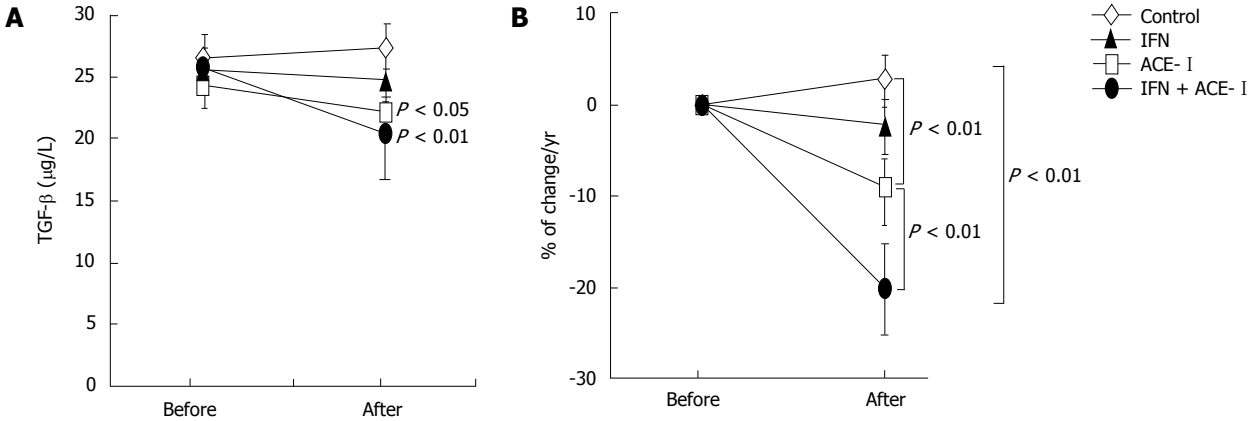


**Figure 5** The effects of IFN and ACE-Ⅰ on the serum TGF-β in the patients with refractory chronic hepatitis C. **A**: TGF-β level before and after the study; **B**: % of change in the TGF-β level in each group. Control: untreated control group. ACE-Ⅰ, IFN: ACE-Ⅰ- and IFN-treated groups, respectively. IFN + ACE-Ⅰ: combination treated group with ACE-Ⅰ and IFN. The data represent the means ± SD.

several serum fibrosis markers along with inhibition of TGF-β in patients with refractory CHC.

After evolvement of the combination therapy with peg-IFN and ribavirin, a significantly higher SVR rate has been achieved as compared with the IFN mono-therapy[2]. Even in the genotype 1b patients, almost half of the patients could reach the SVR status. However, from the opposite aspect, about half of the patients are still resistant

to the IFN-based anti-viral therapy. Also, it is well known that this combination regimen frequently exerts several adverse effects as compared with the IFN mono-therapy, such as hemolytic anemia and potential teratogenecity. Accordingly, an alternative strategy should be developed for patients with both refractory CHC and intolerance to the combination therapy of peg-IFN and ribavirin. One of the clinical goals to be achieved for these patients is prevention of progression to liver cirrhosis and HCC[5]. It has been reported that the improvement of fibrosis stage led to suppression of hepatocarcinogenesis [4]. Taken together, it is likely that the effective anti-fibrotic therapy will significantly improve the prognosis of the patients with refractory CHC.

In addition to the anti-viral effect, several evidences suggested that IFN is also effective in curtaining liver fibrosis both in the experimental animal models and in the clinical practice[6,14]. It has been reported that IFN significantly suppressed the HSC, and it also inhibited the promoter activity of pro-collagen gene[7]. However, in the clinical practice, fibrosis improvement with IFN-based therapy could be observed only in the virological responders[6]. Since it has been reported that the combination treatment with different anti-fibrotic agents exerted a more potent inhibitory effect than single agent therapy, alternative strategies such as combination therapy with other safe agents are required.

It is well known that TGF-β is a central key cytokine in the liver fibrosis development[16]. We previously reported that the clinically used ACE- I even at a clinically comparable low dose significantly attenuated the experimental liver fibrosis development along with suppression of TGF-β[10,17]. In the clinical practice, a retrospective study on liver transplanted patients with CHC, re-infection with HCV revealed that the patients receiving RAS inhibitors, such as ACE- I , had significantly less fibrosis progression than those receiving other types of drugs[12]. In the current study, we found that the combination treatment with ACE- I and IFN significantly suppressed the several serum fibrosis markers along with TGF-β inhibition as compared with ACE- I alone. We have shown similar results in a previous experimental study[14]. The combination treatment with ACE- I and IFN attenuated the liver fibrosis development along with suppression of the activated HSC, pro-collagen mRNA, and TGF-β. It is well known that the activated HSC play a pivotal role in the liver fibrogenesis, and that the activated HSC are one of the main cells producing TGF-β and collagen[16]. The exact mechanism of the inhibitory effect in the current study is still obscure at this time. However, we observed that the serum TGF-β, P-Ⅲ-P, and 7-S-collagen were suppressed almost in parallel by this combination regimen, whereas ALT and HCV status were not altered. These results indicated that, as in the experimental study, the anti-fibrotic effect of this combination regimen was, at least partly, mediated through inhibition of the activated HSC.

It is now widely recognized that angiogenesis plays an important role in many biological phenomena, including fibrosis development[18]. It has been shown that neovascularization significantly increased during the development of liver fibrosis[19]. We previously reported that ACE- I and IFN exerted an anti-angiogenic activit[20]. It would be possible that the anti-fibrotic effect of this combination treatment is, to some extent, mediated by suppression of neovascularization in the liver.

From the clinical point of view, liver biopsy is still considered the "gold standard" to assess the fibrosis development[16]. However, liver biopsy is associated with potential morbidity and mortality and has several limitations, including sampling error and inter-observer variability[21]. Recent studies have shown that evaluation of the serum fibrosis markers can predict the degree of fibrosis to an extent similar to that of liver biopsy[22]. Although we did not perform liver biopsy in the current study, further studies are required in the future to examine the histological and immunohistochemical markers, such as smooth muscle α-actin which is known as a marker of the activated HSC, and to make comparison with the serum fibrosis markers. Furthermore, in the next step, we have to examine whether this IFN+ACE- I regimen is also effective for the CHC patients refractory to the combination therapy of peg-IFN and ribavirin.

In summary, we found in our present study that the combination treatment with ACE- I and IFN significantly attenuated the serum fibrosis markers along with suppression of TGF-β in patients with refractory CHC. This combination treatment may become a new strategy to prevent disease progression in the patients with refractory CHC through its anti-fibrotic effect.

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*VIRAL HEPATITIS*

# Seroprevalence and genotyping of hepatitis B, hepatitis C and HIV among healthy population and Turkish soldiers in Northern Cyprus

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## Abstract

**AIM:** To compare the prevalence of hepatitis viral markers among soldiers from Turkey, blood donors from Northern Cyprus, and soldiers from Northern Cyprus.

**METHODS:** Hepatitis B surface antigen (HBsAg), anti-HCV and anti-human immunodeficiency virus (HIV) seroprevalence, HBV DNA, HCV RNA and HCV genotyping among soldiers from Turkey (group Ⅰ), civil blood donors from Northern Cyprus (group Ⅱ), and soldier candidates from Northern Cyprus (group Ⅲ) were studied and compared to one another. In total, 17545 cases (13546 males and 3999 females with a mean age of 34.5 ± 10.3 year, group Ⅰ = 11234, group Ⅱ = 5057, and group Ⅲ = 1254) were included into the study.

**RESULTS:** Among all cases, HBsAg positivity rates were 2.46%, anti-HCV was 0.46% and anti-HIV was 0.00%. HBV DNA was 2.25%, HCV RNA was 0.33% in all groups. HBsAg positivity rates were 2.16% in group Ⅰ, 3.00% in group Ⅱ and 2.71% in group Ⅲ. There was a significant difference between group Ⅰ and group Ⅱ ($\chi^2$ = 6.11, $P$ = 0.047 < 0.05). Anti-HCV positivity rates were 0.45% in group Ⅰ, 0.45% in group Ⅱ, and 0.56% in group Ⅲ. Genotypes of HCV were 1b and 1a in group Ⅰ, 1b, 1a and 2 in group Ⅱ, and 1b, 1a in group Ⅲ. HBsAg carrier rates were 2.20% in females and 2.53% in males. Anti-HCV prevalence was 0.38% in females and 0.48% in males. HBsAg positivity rates were 2.53% in individuals younger than 50, and 1.47% in older than 50. There was a significant difference between the two groups ($\chi^2$ = 23.48, $P$ = 0.001).

**CONCLUSION:** Prevalences of HBsAg, HCV and HIV infections in Northern Cyprus population are similar to those of Turkey.



**Key words:** Cyprus; Donor; Healthy adults; Hepatitis B; Hepatitis C; Human immunodeficiency virus

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## INTRODUCTION

Worldwide, two billion people have been infected with hepatitis B virus (HBV), 360 million have chronic infection, and 600 000 die each year from HBV-related liver disease or hepatocellular carcinoma[1]. Meanwhile, hepatitis C virus (HCV) infection is the most common chronic blood borne infection in the World. An estimated 3.9 million persons are currently infected and it is responsible for approximately 8000 to 10000 deaths each year in the United States[2]. Both diseases cause serious mortality, morbidity and financial costs, continuing thus to be a major health problem[3]. In addition, prevalence of human immunodeficiency virus (HIV) is increasing everyday and it has become a disaster for humankind in some areas. Prevalence studies of these blood-borne diseases show certain risky groups and risky attitudes that should be studied further. Northern Cyprus population shares many characteristics with Turkey's population. Turkey is among the moderate endemic countries and rates of HBV carriers are 4.4%-12.5% and of HCV positivity 0.3%-4.0%[4,5]. Acquired immune deficiency syndrome (AIDS) is one of the most important mortality causes in the world although it is not endemic in Turkey and Cyprus[6,7]. There is an increasing risk in recent years in this area of this disease. Seroepidemiological studies about blood-borne diseases could have provided us with the opportunity of learning basic knowledge about

epidemiology of these diseases in the community. The aim of this study was to determine prevalence of HBV, HCV and HIV infections among normal population and Turkish soldiers in Northern Cyprus.

## MATERIALS AND METHODS

### Study population

Population of Northern Cyprus is about 175 000 and it has close relation with Turkey. Ethical permission was obtained from Afyon Kocatepe University Human Ethics Committee.

### Study samples

A cross-sectional study was performed using the data of blood centers of two hospitals (Nalbantoglu General Hospital, Nicosa and Akcicek Military Hospital, Kyrenia) in Northern Cyprus between January 2000 and January 2001. There were three different groups in this study: group Ⅰ, Turkish soldiers who came from Turkey and their results represent Turkish population; Group Ⅱ, civilian blood donors who applied to the hospitals from Northern Cyprus and who were inhabitants there, and donors with any history of jaundice in the past were eliminated from the study; Group Ⅲ, Northern Cyprus inhabitants who applied for soldier recruitment and were screened for these diseases.

### Serology

The blood samples were screened for hepatitis serology [Hepatitis B surface antigen (HBsAg), anti-HCV] and anti-HIV. HBsAg, anti-HCV and anti-HIV positivity were investigated by microparticle ELISA (Axsym, Abbott) in blood samples.

### HBV and HCV molecular diagnosis

We screened HBV DNA and HCV RNA markers by reverse transcription polymerase chain reaction (RT-PCR) (7700 Sequence Detection System, AB).

### HCV genotyping

HCV-RNA sequences were amplified by RT-PCR that targeted the 5' non-coding region of the viral genome and were genotyped by line probe assay using INNO-LIPA HCV Ⅱ kit.

### Collection of serum samples

Blood samples were obtained from different groups and stored at -20℃ until use. Prior to RNA extraction, sera were tested on INNO LIA HCV Ab Ⅲ (Innogenetics NV, Antrewp, Belgium) to confirm the presence of anti-HCV antibodies.

### HCV RNA extraction

HCV RNA was extracted from serum or EDTA-plasma. To avoid carryover contamination, the steps of the protocol were carried out at four separate locations. Cotton-plugged tips were generally used, and all buffers, primers, and dNTPs were divided into single-use aliquots. Fifty microliters of serum were mixed with 150 μL of Trizol LS Reagent (Life Technologies Gent, Belgium) at room temperature. After lysis and denaturation, 40 μL of $CHCl_3$ was added. The mixture was vigorously shaken, incubated for 15 min at room temperature, and centrifuged, and the minute amounts of viral RNA were precipitated from the colorless aqueous phase with 20 μL of 1 μg Dextran T500 (Pharmacia, Brussels, Belgium) per μL and 100 μL of isopropanol. The RNA pellet was washed with 200 μL of ethanol and collected by centrifugation. Finally, the RNA pellet was briefly air dried. If the procedure was interrupted at this stage, the RNA was stored at -20℃ as a pellet instead of being dissolved in water.

### cDNA synthesis and PCR amplification

The RNA pellet was dissolved in 15.1 μL random primers (20 mg/L, $pdN_6$; Pharmacia). After denaturation at 70℃ for 10 min, 4.9 μL of a cDNA mixture was added which was composed of 4 μL of 5 × avian myeloblastosis virus reverse transcriptase buffer (250 mmol/L Tris HCl [pH 8.5], 100 mmol/L KCl, $MgCl_2$, 25 mmol/L dithiothreitol), 0.4 μL of 25 mmol/L dNTPs, 0.2 μL or 25 U of RNase inhibitor (HPRI; Amersham, Gent, Belgium), and 0.3 μL or 8 U of avian myeloblastosis virus reverse transcriptase (Stratagene, La Jolla, Calif.). cDNA was synthesized at 42℃ for 90 min. PCR was performed in a volume of 50 μL. Other PCR amplified the cDNA over 40 cycles (1 min at 94℃, 1 min at 50℃, and 1 min at 72℃). One microliter of product was amplified with nested PCR primers for another 40 cycles with the same thermal profile. All primers were tagged with a biotin group at the 5' end.

### Preparation of LIPA strips

A poly (dT) tail was enzymatically added to the 3' end of each oligonucleotide as previously described[8]. The tailed probes were precipitated and washed with ice-cold ethanol. Probes were dissolved at their respective specific concentrations and applied as a positive control (LIPA line 1). The oligonucleotides were fixed to the membrane by baking at 80℃ for 12 h. The membranes were then sliced into 4-mm-wide strips.

### LIPA

Equal volumes (10 μL each) of the biotinylated PCR fragment and the denaturation solution (400 mmol/L NaOH, 10 mmol/L EDTA) were mixed in test troughs by pipetting and incubated at room temperature for 5 min, after which 2 mL of the prewarmed (37℃) hybridization solution (3 × SSC [1 × SSC is 0.15 mol/L NaCl plus 0.015 mol/L sodium citrate], 0.1% sodium dodecyl sulfate) was added, followed by the addition of one strip per trough. Hybridization occurred for 1 h at 50℃ ± 0.5℃ in a closed water bath with back-and-forth shaking. The strips were washed twice with 2 mL of wash solution (3 x SSC, 0.1% sodium dodecyl sulfate) at room temperature for 20 s and once at 50℃ for 30 min. Following this stringent washing, strips were rinsed twice with 2 mL of a standard rinse solution. Strips were incubated on a rotating platform with an alkaline phosphatase-labeled streptavidin conjugate diluted in a standard conjugate solution for 30 min at 20℃ to 25℃. Strips were then washed twice with 2 mL of rinse solution and once with standard substrate buffer, and color development was initiated by addition

**Table 1 Distribution of HBV and HCV markers in various population**

| Group | *n* | HBsAg | HBV DNA | Anti-HCV | HCV RNA |
|---|---|---|---|---|---|
| | | *n* (%) | *n* (%) | *n* (%) | *n* (%) |
| Turkish soldiers | 11234 | 243 (2.16)[a] | 223 (1.98) | 50 (0.45) | 35 (0.31) |
| Civil donors | 5057 | 154 (3.00) | 150 (2.90) | 23 (0.45) | 13 (0.25) |
| Northern cyprus soldiers | 1254 | 34 (2.71) | 23 (1.83) | 7 (0.56) | 5 (0.39) |
| Total | 17545 | 431 (2.46) | 396 (2.25) | 80 (0.46) | 53 (0.33) |

[a]$P = 0.047$ *vs* the group of civil donors (group Ⅱ).

**Table 2 Distribution of HCV genotyping in various population**

| Group | HCV RNA | Genotype 1b | Genotype 1a | Genotype 2 |
|---|---|---|---|---|
| | | *n* (%) | *n* (%) | *n* (%) |
| Turkish soldiers | 35 | 34 (97.1) | 1 (2.8) | |
| Civil donors | 13 | 11 (84.6) | 1 (7.7) | 1 (7.7) |
| Northern Cyprus soldiers | 5 | 4 (80.0) | 1 (20.0) | |
| Total | 53 | 49 (92.4) | 3 (5.7) | 1 (1.9) |

**Table 3 Distribution of HBV and HCV markers by gender**

| Group | *n* | HBsAg | Anti-HCV | Anti-HIV |
|---|---|---|---|---|
| | | *n* (%) | *n* (%) | *n* (%) |
| Female | 3999 | 88 (2.20) | 15 (0.38) | 0 (0) |
| Male | 13546 | 343 (2.53) | 65 (0.48) | 0 (0) |
| Total | 17545 | 431 (2.46) | 80 (0.46) | 0 (0) |

**Table 4 Distribution of HBV, HCV and HIV markers according to age group**

| Age group (yr) | Anti-HIV | HBsAg | Anti-HCV |
|---|---|---|---|
| | *n* (%) | *n* (%) | *n* (%) |
| 20-29 | 10352 (59.0) | 222 (2.14)[b] | 50 (0.48) |
| 30-39 | 4532 (25.8) | 142 (3.13)[b] | 21 (0.46) |
| 40-49 | 1432 (8.2) | 49 (3.42)[b] | 5 (0.35) |
| > 50 | 1229 (7.0) | 18 (1.47) | 4 (0.33) |
| Total | 17545 (100.0) | 431 (2.46) | 80 (0.46) |

[b]$P < 0.001$ *vs* the group of older individuals (> 50 yr).

of 5-bromo-4-chloro-3-indolylphosphate (BCIP) and nitroblue tetrazolium to 2 mL of substrate buffer. After 30 min at room temperature, the color reaction was stopped by aspiration of the substrate buffer and addition of distilled water. Immediately after drying, the strips were interpreted[8].

### *Statistical analysis*

The differences between groups were analyzed by the Chi-square test. $P < 0.05$ was taken as significant.

## RESULTS

Totally 17 545 individuals, aging 20-56 (mean ± SD, 34.5 ± 10.3) years, of whom 13 546 were men and 3999 were women were included into the study. HBsAg positivity rate was 2.46%, anti-HCV was 0.46%, anti-HIV was 0.00%, HBV DNA was 2.25% and HCV RNA was 0.33% in all groups. HBsAg positivity was the lowest in group I (2.16%) among all groups. There was a significant difference between groups I and Ⅱ for HBsAg positivity rates ($\chi^2 = 6.11$, $P = 0.047$). There were no significant differences between groups for anti-HCV prevalence ($\chi^2 = 0.32$, $P = 0.852$) (Table 1).

Three different genotypes were determined such as 1b, 1a, and 2 genotypes. Number and ratios of 1b, 1a, and 2 types were 49 (92.4%), 3 (5.7%), 1 (1.9%), respectively (Table 2). There was no significant difference in the distribution of HCV genotypes with respect to age, sex and transfusion history ($P > 0.05$).

HBsAg prevalence was higher in males (2.53% *vs* 2.20%). There was no significant difference between the groups for HBsAg prevalence ($\chi^2 = 1.42$, $P = 0.234$). Anti-HCV positivity was higher in males (0.48% *vs* 0.38%). There was no significant difference between two groups for anti-HCV prevalence ($\chi^2 = 0.75$, $P = 0.387$) (Table 3).

Positivity of HBsAg had a close relation with age. The HBsAg carrier rate was highest in the 40-49 year old group (3.42%) and there was a significant difference between older and younger than 50 years groups (2.53% in younger than 50 years, and 1.47% in older; $\chi^2 = 23.48$, $P = 0.001$). Anti-HCV prevalence was higher in younger age groups than in the older age groups, however, there was no significant difference among different age groups for anti-HCV prevalence ($\chi^2 = 0.99$, $P = 0.803$) (Table 4).

## DISCUSSION

Epidemiological studies about blood-borne diseases such as hepatitis B, C and HIV are important for revealing the risk groups and risk factors for these infections. Evaluation of the prevalence among blood donors and army recruits is a common and easy method to obtain the epidemiology of these infections in a community. There are some differences between normal population and blood donors or soldiers, however, this approach is very common for screening studies. Screening these groups helps us to solve difficulties in collecting information among healthy populations[9,10].

Northern part of Cyprus has very close relationship with Turkey and both peoples share similar demographic features. At the same time, there is similar close relationship between Southern Cyprus and Greece. Prevalence of HBsAg in Greece has low endemicity level and seropositivity is lower than 1% among volunteer blood donors and military recruits[11,12]. In contrast, HBsAg positivity is reported between 4% and 10% among civil blood donors according to a study including over 10 000 individuals in Turkey[9,10]. Many studies showed that the incidence of HBsAg has a moderate level in Turkey. These data suggest that epidemiology of HBV in Northern

Cyprus population is similar to Turkey. On the other hand, Papaevangelou[13] showed that the carrier rate of HBsAg in the blood donor and army recruit samples ranged between 0.77% and 1.01% in Southern Cyprus. Therefore, Southern Cyprus results are similar to Greece and other south European countries.

At the beginning, our expectation was that the results of Northern Cyprus might have been between Turkey and Southern Cyprus. However, the result has suggested that prevalence of hepatitis B in native population of Cyprus is not lower than in Turkish soldiers. In the last ten years, the intensive efforts on vaccination and prevention precautions might have been effective on prevalence of the disease in Turkey. Our results could be attributed to good infection control and vaccination program against hepatitis B in Turkey. In contrast, there is no effective prevention program against hepatitis B in Northern Cyprus[14].

The incidence of HCV is about 0.01% in North Europe and 1%-1.5% in South Europe[15]. In Turkey, HCV prevalence is between 0.1% and 1.5% in blood donors[9,10,16]. It could be seen easily that HCV prevalence in Turkey and Greece is similar to that of other South European countries. In this study, the incidence of anti-HCV in Northern Cyprus is similar to Turkey and Greece results. In Turkey, prevalence of HCV is increasing in older age.

On the basis of its extensive genetic heterogeneity, HCV has been divided into six major genotypes and at least 100 subtypes. Genotypes 1, 2 and 3 are found throughout the world; but the other genotypes are common in particular geographic regions (genotype 4 is common in North Africa and the Middle East, genotype 5 is common in South Africa, and genotype 6 is common in Southeast Asia)[17]. The predominant genotype in patients with chronic HCV infection in Turkey is genotype 1, followed by genotype 2 and 3[18]. Predominant molecular techniques used (hybridization and direct DNA sequencing) are based on nucleotide differences in the highly conserved 5' UTR among genotypes[17]. The widely used INNO-LIPA HCV Ⅱ assay (Innogenetics, Ghent, Belgium) uses PCR products from the 5' UTR that hybridize to type-specific probes embedded on a nitrocellulose strip[17].

Abacioglu's study showed that the predominant genotype was 1b (75.3%), followed by 1a (19.1%), 2 (3.4%) and 4 (2.2%). In that study, HCV RNA sequences were amplified in the 5' non-coding region and were typed by restriction fragment length polymorphism analysis[19]. These results, together with the results of two previous studies, indicate that HCV genotypes 1, 2, 3 and 4 are prevalent at different frequencies in the Turkish population. In the present study, three different genotypes were determined such as 1b, 1a, and 2 genotypes. There was no significant difference in the distribution of HCV genotypes with respect to groups, age, sex, transfusion history ($P > 0.05$) (Table 2).

In this study, male gender had a higher rate for HBsAg carrier status but the difference was not significant (2.20% in females *vs* 2.54% in males). Koulentaki's[20] study from Greece showed that a greater number of males than females were HBsAg positive (0.41% *vs* 0.28%, respectively). In many studies, males are more frequently exposed to HBV and become carriers more often than females. For HCV, an opposite gender trend was reported that females being infected more frequently than males (0.49% in females *vs* 0.37% in males). In our study, prevalence of HCV was higher in males.

Another agent, which has similar transmission ways to HBV, is HIV. There is a potential hazard for everybody in the world because of the characteristics of the diseases. Although we did not detect any HIV positive case in this study, Cyprus is a sensitive area because of visitors from other countries. There is a young population and many sea workers, and individuals are not well informed about the subject, and drug-users may increase.

In conclusion, seroprevalence of blood-borne disease in Northern Cyprus is similar to Turkey. This study suggests that it is necessary to investigate risk factors and risk groups for these infections in Northern Cyprus. In the light of this result, an effective control and training program for soldiers and civilians should be implemented.

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*BASIC RESEARCH*

# Cloning and expression of *SLC10A4*, a putative organic anion transport protein

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## Abstract

**AIM:** To determine if novel bile acid transporters may be expressed in human tissues.

**METHODS:** *SLC10A1* (NTCP) was used as a probe to search the NCBI database for homology to previously uncharacterized ESTs. The homology search identified an EST (termed *SLC10A4*) that shares sequence identity with *SLC10A1* and *SLC10A2* (ASBT). We performed Northern blot analysis and RT-PCR to determine the tissue distribution of *SLC10A4*. *SLC10A4* was cloned in frame with an epitope tag and overexpressed in CHO cells to determine cellular localization and functional analysis of bile acid uptake.

**RESULTS:** Northern analysis revealed that *SLC10A4* mRNA is ubiquitously expressed in human tissues with the highest levels of mRNA expression in brain, placenta, and liver. In *SLC10A4*-transfected CHO cells, immunoblotting analysis and immunofluorescence staining demonstrated a 49-kDa protein that is expressed at the plasma membrane and intracellular compartments. Functional analysis of *SLC10A4* showed no significant taurocholate uptake in the presence of sodium when compared to untransfected CHO cells.

**CONCLUSION:** To date, we have shown that this protein has no capacity to transport taurocholate relative to *SLC10A1*; however, given its ubiquitous tissue distribution, it may play a more active role in transporting other endogenous organic anions.



**Key words:** *SLC10A4*; Bile acid transport; Biliary epithelium; Taurocholate; Plasma membrane

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## INTRODUCTION

Bile acids, the major solute in bile, are physiologically important for promoting bile flow and facilitating the absorption of dietary lipids[1,2]. Bile acids are also involved in cholesterol homeostasis, xenobiotic excretion, as well as apoptotic and cell signal transduction pathways[1-6]. Unlike most other biliary solutes, bile acids are efficiently conserved and cycle in the enterohepatic circulation (EHC). This EHC is maintained by the vectorial transport of bile acids *via* specific bile transport proteins located on the plasma membranes of liver and intestinal epithelia[1].

Within the liver, hepatocytes express the sodium dependent taurocholate transporting protein (*SLC10A1*) and several sodium independent organic anion-transporting proteins at the sinusoidal membrane[6-9]. On their canalicular membrane, hepatocytes express the bile salt export pump (*ABCB11*)[10]. Cholangiocytes, the epithelial cells that line the bile ducts, express the apical sodium dependent bile acid transporter (*SLC10A2*) on lumenal surface[11]. Rat cholangiocytes express a truncated form of ASBT, that may export bile acids across the basolateral membrane[12]. To complete the EHC of bile acids, the enterocytes of the distal ileum express *SLC10A2*[13]. After *SLC10A2*-mediated uptake, bile acids are directed across the ileal enterocyte and secreted into the portal circulation, allowing for efficient recycling of the bile acids from the intestine to the liver[13].

Given the redundancy of biological systems, we hypothesize that additional bile acid transporters may exist within the human genome. In this study, we identified a novel member termed *SLC10A4* of the SLC10A family of sodium bile acid symporter related proteins. We also examined the expression of *SLC10A4* mRNA presence in human tissues and have over expressed this protein in CHO cells.

## MATERIALS AND METHODS

### Materials

All chemicals were of highest commercially available purity and were purchased from Sigma, St. Louis, MO Chemical C. (St. Louis, MO) unless otherwise indicated.

### Cell culture

Cells were maintained at 37℃ in a humidity-controlled incubator with $CO_2$. The cholangiocarcinoma cell-lines (Witt, KMC, and KMCH) were grown in Dulbecco's modified Eagle's medium containing F12 (Sigma, St. Louis, MO) supplemented with 5% (v/v) FBS (Mediatech, Herndon, VA), Penicillin (100 U/mL)/Streptomycin 100 μg/mL (Sigma, St. Louis, MO), and 2 mmol/L-glutamine (Sigma, St. Louis, MO). H69 cells are SV40 transformed normal human biliary epithelial cells (a gift from Dr. Douglas Jefferson, Tufts University) and were grown as previously described. T84 cells, derived from a human adenocarcinoma, were grown in Dulbecco's modified Eagle's medium containing F12 (Sigma, St. Louis, MO) supplemented with 5% (v/v) FBS (Mediatech, Herndon, VA), Penicillin (100 U/mL)/Streptomycin 100 μg/mL (Sigma, St. Louis, MO). Chinese hamster ovary (CHO) cells were grown in Dulbecco's modified Eagle's medium containing F12 (Sigma, St. Louis, MO) supplemented with 10% (v/v) FBS (Mediatech, Herndon, VA), Penicillin (100 U/mL)/Streptomycin 100 μg/mL (Sigma, St. Louis, MO), 1 × MEM vitamin solution (Sigma, St. Louis, MO), and 2 mmol/L L-glutamine (Sigma, St. Louis, MO).

### Identification of the SLC10A4 EST

To initially identify novel members of the SLC10 family of proteins, a bioinformatic approach was used. Amino acids 1-200 of *SLC10A1* (GenBank accession no. AAA36381) were used to perform a tBlastN search of the NCBI database. The candidate EST (Htm1-361F, GenBank accession no. BE39690) sequence was then used for BlastN and BlastX database searches to confirm that it had not been previously identified and as a template to design PCR primers.

### RNA isolation

Total RNA was extracted from the tissue culture cell-lines using Tri-Reagent (Sigma, St. Louis, MO). The cells were lysed using 1 mL of Tri-Reagent/1.0 × $10^6$ cells and stored for 5 min at room temperature. Next, 0.1 mL of 1-bromo-3-chloropropane was added, the samples were vortexed, incubated at room temperature for 15 min, and centrifuged at 12000 × *g* for 15 min at 4℃. RNA was subsequently precipitated with isopropanol. The RNA pellet was resuspended with RNA Secure (Ambion, Austin, TX), and the concentration and purity was assessed by spectroscopy.

### Reverse transcription-polymerase chain reaction and pCRII cloning

5 μg of total RNA was reverse transcribed using a SuperScript™ II reverse transcritpase (In Vitrogen, Carlsbad, CA). The reaction mixture contained total RNA, 10 mmol/L deoxynucleotide triphosphates and random hexamers in a final volume of 10 μL. This mixture was incubated 5 min at 65℃. Reverse transcription buffer, 25 mmol/L $MgCl_2$, 0.1 mmol/L dithiothreitol (DTT), and RNase inhibitor was then added to the reaction mixture and incubated for 2 min at 25℃. Finally, the reverse transcriptase was added to the reaction mixture and incubated for 50 min at 42℃. The reverse transcriptase reaction was terminated at 70℃ for 15 min and chilled on ice. The *SLC10A4* cDNA was PCR amplified using *SLC10A4* specific primers; sense (5'-GGCAATCTCTC CAATCTTATGTC-3') and antisense (5'-CAGTTGGTG GAGATGAAGAGAGT-3'). The 441 bp PCR amplicon was electrophoresed on a 1% agarose gel and the bands were visualized by ethidium bromide staining. The PCR amplicons were then cloned into the pCRII dual promoter vector (In Vitrogen, Carlsbad, CA) according to the manufacturers protocol. Briefly, 2 μL of the PCR product was mixed with 10 × ligation buffer, 50 ng of pCRII vector, water, and T4 DNA ligase. The ligation reaction was incubated overnight at 14℃ and transformed into competent *Escherichia coli* (*E. coli*). Following the isolation of plasmid DNA, the pCRII (*SLC10A4*) was sequenced to confirm identity of the DNA insert (Mayo Molecular Core Facility, Rochester, MN).

### Random priming and Northern blot analysis

The pCRII (*SLC10A4*) plasmid was linearized and random primed using radiolabeled [α-$^{32}$P] dCTP. The random primer protocol was followed per manufactures (In Vitrogen, Carlsbad, CA) directions. Briefly, 25 ng of pCRII (*SLC10A4*) template DNA was denatured by boiling for 5 min and cooled on ice. To the template DNA, the following was added; 2 μL of dATP, 2 μL of dGTP, 2 μL of dTTP, 15 μL of random primer buffer, 5 μL of [α-$^{32}$P] dCTP (10 Ci/L). This reaction mixture was brought up to 49 μL. The 1 μL of Klenow fragment was added, mixed, and centrifuged briefly. This reaction mixture was incubated for 1 h at 25℃. Following the incubation, 5 μL of stop buffer was added and the $^{32}$P-labeled *SLC10A4* cDNA probe was purified using a NAP-25 column. Next, the Human MTN Blot (Clontech, Palo Alto, CA) and the Human Digestive System MTN Blot (Clontech, Palo Alto, CA) was prehybridized in ExpressHyb solution (Clontech, Palo Alto, CA) for 30 min at 68℃ and then 1 × $10^6$ cpm/mL of the labeled probe was added and incubated for an additional 60 min at 68℃. Following hybridization, the blots were washed several times and exposed to x-ray film (Kodak, Rochester, NY) and developed. Subsequently, the membranes were stripped with 0.5% SDS at 95℃ for 10 min and reprobed with β-actin to normalize for mRNA loading.

### Cloning the open reading frame (ORF) of SLC10A4

We obtained IMAGE clone 3502817 (ATCC, Manassas, VA) that contains the complete ORF of *SLC10A4* in the pOTB7 vector. The IMAGE clone was used as template DNA for PCR to delete the stop codon using specific PCR primers; sense (5'-GGATCCCCAAGTAACTATAACGG TCC-3') and antisense (5'-GAATTCGTATCTCCACAT TTGGAGAGAAGTCTG -3'). This fragment was cloned into pCRII (In Vitrogen, Carlsbad, CA) using the method

described above. The pCRII (*SLC10A4*) was digested with *Eco*RI and *Bam*HI restriction enzymes, the *SLC10A4* fragment was isolated by gel purification, and subcloned into the *Eco*RI and *Bam*HI sites of pcDNA4 V5-HisA (In Vitrogen, Carlsbad, CA), a mammalian expression vector which expresses the V5 epitope tag. The *SLC10A4* insert was sequenced to confirm identity of the DNA insert (Mayo Molecular Core Facility, Rochester, MN).

### Transfection of CHO cells

Transfections were performed using Lipofectamine reagent (In Vitrogen, Carlsbad, CA) according to the manufactures directions. The day of transfection, the CHO cells were 50%-80% confluent. The media was replaced with Optimem (In Vitrogen, Carlsbad, CA) and the cells were transfected with 3 μg of pcDNA V5-HisA (*SLC10A4*) or empty vector pcDNA V5-HisA plasmid DNA for 4 h after which the transfection media was replaced with complete CHO media. After 48 h, Zeocin (500 μg/mL) was added to the complete media and the cells were selected. Individual stable clones were isolated using cloning cylinders and confirmed by RT-PCR and immunoblotting to confirm the expression of *SLC10A4*.

### Immunofluorescent staining

CHO cells were grown on collagen-coated coverslips and fixed by 0.1 mol/L PIPES, pH 6.95, 1 mmol/L ethylene glycolbis (β-amino-ethylether-N, N, N', N'-tetra acetic acid (EGTA), and 2% paraformaldehyde in 1X phosphate buffered saline (PBS) for 20 min at room temperature. The cells were then permeabilized in 0.2% Triton for 2 min and incubated for 1 h with V5 epitope tag (1:500; In Vitrogen, Carlsbad, CA) monoclonal antibody. The cells were washed with PBS and subsequently incubated Texas Red-conjugated goat anti-mouse secondary antibody (Molecular Probes, Eugene, OR). The coverslips were washed with PBS and then mounted using Prolong Antifade mounting medium (Molecular Probes, Eugene, OR) and analyzed using a confocal microscope.

### Immunoblotting for the V5 epitope tag

For the immunoblotting assay, cell lysates and mixed plasma membranes were prepared as previously described[14]. Cell lysates and MPM were heated to 95℃ for 10 min in sample buffer containing 0.8 mol/L dithiothreitol (Sigma, St. Louis, MO) and 10% SDS (Sigma, St. Louis, MO) for protein denaturation and solubilization. These protein samples were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes. After blocking, blots were incubated with the V5 epitope tag antibody (1:2000 dilution; In Vitrogen, Carlsbad, CA) and incubated over night at 4℃. The blots were washed and incubated for 1 h at room temperature with horseradish peroxidase conjugated secondary antibody (1:2000 dilution), and bands were detected using the enhanced chemiluminescent plus detection system (ECL Plus; Amersham, Arlington Heights, IL). Autoradiographs were obtained by exposing the nitrocellulose to Kodak XAR film (Rochester, NY).

### Bile Acid Uptake Study

Uptake experiments were performed in triplicate using un-transfected, empty vector transfected or *SLC10A4*-transfected CHO cells. CHO cells transfected with *SLC10A1* was used as the positive control for the uptake experiments. [$^3$H]Taurocholate (specific activity 2.0 Ci/mmol) of > 95% purity was purchased from PerkinElmer (Boston, MA). The uptake experiments were performed as previously described[15].

## RESULTS

### Identification of SLC10A4 and sequence analysis

Using a bioinformatics approach, numerous expressed sequence tags (EST), including (Htm1-361f [GenBank accession no. BE439690] and Htm1-302f [GenBank accession no. BE439637]) were identified within the NCBI database. Upon further analysis it was determined that these ESTs represented a novel gene (*SLC10A4*) belonging to the sodium bile acid symporter family. The DNA and deduced amino acid sequence are shown in Figure 1. The human *SLC10A4* cDNA encodes a protein of 437 amino acids with a predicted molecular mass of 46.5 kDa. The cDNA encompasses a consensus methionine start codon[16] and an in-frame stop codon (Figure 1). The cDNA also encompasses a 5' untranslated region (UTR) of 183 bp and a 271 bp 3' UTR which contains a poly A tail. Kyte-Doolittle hydropathy analysis suggests that SLC10A4 contains 8 putative transmembrane domains (Figure 1). Furthermore, the amino terminus is predicted to contain three potential N-linked glycoslylation sites.

### Comparison of SLC10A4 with other species and with other members of the sodium bile acid symporter family

The *SLC10A4* deduced amino acid sequence for human compared to rat (accession no. XP_579196) share the greatest evolutionary conservation of *SLC10A4* with 89% identity (Figure 2A). In contrast the amount of identity shared between human compared to mouse (XP_775579) or bovine (XP_591836) was equal, 73%.

To further our understanding between the relationships of *SLC10A4* and the other members of the SLC10 family, we performed multiple amino acid alignments and examined the evolutionary relationship between the SLC10 proteins. At the amino acid level, *SLC10A4* shares 30% identity with *SLC10A3* (P3; a SLC10A family member of unknown function), 27% identity with *SLC10A5*, 27% identity with *SLC10A2*, and 24% identity with *SLC10A1* (Figure 2B). We have also looked for relationships of this *SLC10A4* protein compared with anion transporters but no conservation was found (data not shown).

### RT-PCR amplification of the SLC10A4 fragment

RT-PCR was performed using *SLC10A4*-specific primers and cDNA prepared from human colon, T84 cells, and cholangiocyte cell lines. This analysis identified *SLC10A4* mRNA in T84 cells, a normal human cholangiocyte cells (H69), and the cholangiocarcinoma cell-lines Witt, KMC and KMCH (Figure 3). In each case, the PCR amplicon was sequenced to confirm the identity of the PCR product.

```
1     ATTGCACGATCCACAAGTTTGTACAAAAAAGCAGGCTTGTAAACGCGGCCAAGTAACTAT
61    AACGGTCCTAAGGTAGCGAGGCCTGGGTGGCGAATTCGGCACGAGGGCGGCGACTGCGGC
121   GACCGCGGGACGGCGAGAGGCACGCGGCGGGAGGGGACCGGAATCCGCAGCTCCGGCCGC
181   GCCATGGACGGCAACGACAACGTGACCCTGCTCTTCGCCCCTCTGCTGCGGGACAACTAC
        M  D  G  N  D  N  V  T  L  L  F  A  P  L  L  R  D  N  Y      19
241   ACCCTGGCGCCCAATGCCAGCAGCCTGGGCCCCGGCACGGACCTCGCCCTCGCCCCTGCC
        T  L  A  P  N  A  S  S  L  G  P  G  T  D  L  A  L  A  P  A      39
301   TCCAGCGCCGGCCCCGGCCCTGGGCTCAGCCTCGGGCCGGGTCCGAGCTTCGGCTTCAGC
        S  S  A  G  P  G  P  G  L  S  L  G  P  G  P  S  F  G  F  S      59
361   CCCGGCCCCACTCCGACCCCGGAGCCCACGACCAGCGGCCTCGCGGGCGGCGCGGCGAGC
        P  G  P  T  P  T  P  E  P  T  T  S  G  L  A  G  G  A  A  S      79
421   CACGGCCCTTCCCCGTTCCCTCGGCCCTGGGCGCCCCACGCGCTCCCGTTCTGGGACACG
        H  G  P  S  P  F  P  R  P  W  A  P  H  A  L  P  F  W  D  T      99
481   CCGCTGAACCACGGGCTGAACGTGTTCGTGGGCGCCGCCCTGTGCATCACCATGCTGGGC
        P  L  N  H  G  L  N  V  F  V  G  A  A  L  C  I  T  M  L  G     119
541   CTGGGCTGCACGGTGGACGTGAACCACTTCGGGGCGCACGTCCGTCGGCCCGTGGGCGCG
        L  G  C  T  V  D  V  N  H  F  G  A  H  V  R  R  P  V  G  A     139
601   CTGCTGGCAGCGCTCTGCCAGTTCGGCCTCCTGCCGCTGCTGGCCTTCCTGCTGGCCCTC
        L  L  A  A  L  C  Q  F  G  L  L  P  L  L  A  F  L  L  A  L     159
661   GCCTTCAAGCTGGACGAGGTGGCCGCCGTGGCGGTGCTCCTGTGTGGCTGCTGTCCCGGC
        A  F  K  L  D  E  V  A  A  V  A  V  L  L  C  G  C  C  P  G     179
721   GGCAATCTCTCCAATCTTATGTCCCTGCTGGTTGACGGCGACATGAACCTCAGCATCATC
        G  N  L  S  N  L  M  S  L  L  V  D  G  D  M  N  L  S  I  I     199
781   ATGACCATCTCCTCCACGCTTCTGGCCCTCGTCTTGATGCCCCTGTGCCTGTGGATCTAC
        M  T  I  S  S  T  L  L  A  L  V  L  M  P  L  C  L  W  I  Y     219
841   AGCTGGGCTTGGATCAACACCCCTATCGTGCAGTTACTACCCCTAGGGACCGTGACCCTG
        S  W  A  W  I  N  T  P  I  V  Q  L  L  P  L  G  T  V  T  L     239
901   ACTCTCTGCAGCACTCTCATACCTATCGGGTTGGGCGTCTTCATTCGCTACAAATACAGC
        T  L  C  S  T  L  I  P  I  G  L  G  V  F  I  R  Y  K  Y  S     259
961   CGGGTGGCTGACTACATTGTGAAGGTTTCCCTGTGGTCTCTGCTAGTGACTCTGGTGGTC
        R  V  A  D  Y  I  V  K  V  S  L  W  S  L  L  V  T  L  V  V     279
1021  CTTTTCATAATGACCGGCACTATGTTAGGACCTGAACTGCTGGCAAGTATCCCTGCAGCT
        L  F  I  M  T  G  T  M  L  G  P  E  L  L  A  S  I  P  A  A     299
1081  GTTTATGTGATAGCAATTTTTATGCCTTTGGCAGGCTACGCTTCAGGTTATGGTTTAGCT
        V  Y  V  I  A  I  F  M  P  L  A  G  Y  A  S  G  Y  G  L  A     319
1141  ACTCTCTTCCATCTTCCACCCAACTGCAAGAGGACTGTATGTCTGGAAACAGGTAGTCAG
        T  L  F  H  L  P  P  N  C  K  R  Y  V  C  L  E  T  G  S  Q     339
1201  AATGTGCAGCTCTGTACAGCCATTCTAAAACTGGCCTTTCCACCGCAATTCATAGGAAGC
        N  V  Q  L  C  T  A  I  L  K  L  A  F  P  P  Q  F  I  G  S     359
1261  ATGTACATGTTTCCTTTGCTGTATGCACTTTTCCAGTCTGCAGAAGCGGGGATTTTTGTT
        M  Y  M  F  P  L  L  Y  A  L  F  Q  S  A  E  A  G  I  F  V     379
1321  TTAATCTATAAAATGTATGGAAGTGAAATGTTGCACAAGCGAGATCCTCTAGATGAAGAT
        L  I  Y  K  M  Y  G  S  E  M  L  H  K  R  D  P  L  D  E  D     399
1381  GAAGATACAGATATTTCTTATAAAAAACTAAAAGAAGAGGAAATGGCAGACACTTCCTAT
        E  D  T  D  I  S  Y  K  K  L  K  E  E  E  M  A  D  T  S  Y     419
1441  GGCACAGTGAAAGCAGAAAATATAATAATGATGGAAACCGCTCAGACTTCTCTCTAAATG
        G  T  V  K  A  E  N  I  I  M  M  E  T  A  Q  T  S  L  *
1501  TGGAGATACACAGGAGCTTCTATCTTGCTGAAATATTGCTTCATATTTATAGCCTGTGGT
1561  AGTGCACATGGTTAACATAAAAGATAACACTGGTTCACATCATACATGTGACAATTCTGA
1621  TCTTTTTAAGGGTCACTGGGGTATTAACCAAACGTTGTCACAAATTCAAATCAATGCTGT
1681  TATATAATTTGCACCCGGAATGGCTTACGCGAAGACTGAATTCAAAGTGGGTTTTACGTT
1741  TTACCAGCCCAAAAAAAAAAAAAAAAT
```

**Figure 1** Analysis of the *SLC10A4* DNA and protein sequence. Nucleotide and deduced amino acid sequences of *SLC10A4* cDNA. The nucleotide sequence is numbered on the left and the amino acid sequence is numbered on the right. The 5' UTR encompasses nucleotides (1 to 183) and the initiator methionine lies in an appropriate Kozak sequence (underline). The transmembrane domain is highlighted in bold font. The location of the stop codon is indicated by the asterisk.

### *Tissue distribution of SLC10A4 mRNA*

The *SLC10A4* transcript was detected by Northern blot analysis in a wide variety of human tissues (Figure 4A). The highest *SLC10A4* mRNA expression was observed in brain, placenta, and pancreas. Lower levels of expression were also observed in liver and kidney. While the major *SLC10A4* transcript is approximately 2.4 kb, an additional prominent 0.7 kb transcript was observed in pancreas, and a minor 1.4 kb transcript was observed in liver and kidney. β-actin expression appears to be comparable in each tissue suggesting equal loading of mRNA (Figure 4B).

### *Analysis of SLC10A4-transfected CHO cells*

CHO cells lacking endogenous expression of *SLC10A4* were stably transfected with the human *SLC10A4* cDNA. Figure 5A shows that the pcDNA V5-HisA (*SLC10A4*) transcript is expressed in the stably transfected CHO cells. In contrast, the *SLC10A4* mRNA was not detected in non-transfected CHO cells or in CHO cells transfected with the expression vector (pcDNA4 V5-HisA) alone.

### *Subcellular localization of SLC10A4-V5 in transfected CHO cells by immunofluorescent staining*

To address the cellular localization of this novel protein, transfected cells were stained using an affinity-purified mouse monoclonal antibody raised against the V5 epitope tag. We observed strong staining for *SLC10A4* in the transfected CHO cells, confirming its expression (Figure 5B). The transfected V5-epitope tagged *SLC10A4* protein was expressed primarily in intracellular compartments and to a lesser degree on the plasma membrane.

### *Immunoblot analysis of the SLC10A4-V5 protein*

Protein extracts from untransfected CHO cells, empty vector transfected CHO cells, and *SLC10A4*-transfected CHO cells were fractionated into mixed plasma membranes and an intracellular fraction and used for immunoblotting analysis. This analysis detected a 49-kDa protein and larger apparent aggregate, possibly a glycosylated form of this protein, in the *SLC10A4*-transfected cells, whereas no protein was detected in the non-transfected or empty

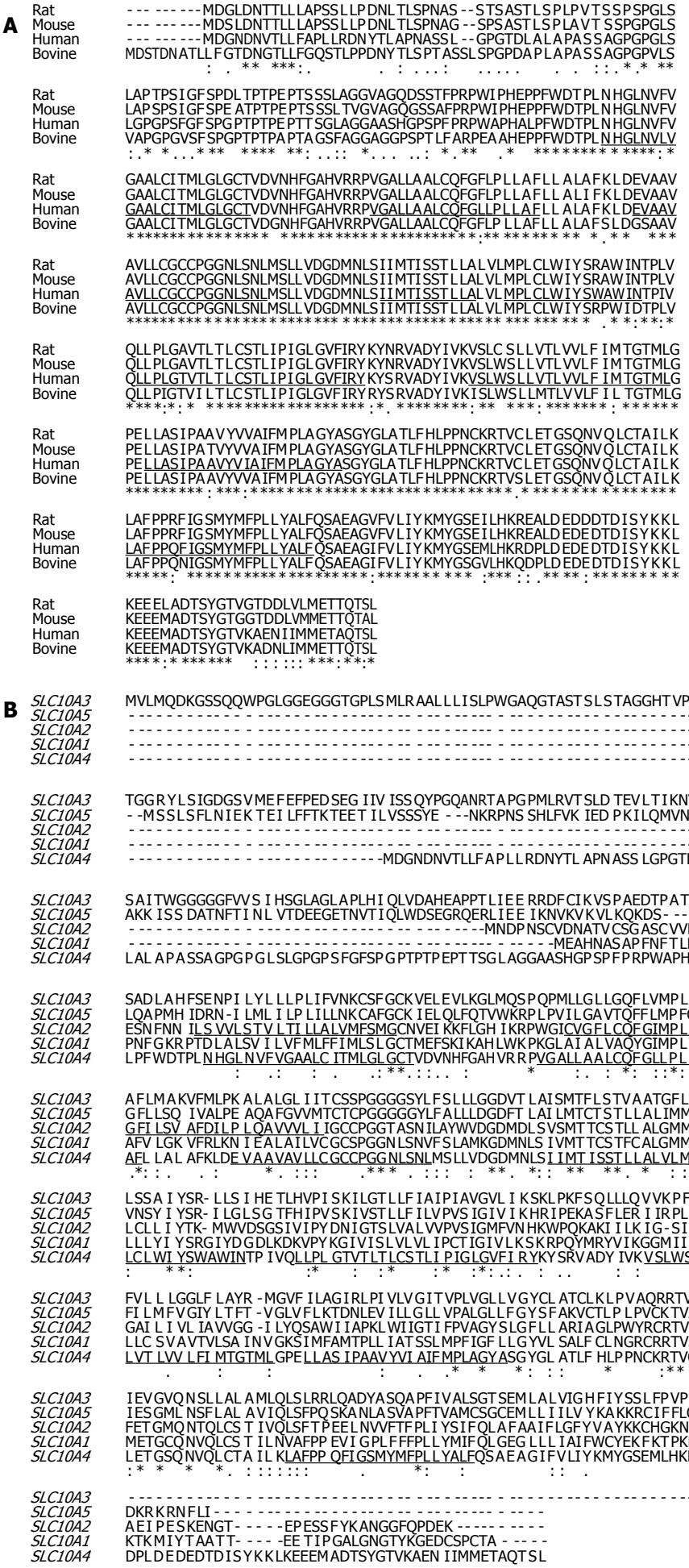


**Figure 2** Multiple alignment of the deduced amino acid sequence for SLC10A4 with sequences from rat (XP_579196), mouse (XP_775579), and bovine (XP_591836) and sequences with other SLC10A4 family members (**A**). The deduced amino acid sequence of *SLC10A4* was aligned with human *SLC10A3* (P09131), human *SLC10A5* (XP_376781), human *SLC10A2* (NP_000443) and *SLC10A1* (AAH74724)(**B**). The SLC10A4 putative transmembrane domains are underlined.

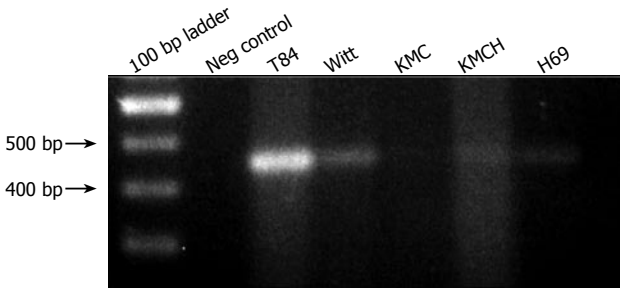


**Figure 3** Expression of *SLC10A4* mRNA in cultured cholangiocyte. Positive bands were detected in normal human cholangiocytes (H69) cells, in cholangiocarcinoma cell-lines (Witt, KMC, and KMCH) and the positive control (T84 cells). No bands were detected in the negative control (i.e., no template cDNA).

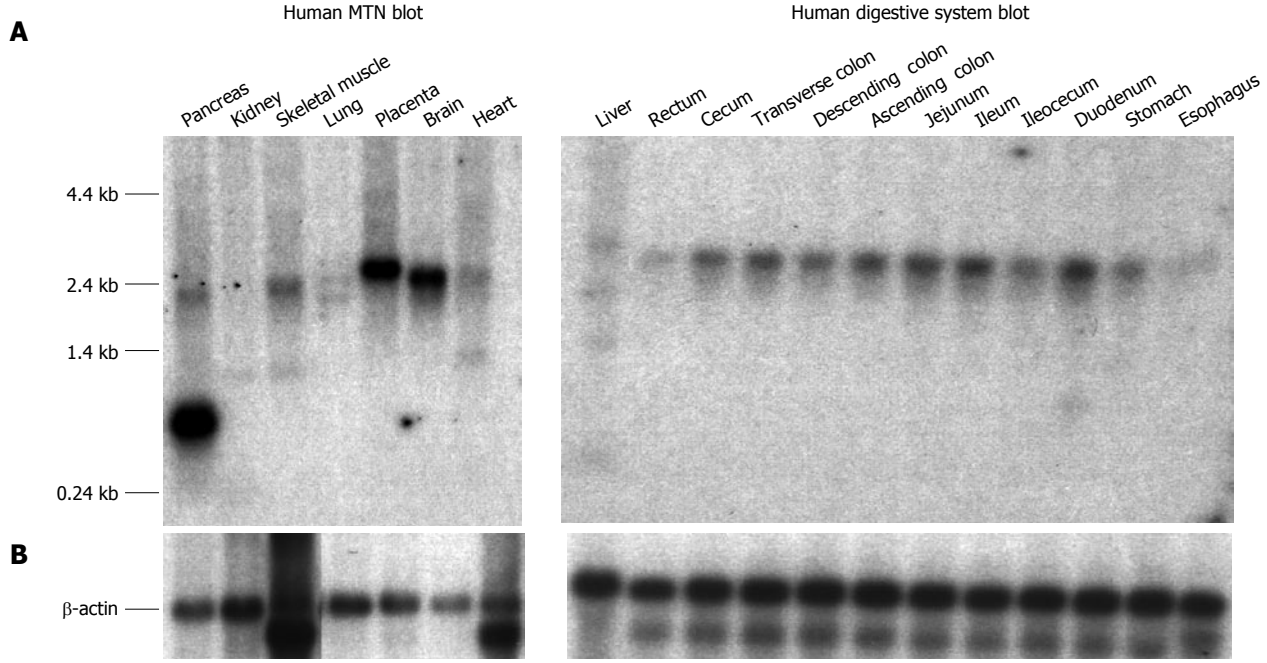


**Figure 4** Northern blot analysis of *SLC10A4* mRNA expression in human tissues. (**A**) The Human MTN and Human Digestive System blots contain 2 μg and 1 μg of poly A+ mRNA per lane, respectively. Each blot was hybridized with a $^{32}$P-labeled *SLC10A4* specific probe. (**B**) Hybridization using a $^{32}$P-labeled β-actin specific probe to confirm equal loading of mRNA.

vector-transfected CHO cells (Figure 5C). The 49-kDa band was detected in the lysate, plasma membrane and vesicles of the *SLC10A4*-transfected CHO cells.

### *Taurocholate functional uptake study*

To examine the possible functional capability of *SLC10A4*, we performed taurocholate uptake studies using stably transfected CHO cells (Figure 6). In functional uptake the positive control, namely, CHO cells stably transfected with *SLC10A1*, exhibited an approximate seven fold increase in taurocholate uptake compared to untransfected and empty vector transfected cells in the presence of sodium (Figure 6). In the same experiment, *SLC10A4*-transfected cells showed no significant increase in taurocholate uptake when compared to the untransfected cells (Figure 6). In the absence of sodium, neither transporter (i.e. *SLC10A1* or *SLC10A4*) showed a significant bile acid uptake when compared to control cells (Figure 6).

## DISCUSSION

The major findings described here are: (a) *SLC10A4* shares amino identity with other members of the sodium bile acid symporter family (*SLC10A2*, *SLC10A1*, *SLC10A3*, and *SLC10A5*); (b) the *SLC10A4* mRNA is widely expressed in human tissues, including cultured human cholangiocytes; and (c) the *SLC10A4* protein is expressed in intracellular compartments as well as on plasma membrane in transfected CHO cells.

As a result of the recent sequencing of the human genome, many novel genes have been identified through the cloning of ESTs (i.e., G protein coupled receptors)[17-20]. We utilized a bioinformatic approach to identify a novel SLC10A family member, termed *SLC10A4*. Prior to the completion of this work, two cDNA clones (accession no. AAH 12048 and AAH19066) have been sequence and listed in the NCBI database as SLC10a4 derived from neuroblastoma brain tissue. To date, only minimal bioinformatics observations have been made regarding *SLC10A4*[21]; however to our knowledge this is the first report to have experimentally analyzed *SLC10A4* mRNA distribution in various tissues, including cholangiocytes. Furthermore we have expressed the *SLC10A4* protein in a CHO cell-line to determine its subcellular localization.

The *SLC10A4* amino acid sequence is conserved amongst various mammalian species. This suggests that this protein may have an important role in substrate transport within various mammal species. *SLC10A4* protein

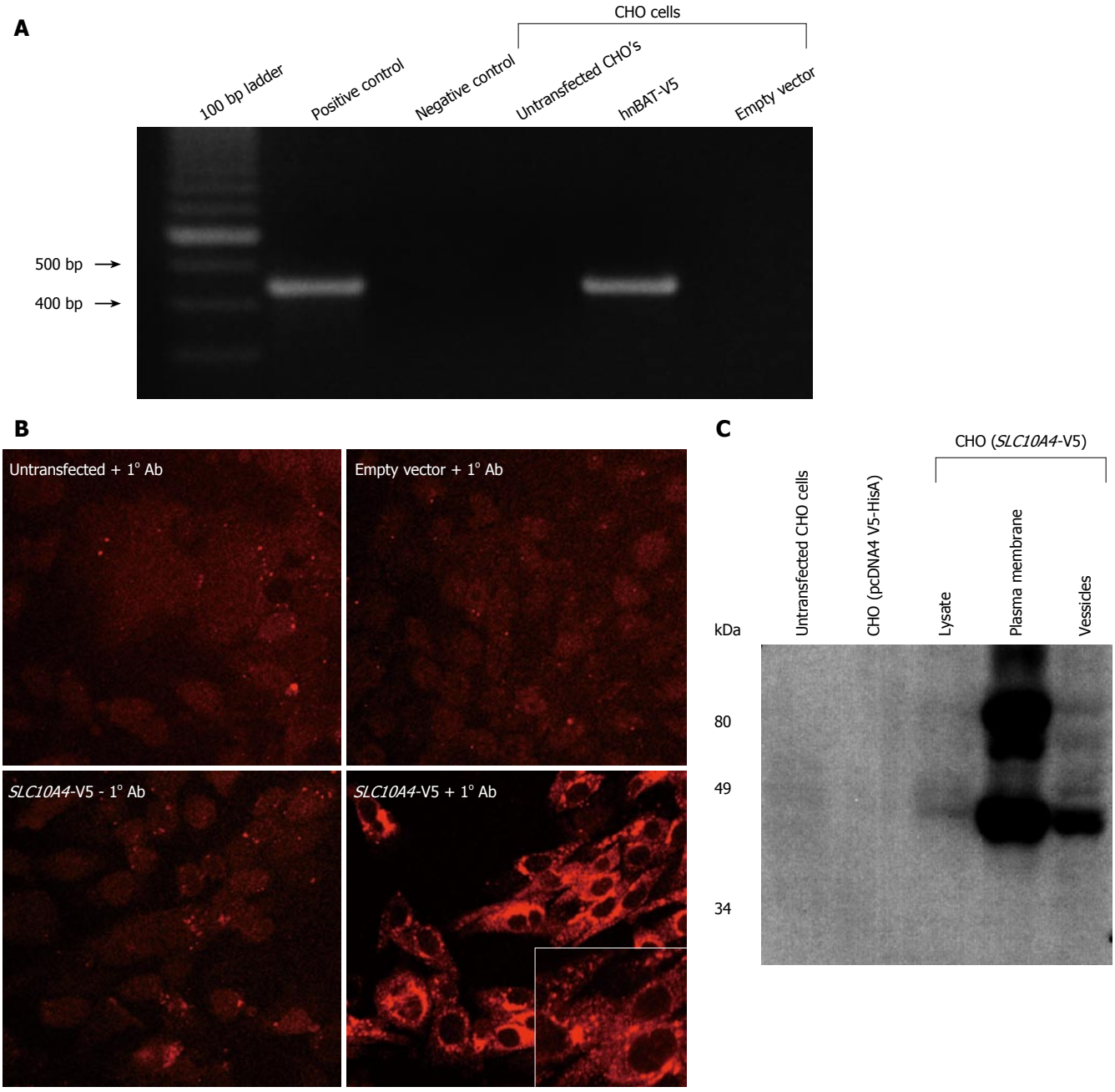


**Figure 5** Analysis of the *SLC10A4* stably transfected CHO cells. (**A**) Expression of *SLC10A4* mRNA in transfected CHO cells. *SLC10A4* mRNA was detected by RT-PCR in the *SLC10A4* transfected cells but not in untransfected CHO cells or the negative control (i.e., no template cDNA). (**B**) Protein expression of *SLC10A4* in stably transfected CHO cells. No staining was found in untransfected CHO cells or CHO cells stably transfected with the empty vector (pcDNA4 V5-HisA) alone in the presence of the affinity-purified monoclonal V5 antibody, or in the CHO cells transfected with *SLC10A4* in the absence of the primary antibody. Robust staining was detected in CHO cells transfected with *SLC10A4* in the presence of the V5 antibody (× 40). (**C**) Immunoblotting analysis of *SLC10A4* in transfected CHO cells. *SLC10A4* is detected in the cell lysate (20 μg), mixed plasma membranes (40 μg) and vesicle (40 μg) fractions of the cells. No bands were seen in cell lysates (40 μg) from untransfected CHO cells or empty vector (pcDNA4 V5-HisA) stably transfected CHO cells.

has a long extracellular amino terminus (shared amongst species) relative to that of *SLC10A1* and *SLC10A2* which may suggest a unique physiological process and role that this protein may have.

*SLC10A4* shares many characteristics of the human sodium dependent bile acid symporter family members. The SLC10 family is composed of five family members that show approximately 30% identity at the amino acid level. Within the SLC10 family, *SLC10A1* and *SLC10A2* share the greatest amino acid identity (about 36%) and are the only proteins shown to transport bile acids. From an evolutionary perspective, *SLC10A4* appears to be an intermediate between *SLC10A1* and the uncharacterized *SLC10A5* protein. To this end, the exon structure of the sodium dependent bile acid symporter family is of interest. *SLC10A5* has 1 predicted exon; *SLC10A4* and *SLC10A3* are composed of 3 predicted exons, *SLC10A1* is encoded by 5 exons, and *SLC10A2* is encoded by 6 exons. The simpler genomic organization of *SLC10A4*, *SLC10A5* and *SLC10A3* allows us to hypothesize that the genes may be ancesteral in nature. SCL10A4 is composed of 437 amino acids compared to *SLC10A1* and *SLC10A2* that are made of 349 and 348 amino acids, respectively. Furthermore, *SLC10A1* and *SLC10A2* are glycosylated and *SLC10A4*

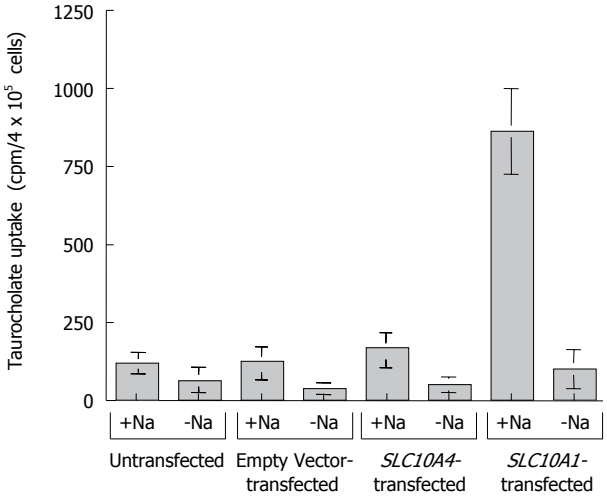


**Figure 6** Taurocholate uptake studies by *SLC10A4*. The cells were exposed to 200 μmmol/L of taurocholate (a mixture of [$^3$H]taurocholate and unlabeled taurocholate) in the presence or absence of $Na^+$. After 5 min at 37℃, the cells were washed with ice-cold wash solution, lysed, and the amount of [$^3$H] taurocholate was measured. The results are expressed as mean ± SE ($n$ = 3).

is predicted to have numerous glycosylation sites on the amino terminus. The predicted size of *SLC10A4* is slightly smaller than the experimental molecular weight, which may be due to addition of the V5 epitope tag on its carboxyl terminus. Additionally, the immunoblot shows a larger band of about 80 kDa that is possibly due to the glycosylation of the native protein, which is expected to occur based on the bioinformatics predictions. In contrast to *SLC10A1* and *SLC10A2*, the hydropathy plot for *SLC10A4* suggests eight potential transmembrane-spanning domains whereas the more characterized models of *SLC10A1* and *SLC10A2* have only seven again suggesting that this protein may have diverged from the well studied *SLC10A1* and *SLC10A2* genes. This discrepancy will require further experimental analysis to determine the actual number of transmembrane domains that reside in *SLC10A4*. The major sodium bile acid symporters involved in the secretion and absorption of bile acids are *SLC10A1* and *SLC10A2*. *SLC10A1* is primarily found in the liver where it is localized in the hepatocytes[6]. Within the hepatocytes, the *SLC10A1* protein resides on the sinusoidal membrane[6]. In contrast, *SLC10A2* mRNA has been shown in the cholangiocytes, ileal enterocytes, and in the proximal tubular cells of the kidneys[11,15,22,23]. *SLC10A2* is an apical oriented protein in the afore mentioned cells. Both *SLC10A1* and *SLC10A2* have been shown to transport various bile acids in a sodium dependent manner[6,15,22,23]. Much less is known about the remaining members of the SLC10 family of sodium bile acid symporters. *SLC10A3* is ubiquitously expressed in tissues and has been localized to various ESTs including placenta[21] (GenBank accession no. BX377672, brain (GenBank accession no. BM559383), lung (GenBank accession No. BM981524), kidney (GenBank accession no. BG249893) and stomach (GenBank accession no. BM747336). Far less is known for *SLC10A5* mRNA tissue distribution that has been limited to fetal brain (GenBank accession no. XM_294493)[21]. *SLC10A4* is also ubiquitously expressed in all tissues we have tested. Interestingly, *SLC10A4* mRNA shows slight difference in mRNA size amongst tissue by Northern blotting suggesting that this may be the result of tissue specific alternative transcription.

To our knowledge, the protein localization and substrate specificity have not been determined for either *SLC10A3* or *SLC10A5*, and these genes remain orphan transport proteins. In CHO cells expressing *SLC10A4*, we were not able to show transport of taurocholate, suggesting that *SLC10A4* is also orphan transport protein.

We have found that the *SLC10A4* mRNA is widely expressed in human tissues and is comparable to distribution pattern of *SLC10A3*. In fact, both *SLC10A3* and *SLC10A4* mRNA are expressed in similar tissues, suggesting both proteins may serve a ubiquitous function in the cell machinery. To define the localization of *SLC10A4* in CHO cells we created a chimeric protein in which the coding sequence of *SLC10A4* was cloned in frame with the V5-epitope tag. This construct was stably transfected in CHO cells to characterize the *SLC10A4* protein. We found that the novel protein localizes to both intracellular and plasma membrane of CHO cells. The intracellular localization infers that the protein is a regulated protein by some intracellular messenger. It has been shown the *SLC10A1*-GFP is regulated by cAMP when this construct was transfected into HepG2 cells[24]. It is possible that *SLC10A4* is regulated in a similar manner as *SLC10A1*. *SLC10A4* may be a regulated protein that requires an agonist to translocate the protein to the plasma membrane.

The physiological relevance of this novel transporter is currently unclear. Although the protein does not transport taurocholate and chenodeoxycholic acid, we cannot rule the transport capacity of other bile acids or other polar solutes. Nevertheless, *SLC10A4* protein shares homology with *SLC10A1* and *SLC10A2*. We have cloned and characterized the human *SLC10A4* in hoped that they data will improve our understanding on the phylogeny, origin and evolution of the SLC10A family of transport proteins which may provide important insight into which protein regions are involved in substrate specificity.

## ACKNOWLEDGMENTS

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*BASIC RESEARCH*

# Dexamethasone mediates protection against acute pancreatitis *via* upregulation of pancreatitis-associated proteins

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## Abstract

**AIM:** To examine the influence of dexamethasone on pancreatitis-associated protein (PAP) gene expression using both *in vitro* and *in vivo* models of acute pancreatitis and to study how PAP gene expression correlates with severity of pancreatitis.

**METHODS:** *In vitro*, IL-6 stimulated pancreas acinar AR42J cells were cultured with increasing concentrations of dexamethasone and assayed for PAP expression (RT-PCR). *In vivo*, pancreatitis was induced in rats by retrograde injection of 40 g/L taurocholate into the pancreatic duct. Animals were pretreated with dexamethasone (2 mg/kg) daily or saline for 4 d. Pancreata and serum were harvested after 24 h and gene expression levels of PAP Ⅰ, Ⅱ and Ⅲ were measured by RT-PCR. Severity of pancreatitis was based on serum amylase, pancreatic wet weight, and histopathological score.

**RESULTS:** *In vitro*, dexamethasone and IL-6 induced a marked transcription of PAP Ⅰ, Ⅱ and Ⅲ genes in AR42J cells at 24 h ($P < 0.05$ for all comparisons). *In vivo*, pancreas mRNA levels of PAP Ⅰ, Ⅱ or Ⅲ increased by 2.6-fold, 1.9-fold, and 1.3-fold respectively after dexamethasone treatment, compared with saline treated animals. Serum amylase levels and edema were significantly lower in the dexamethasone group compared with the saline group. Histopathologic evaluation revealed less inflammation and necrosis in pancreata obtained from dexamethasone treated animals ($P < 0.05$).

**CONCLUSION:** Dexamethasone significantly decreases the severity of pancreatitis. The protective mechanism of dexamethasone may be *via* upregulating PAP gene expression during injury.



**Key words:** Pancreatitis; Pancreatitis-associated protein; Pancreatitis-associated protein; Dexamethasone

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## INTRODUCTION

Acute pancreatitis is an acute inflammatory response to pancreatic injury and induces important changes in the expression of a number of genes in the pancreas[1,2]. Among these, the most profound change is that of the pancreatitis-associated protein (PAP) family, the expression of which is very low in the normal pancreas and becomes strongly overexpressed after even mild pancreatic inflammation[1,3-5]. Multiple functions have been ascribed to PAP. It has been shown to be antibacterial, anti-apoptotic, and mitogenic *in vitro*, and sequence analysis of PAP reveals the presence of a carbohydrate recognition domain in the protein, suggesting that PAP Ⅰ might act as a carbohydrate-binding lectin and can aggregate bacteria in suspension[6]. Therefore, PAP may function as an endogenous anti-bacterial agent and be protective against infectious complications of pancreatitis, which can otherwise lead to severe disease with a high mortality. In addition, PAP expression is upregulated by free radicals or cytokines, and such upregulation confers cellular resistance to apoptosis[7]. Previous results from our laboratory also showed that reg Ⅲ (PAP) isolated from cow is mitogenic for pancreatic-derived cells, thus implicating PAP in the proliferative response of pancreas to injury[8]. An anti-inflammatory effect of PAP has been found which protects the lung from leukocyte-induced injury[9]. In addition, we recently demonstrated that antisense knockdown of PAP gene expression exacerbates the severity of pancreatitis, suggesting a protective function of PAP in acute pancreatitis[10].

Pancreatitis is an important clinical problem, for which

**Table 1 Taqman primers and probes for real-time quantitative PCR**

| Gene (Accession No.) | Primer/Probe sequences (Forward/Reverse/Probe) | Position (nt No.) | Size (bp) |
|---|---|---|---|
| PAP Ⅰ | F 5'-AAAATACCCTCTGCACGCATTAG-3' | 153-171 | 67 |
| (NM_053289) | R 5'-GGGCATAGCAGTAGGAGCCATA-3' | 219-198 | |
| | P 5'-FAM-TGCCCCAAAGGCTCCCAGGC-TAMRA-3' | 177-196 | |
| PAP Ⅱ | F 5'-CCAGAAGGCAGTGCCCTCTA-3' | 240-259 | 67 |
| (L10229) | R 5'-GCAGTAAGAACGATAAGCCTTGGA-3' | 306-283 | |
| | P 5'-FAM-ACGAACCAGCTGCCCCATGG-TAMRA-3' | 261-280 | |
| PAP Ⅲ | F 5'-TGTGCCCACTTCACGTATCAG-3' | 121-140 | 64 |
| (L_20869) | R 5'-GGCATAGCAATAGGAGCCATAGG-3' | 184-162 | |
| | P 5'-FAM-TGCCCCAAGGGCTCGCG-TAMRA-3 | 143-159 | |
| β-actin | F 5′-TTCAACACCCCAGCCATGT-3′ | 379-397 | 68 |
| | R 5′-GTGGTACGACCAGAGGCATAC A-3′ | 446-425 | |
| | P 5′- FAM-CGTAGCCATCCAGGCTGTGTTGTCC-MGBNFQ-3′ | 399-422 | |

treatment remains largely supportive. Corticosteroids have been shown to be beneficial in treatment of acute pancreatitis[11-19]. Although it is thought that steroid administration exerts an anti-inflammatory effect of the inflamed pancreas, the mechanism of how this occurs remains unclear. We postulate that during acute pancreatitis, the pancreas turns on a defense mechanism that includes expression of PAP and other stress proteins that enable the survival of pancreas under conditions of acute stress and that corticosteroid treatment augments the PAP response.

In the present study, the influences of dexamethasone on PAP Ⅰ, Ⅱ and Ⅲ gene expression using both *in vitro* cellular analysis and an *in vivo* model of acute pancreatitis were examined and correlated with severity of pancreatitis. It is our hypothesis that dexamethasone mediates protection against acute pancreatitis *via* PAP gene induction.

## MATERIALS AND METHODS

### Materials

IL-6 (1-10 MU/L) stimulated pancreas acinar AR42J cells were cultured with dexamethasone (100 nmol/L) and assayed for PAP expression. Sprague Dawley rats obtained from Harlan Sprague Dawley (Indianapolis, IN) and weighing 175-200 g at onset of studies served as subjects. They were fed standard laboratory chow, given water *ad libitum*, and randomly assigned to control or experimental groups.

### Pancreatitis induction and dexamethasone treatment

Pancreatitis was induced in rats by retrograde injection of 40 g/L sodium taurocholate (NaT) (Sigma, St. Louis, MO) into the pancreatic duct as previously described[20-21]. Briefly, under pentobarbital (Abbott Laboratories, North Chicago, IL) anesthesia (50 mg/kg ip), a midline incision was performed. The common bile duct was identified and cannulated in an antegrade direction with PE-10 tubing (Fisher Scientific, Pittsburgh, PA) so that the proximal end of the tube was beyond the ampulla of Vater in the duodenum. The bile duct was then ligated to prevent the flow of bile and 40 g/L NaT in sterile saline was infused into the pancreatic duct at a rate of 1 mL/kg over 10 min[22]. Animals were pretreated with daily intraperitoneal injection of dexamethasone (2 mg/kg) or saline for 4 d prior to pancreatitis induction ($n$ = 8 per group). Pancreata and serum were harvested 24 h after pancreatitis induction. Severity of pancreatitis was based on serum amylase, pancreatic wet weight, and histopathological score. All animal studies have been approved by the Division of Animal and Laboratory Resources, SUNY Downstate Medical Center.

### Biochemical analysis and evaluation of pancreatic morphology

Serum amylase activity (U/L) was measured using 4,6-ethylidene ($G_7$)-*p*-nitrophenyl ($G_1$)-$\alpha_1$D-maltoheptaoside as the substrate[23]. The extent of pancreas edema was quantitated by the ratio of pancreas wet weight over rat's total (mg/g) body weight[21]. For morphologic analysis, 5-μm-thick paraffin sections of pancreas samples were stained with HE. Ten randomly chosen microscopic fields were examined for each tissue sample, and inflammation as well as necrosis, were scored as follows: none = 0; mild = 1; moderate = 2; and severe = 3[24].

### Analysis of PAP gene expression

PAP Ⅰ, Ⅱ and Ⅲ levels were measured, *in vitro* and *in vivo*, by real-time quantitative reverse transcription-polymerase chain reaction (RT-PCR), based on the fluorogenic 5'-nuclease assays previously described[25,26]. The assay, which confers very high specificity, was carried out using a GeneAmp 5700 sequence-detection system (Applied Biosystems, Foster City, CA), with β-actin as an internal control to standardize the amount of sample RNA added to a reaction. Primers and probes were designed using Primer Express software (PE Biosystems). Sequences for all primers and probes used in these analyses are listed in Table 1.

All primers and probes and other reagents for real-time quantitative PCR were purchased from Applied Biosystems. One hundred ng of total RNA was used to set up 25-μL real-time quantitative PCRs that consisted of 1 X TaqMan Universal PCR Master Mix, 500 nmol/L forward and reverse primers, and 200 nmol/L TaqMan probe. PCR amplification was carried out with the following temperature profile: 30 min at 48℃; 10 min at 95℃;

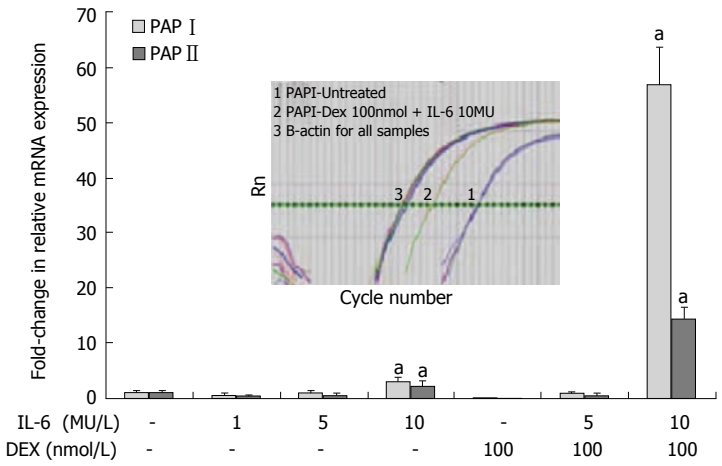


**Figure 1** PAP expression was upregulated by treatment of dexamethasone and IL-6. AR42J cells were treated with indicated dose of dexamethasone (Dex) and IL-6. PAP Ⅰ and Ⅱ mRNA levels were determined by real-time quantitative RT-PCR analysis, using PAP Ⅰ, or Ⅱ-specific TaqMan primers and probe. Amount of mRNA in each group was normalized to β-actin. Results are presented as fold change in PAP Ⅰ or Ⅱ mRNA in treated cells relative to the levels observed in untreated/control cells and similar results were obtained with PAPⅢ (data not shown). Bars represent mean ± SD from 3 independent experiments ($^aP < 0.05$ *vs* untreated/control cells, ANOVA, Tukey). Insert shows representative amplification plots.

and 40 cycles of 15 s at 95℃ and 1 min at 60℃. Assays were performed in triplicate. Data were analyzed with the relative standard curve method[27]. Standard curves of the genes of interest and β-actin were prepared with three 1:2 dilutions (four points, eightfold range) of total RNA from one of the samples that was expected to have the highest amount of mRNA for the gene of interest. For each reaction tube, the amount of target or internal reference was determined from the standard curves. The mean amount of each sample was calculated from the triplicate data and was normalized by division by the mean quantity of β-actin RNA for the same sample. The mean and SD of each treated group were calculated from the normalized value for each rat in that group.

### *Statistical analysis*

Values for results were expressed as means ± SD obtained from multiple determinations in 3 or more separate experiments. *P* values computed were two-tailed, and $P < 0.05$ was considered statistically significant (Student's *t*-test, ANOVA with Tukey post hoc correction).

## RESULTS

When AR42J cells which were cultured in the presence of IL-6 were exposed to increasing amounts of dexamethasone, increased gene expression of PAP Ⅰ, Ⅱ, and Ⅲ was observed at 24 h ($P < 0.05$ when compared with controls) (Figures 1 and 2).

Rats which were treated with dexamethasone prior to pancreatitis induction demonstrated upregulation of pancreas mRNA levels of PAP Ⅰ, Ⅱ or Ⅲ when compared with saline treated controls (PAP Ⅰ: 2.6-fold, PAP Ⅱ: 1.9-fold and PAP Ⅲ: 1.3-fold respectively) ($P < 0.05$ for PAP Ⅰ and PAP Ⅱ) (Figure 3).

Furthermore, serum amylase levels and edema were significantly lower in the dexamethasone group compared with the saline group (Figure 4A and B) and histopathologic evaluation revealed less inflammation and necrosis in pancreata obtained from dexamethasone treated rats when compared with controls (Figure 4C) ($P < 0.05$).

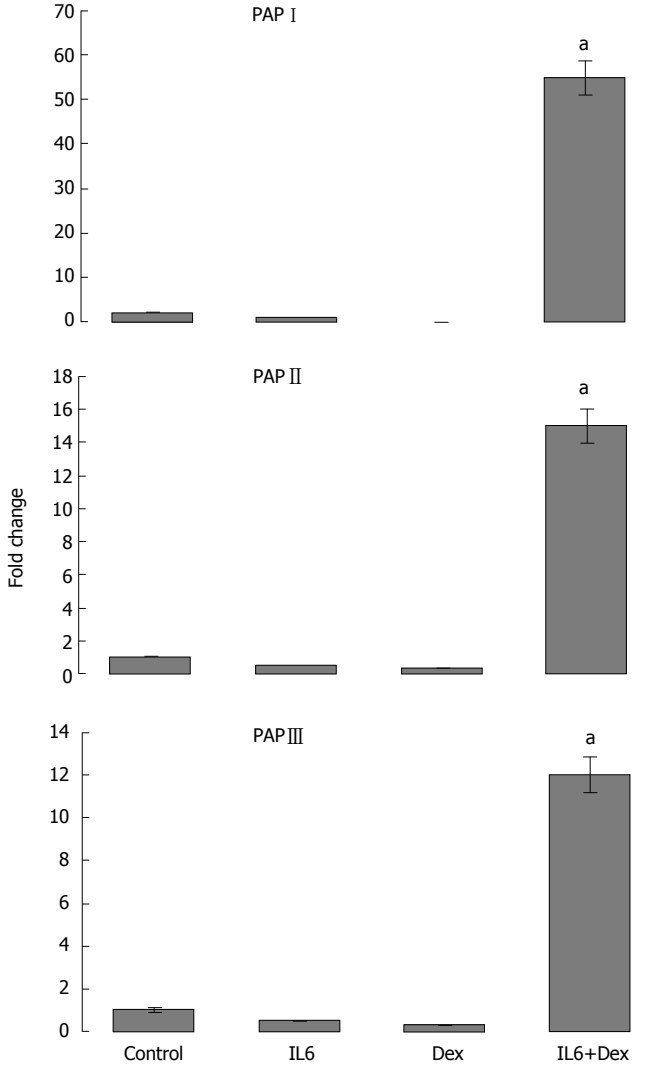


**Figure 2** PAP expression was upregulated by treatment of dexamethasone and IL-6: *In vitro*, dexamethasone (Dex) and IL-6 induced a marked transcription of PAP Ⅰ, Ⅱ and Ⅲ genes in AR42J cells at 24 h when compared with untreated (control) cells. Data represent fold change of untreated/unstimulated cells as determined by real-time PCR analysis (see materials and methods). Bars represent mean ± SD from 3 independent experiments ($^aP < 0.05$ *vs* controls, ANOVA, Tukey).

## DISCUSSION

In the current study, the influence of dexamethasone on PAP gene expression using both *in vitro* cellular analysis and an *in vivo* model of acute pancreatitis was investigated. Our results suggest that dexamethasone has an anti-

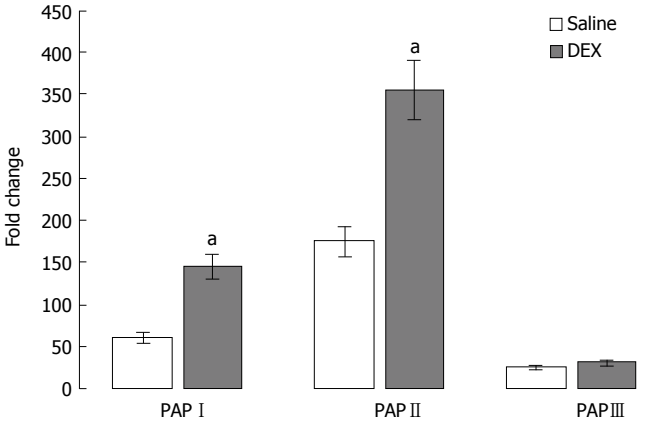


**Figure 3** PAP expression is upregulated by dexamethasone: Rats (8/group) were treated with daily saline (white bars) or dexamethasone (grey bars) prior to pancreatitis induction. Pancreata were harvested 24 h after pancreatitis induction and PAP levels were determined by real time PCR and presented as fold change (normalized to β-actin) as described in materials and methods, and described in text as a ratio of PAP mRNA from NaT treated/saline treated pancreas mRNA ($^aP < 0.05$ *vs* saline treated controls, Student's *t*-test).

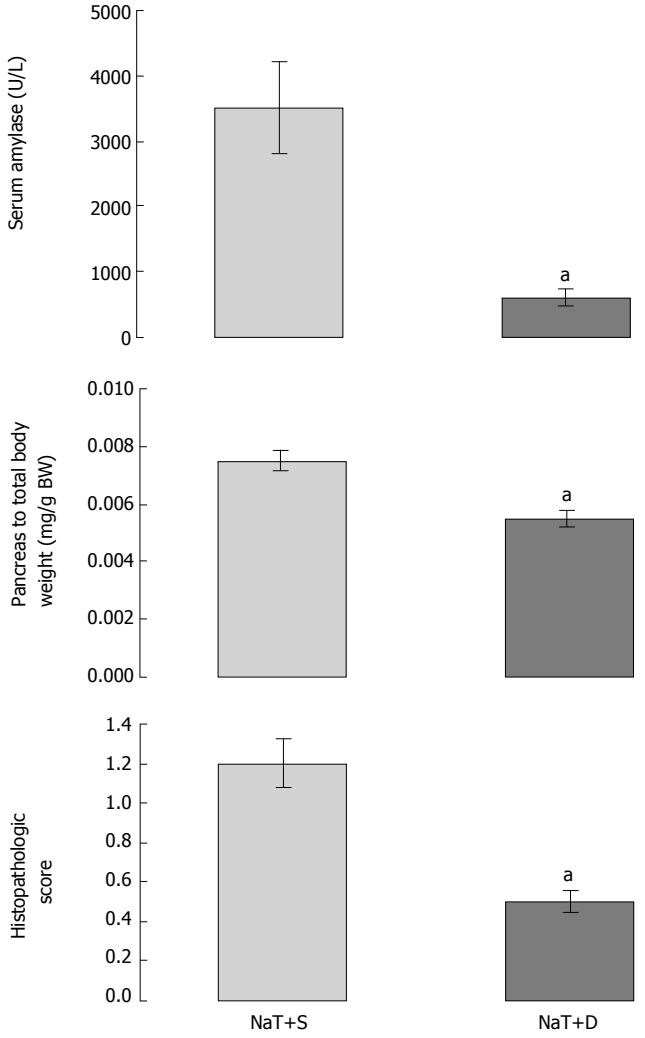


**Figure 4** Dexamethasone treatment decreased pancreatitis severity *in vivo*. Rats were treated with saline (S) or dexamethasone (D) for 4 d prior to pancreatitis induction with sodium taurocholate (NaT). Pancreata were harvested 24 h after pancreatitis induction and severity of pancreatitis was based on serum amylase (U/L), pancreatic wet weight (mg/g BW), and histopathological score. The bars represent the mean ± SD ($n = 8$, $^aP < 0.05$ *vs* the saline-treated control group).

inflammatory effect in acute pancreatitis *via* upregulating PAP gene expression, which is inversely correlated with local pancreatic inflammation such as pancreatic edema and neutrophilic infiltration. Additionally, recent data from our laboratory demonstrates a protective role for PAP at the protein level since administration of anti-PAP antibodies worsened pancreatitis severity *in vivo*[28,29]. PAP is a member of the family of secretory proteins expressed in the gastrointestinal tract and was originally isolated from the pancreatic juice of rats with acute pancreatitis[3]. Currently, three PAP genes have been characterized in human[5] and rat[6,30,31]. mRNA for PAP isoforms (PAP Ⅰ, Ⅱ, and Ⅲ) are expressed in the pancreas and small intestine among other tissues[30,32]. Results of PAP gene regulation studies suggest that PAP is an acute phase stress protein secreted from pancreas. PAP protein, which is not detectable in the healthy pancreas, is significantly expressed six hours after induction and reaches maximal expression after 48 h[21,32]. PAP accounts for about 5% of the secretory proteins during acute pancreatitis and can be detected in blood within 48 h of induction of acute pancreatitis[21]. We have previously reported a direct relationship between the severity of pancreatitis and serum levels of PAP[21]. We have also demonstrated that antisense mediated gene knockdown of PAP expression correlated with worsening pancreatitis *in vivo*[10]. We postulate that PAP proteins serve a protective role in acute pancreatitis and that the protective effect may be *via* the downregulation of acute phase cytokine gene expression in the peripheral immune system[33].

In the present study we showed that AR42J, a rat acinar cell line can be induced and used as a model to express all three PAP isoforms (PAP Ⅰ, Ⅱ and Ⅲ) when treated with combination of IL-6 with dexamethasone. Although previous *in vitro* studies revealed that induction of PAP Ⅰ gene expression could be obtained *via* dexamethasone treatment[34], here we demonstrate that all PAP isoforms are upregulated when cultured with increasing amount of dexamethasone and this effect is potentiated with addition of IL-6. Although many cytokines, including IL-6 are upregulated in many inflammatory states including pancreatitis[35-37], it is possible that IL-6, in conjunction with other mediators, contributes to PAP upregulation and disease resolution *in vivo*. This is likely since PAP/Reg genes possess IL-6 and glucocorticoid response elements[20,34]. We have previously demonstrated that antisense knockdown of PAP correlates with disease progression *in vivo*[10] and that more recently, plasmid and linear based siRNA gene knockdown of one PAP isoform impacts the expression of other PAP isoforms[38], suggesting that expression of PAP isoforms is contingent on one another rather than being redundant genes of common ancestry. Sequence comparisons of PAP isoforms demonstrate that they are closely related[39,40], although differing in their expression pattern and contain a concensus sequence coding for a bioactive protein component common to all three PAP isoforms (GGWEWSN)[41], which is able to knockdown gene expression of PAP Ⅰ, PAP Ⅱ, and PAP Ⅲ. It is our

hypothesis that this highly homologous sequence common to all three isoforms may harbor important genetic information and may encode a bioactive fragment common to all PAP isoforms. To this end, Bodeker and colleagues have shown that PAP Ⅰ interacts with PAP Ⅱ, PAP Ⅲ and lithostatin (RegIα) as well as itself to form homo/heterodimers[42], suggesting that PAP proteins may provide overlapping function for other members of the Reg protein family.

To evaluate the role of dexamethasone-induced upregulation of PAP expression in limiting the severity of pancreatitis, dexamethasone was administered before pancreatitis induction with NaT, an experimental model of necrotizing acute pancreatitis[43]. It is well known that corticosteroids are immunological moderators and influence a number of factors involved in the process of tissue inflammation and edema[11,44]. There is evidence that endogenous glucocorticoids may protect acinar cells in acute pancreatitis by decreasing their sensitivity to the induction of cell death[20]. The adrenocortical function is stimulated during acute pancreatitis and it has been suggested that the secretion of endogenous glucocorticoids may play an important role in mitigating the progress of this disease, probably by inhibiting cytokine production[45-48]. Additionally, hydrocortisone therapy was shown to be effective and beneficial at a dose of 4-25 mg/kg given 30 min before inducing acute pancreatitis, depending on the experimental system[14,44]. Furthermore, amelioration of acute pancreatitis by glucocorticoid treatment is related to the dose and time factor to achieve optimal therapeutic results[44]. The present study indicated that dexamethasone significantly improved both the local pancreatic inflammatory response as well as systemic inflammatory parameters and correlated with upregulation of PAP gene expression. Similarly, studies by Paszt *et al*[49], have also demonstrated a reduction in serum amylase and pancreatic weight/body weight ratios in pancreatitic rats after treatment with dexamethasone. The beneficial effects of dexamethasone treatment may be directly related to PAP upregulation, possibly through utilization of a glucocorticoid response element found in the PAP/Reg gene family[20]. PAP Ⅲ gene expression did not differ between dexamethasone and control groups at 24 h. It could be that PAP Ⅲ responds to corticosteroid treatment at earlier or later time points and may interact with other PAP isoforms and affect their expression, as has been observed with PAP Ⅰ[42].

In conclusion, the present study demonstrates a protective function of dexamethasone in acute pancreatitis which may be *via* upregulation of PAP gene expression during injury. In clinical practice, it is well known that the development of severe acute pancreatitis leads to multiple organ failure. Based on the present results, dexamethasone therapy may have the potential to help prevent the progression of acute pancreatitis. Dexamethasone treatment reduces edema, leukocyte infiltration and fat necrosis in the pancreas which is likely due to the upregulation of PAP. Future studies need to generate recombinant PAP and bioactive PAP peptides and explore their protective role against pancreatitis.

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Emad Kandil and Yin-Yao Lin contributed equally to this manuscript.

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*BASIC RESEARCH*

# Reduction of ischemia reperfusion injury after liver resection and hepatic inflow occlusion by α-lipoic acid in humans

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## Abstract

**AIM:** To evaluate the protective effects of preconditioning by α-lipoic acid (LA) in patients undergoing hepatic resection under inflow occlusion of the liver.

**METHODS:** Twenty-four patients undergoing liver resection for various reasons either received 600 mg LA or NaCl 15 min before transection performed under inflow occlusion of the liver. Blood samples and liver wedge biopsy samples were obtained after opening of the abdomen immediately after inflow occlusion of the liver, and 30 min after the end of inflow occlusion of the liver.

**RESULTS:** Serum levels of aspartate transferase and alanine transferase were reduced at all time points in patients who received LA in comparison to those who received NaCL. This was accompanied by reduced histomorphological features of oncosis. We observed TUNEL-positive hepatocytes in the livers of the untreated patients, especially after 30 min of ischemia. LA attenuated this increase of TUNEL-positive hepatocytes. Under preconditioning with LA, ATP content was significantly enhanced after 30 min of ischemia and after 30 min of reperfusion.

**CONCLUSION:** This is the first report on the potential for LA reducing ischemia/reperfusion injury (IRI) of the liver in humans who were undergoing liver surgery. Beside its simple and rapid application, side effects did not occur. LA might therefore represent a new strategy against hepatic IRI in humans.



**Key words:** Liver ischemia; Pringle manoeuvre; Pharmacological pre-treatment; Liver preconditioning; Apoptosis; Adenosine triphosphate

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## INTRODUCTION

Excessive blood loss during surgery and the need for transfusion have been shown to hinder the post-operative course of patients[1]. In order to minimize blood loss, clamping of the portal triad (inflow occlusion), also called pringle maneuvre, is used during liver surgery[2]. However, the risk of ischemia/reperfusion injury (IRI) of the liver is increased under these conditions[3].

Ischemia reperfusion injury of the liver is still a major cause of morbidity and mortality in patients undergoing liver surgery and transplantation[4]. A period of ischemia followed by a brief period of ischemia (ischemic preconditioning, IP) prior to sustained ischemia is a method to reduce IRI of the liver in animal models as well as in humans[5]. However, the protective effects are lost in older patients and in patients undergoing major tissue loss (> 50% of the liver)[6].

In animal models there are numerous pharmacological agents protecting the liver from IRI[7]. However, there is a lack of clinically proven strategies to pharmacologically reduce IRI of the liver in humans. We have previously described the potential of α-lipoic acid (LA) to protect from IRI of the isolated perfused rat liver, which could be confirmed *in vivo* for livers undergoing 90 min of warm ischemia[8].

α-Lipoic acid is a naturally occurring compound present in the majority of pro- and eukaryotic cells. For many years LA has been used as a pharmacological agent against diabetic polyneuropathy and is known to be without serious side effects[9]. Therefore, oral and intravenous application as well as the bioavailability of LA

is well established[10]. The aim of the present study was to evaluate the influence of LA in ischemia/reperfusion injury of the liver in patients who are undergoing liver resection.

## MATERIALS AND METHODS

### Experimental design

Informed consent was obtained from each patient before surgery. Patients scheduled to undergo liver resection were included. Characteristics of patients are shown in Table 1. Baseline wedge biopsy samples were taken from the part of the liver to be resected. Clamping of the portal triad was performed with the torniquet technique. Samples of liver tissue (wedge biopsy) obtained during surgery were immediately transferred into liquid nitrogen or stored in 4% paraformaldehyde. Separate clamping of aberrant left hepatic arteries was carefully performed when present. Transection of the liver was immediately performed after complete hepatic inflow occlusion; if necessary vessels and bile duct were ligated with 2-0 silk suture. After exactly 30 min, the portal clamping was released and hemostasis was obtained. Each surgery was performed with a low central venous pressure to minimize blood loss and was carried out by using the same anesthetic combinations. Surgery was performed by one surgeon.

Our protocol is based on the data we obtained in rodents, where an amount of 500 μmol (120 μg) LA strongly protected the liver against IRI after a prolonged period of ischemia[8]. Previous studies have shown that ischemic injury in humans becomes detectable after 30 min of ischemia[11]. Twenty-four patients were subjected either to preconditioning with 600 mg LA (in 50 mL NaCl) intravenously 15 min before inflow occlusion of the liver or to receive 50 mL NaCl intravenously 15 min before inflow occlusion of the liver. We performed ischemia 15 min after ending the LA injection because its bioavailability is sufficient 15 min after intravenous application[10].

In order to minimize bias, LA preconditioning was performed in an alternate fashion. Patients with cirrhosis, elevated liver enzymes as well as high operation risk were excluded. Blood samples and wedge biopsies were taken after opening the abdomen (LAP), after 30 min of ischemia (pP0´), and after 30 min of reperfusion (pP30`). Blood samples were taken every day until discharge. All blood samples were analyzed using a serum analyzer (Olympus AU Connector 640, Hamburg, Germany). Wedge biopsy samples were immediately stored in liquid nitrogen or fixed for histological examination. Liver tissue was weighed prior to homogenization and diluted 1:5 with 0.01 mmol/L PBS (phosphate buffered saline). Homogenization was then carried out with a Potter liver homogenizer at 800 revolutions/mine. The mixture was centrifuged at 1000 × *g* for 10 min, the supernatant was transferred into an Eppendorf cup and centrifuged at 10000 × *g* for 20 min. The clear supernatant was pipetted into a fresh Eppendorf cup, and stored until further measurement.

### ATP content in liver tissue

Measurement of adenosine triphosphate (ATP) in liver tissue was carried out using a standardized method[12]. The liver slices were weighed and diluted in 4 % $HClO_4$ (1:10) prior to homogenization with a Potter liver homogenizer at 850 revolutions/min. The mixture was centrifuged at 10000 × *g* for 5 min at 4℃. The supernatant was pipetted into an Eppendorf cup and neutralized with 150 μL 2 mol/L Tris, 150 μL KOH, and phenol-red. 5 mol/L KOH was then added until a blew-lilac complex was observed. The mixture was again centrifuged at 10000 U/min for 5 min at 4℃. The supernatant was measured immediately as described by Trautschold et al. with a hexokinase/glucose-6-phosphat dehydrogenase reaction by UV photometry[13].

**Table 1 Patients characteristics [mean (range)]**

| characteristics | Vehicle | LA |
|---|---|---|
| Age (yr) | 61 (34-79) | 63 (46-79) |
| Sex (M/F) | 5/7 | 6/6 |
| Resected liver tissue (g) | 434 (40-1100) | 588 (112-1365) |
| Blood loss (mL) | 1175 (400-2000) | 825 (200-2200) |
| RC-transfusion intraoperative (mL) | 300 (0-1000) | 265 (0-1000) |
| Intensive care unit (d) | 1 (0-7) | 2 (0-4) |
| Hospital stay (d) | 13 (6-21) | 14 (11-25) |
| Post-OP complications | 1/12 | 1/12 |
| Mortality | 1/12 | 0/12 |
| Indications, malignant | 11/12 | 11/12 |

### Histology and Immunhistochemistry

Formalin-fixed tissue samples were embedded in paraffin, and 5 μm sections were cut. Replicate sections were stained with hematoxylin and eosin (H&E) for the evaluation of oncosis. Morphological criteria of oncosis, such as increased swelling, vacuolization and blebbing were assessed. The terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay was employed for the evaluation of apoptotic related cell injury. For TUNEL staining, livers were fixed with freshly prepared 4% paraformaldehyde in neutral buffered saline (PBS). Frozen sections (5 μm) of the fixed tissue were prepared and stained with the TUNEL method using a commercially available kit (Chemicon International, ApopTag peroxidase apoptosis detection kit, Hampshire, UK). The number of TUNEL-positve hepatocytes was counted in 10 high power fields (400 fold magnification). All histological evaluations were done in a blinded fashion.

### Statistical analysis

All data are expressed as means ± standard deviation. Statistical differences between experimental groups were calculated by Graph Pad Prism version 4.00 for Windows, GraphPad Software, San Diego California USA 1994-1999, and $P < 0.05$ was considered significant. One-way analysis of variance (ANOVA) with subsequent post hoc Tukey-test was used.

## RESULTS

### Clinical characteristics

There was no statistical difference between the two groups indicating that they were homogeneous and comparable (Table 1, data were expressed with median values with ranges [minimum-maximum]). One patient in the untreated

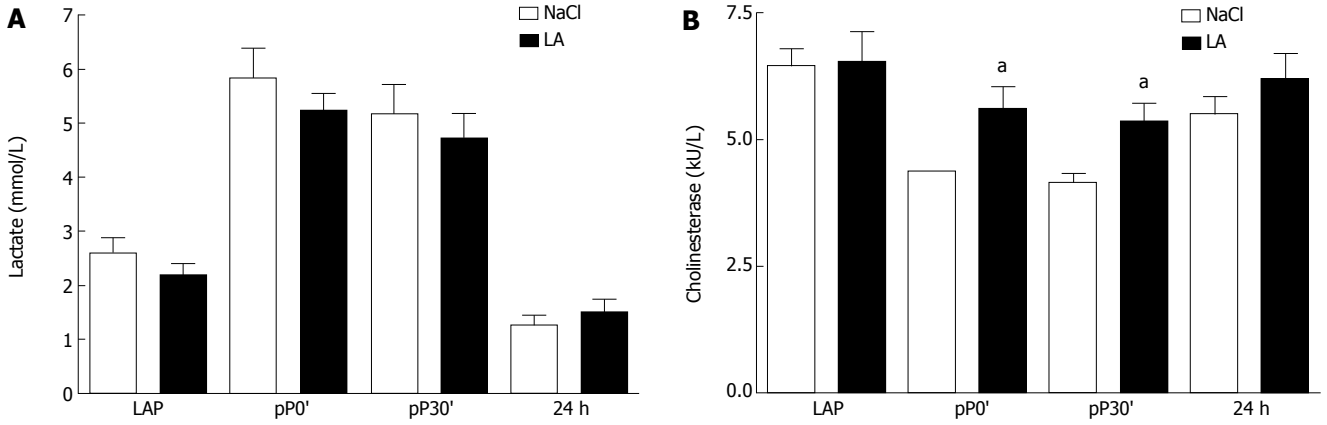


**Figure 1** Lactate (**A**) and cholinesterase (**B**) levels after laparotomy (LAP), 30 min of ischemia (pP0´), 30 min of ischemia with 30 min of reperfusion (pP30´) and 24 h after operation with and without 600 mg LA 15 min prior to ischemia, values as means ± SD, $n = 12$ each group, $^{a}P < 0.05$ *vs* NaCl.

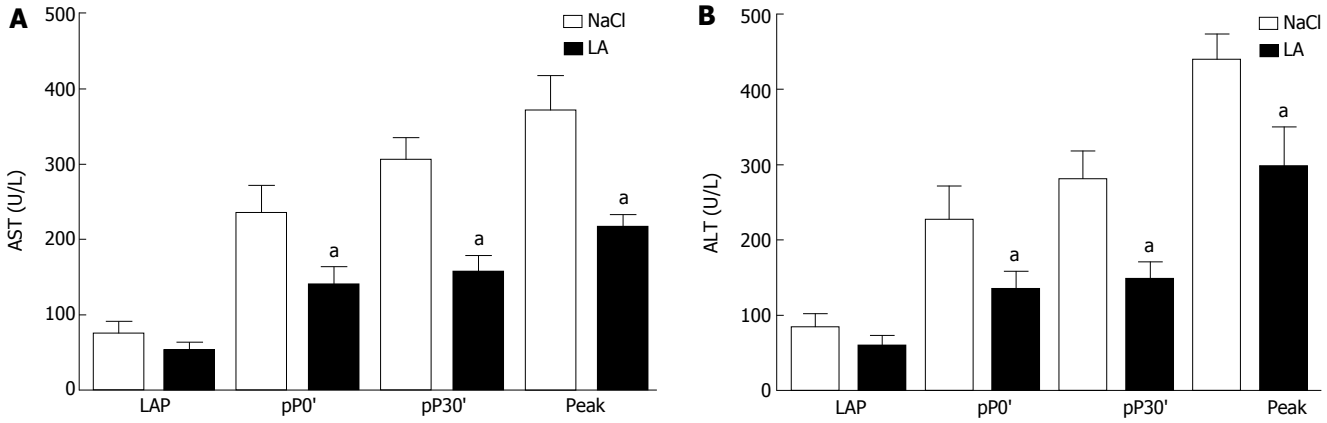


**Figure 2** AST (**A**) and ALT (**B**) levels after laparotomy (LAP), after 30 min of ischemia (pP0´), after 30 min of ischemia with 30 min of reperfusion (pP30´) and after d 1-3 (peak level) with and without 600 mg LA 15 min prior to ischemia, values as means ± SD, $n = 12$ each group, $^{a}P < 0.05$ *vs* NaCl.

group died because he suffered mesenteric ischemia at day 10 after liver resection. During revision of the abdomen we saw great areas of necrotic small intestine whereas the portal vein was not occluded. Operative morbidity was low in both groups. One patient in each group suffered bile leakage; both were cured by local drainage.

### *Lactate in serum*

To evaluate inflow occlusion in both groups we determined lactate levels in serum. We observed no difference of lactate between both groups directly after opening of the abdomen (LAP), after 30 min of ischemia, after 30 min of reperfusion, and 24 h after inflow occlusion (Figure 1A).

### *Cholinesterase in serum*

Cholinesterase (CHE) in serum was decreased in the untreated group at pP0´and pP30` compared to LAP while levels of CHE were significantly increased at both time points in LA-treated patients compared to controls (Figure 1B).

### *AST/ALT in serum*

After opening the abdomen AST levels in serum were low in both groups. After 30 min of ischemia, at 30 min of reperfusion, as well as at days 1-3 after the operation (peak level) the value increased in the untreated patients. In the LA-pretreated group we measured significantly lower levels of AST at each time point (Figure 2A). AST and ALT levels in both groups reached baseline values until discharge (Figure 2B). Total bilirubin (tBIL) and glucose (GC) did not differ from baseline levels neither in controls nor in LA-treated patients (data not shown).

### *ATP content in liver tissue*

After pretreatment by LA we observed significantly increased ATP content in post-ischemic liver tissue compared to the untreated group (Figure 3). During reperfusion ATP content was also increased in the LA group but did not reach statistical significance.

### *Histology/Immunhistochemistry*

Our histological findings strongly support our results regarding the enzyme release of AST and ALT. H&E-stained liver tissue of untreated patients clearly showed histomorphological features of oncocis (Figure 4). In contrast, in livers of the LA-pretreated patients features of oncocis were rare. We observed TUNEL-positive hepatocytes after 30 min of ischemia (pP0´) in the

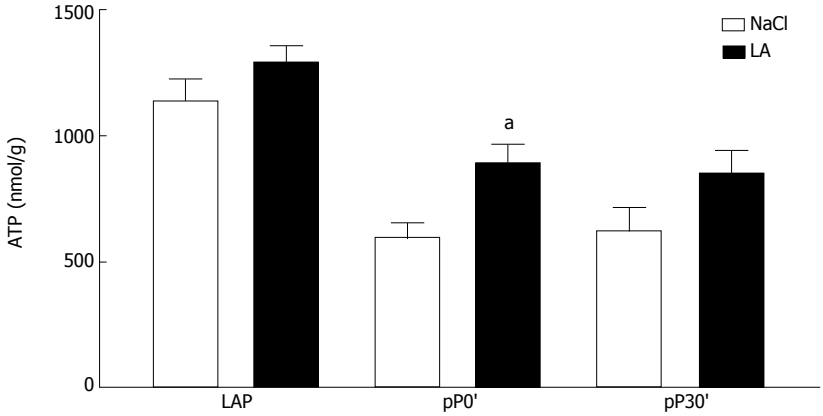


**Figure 3** ATP-content in liver tissue after laparotomy (LAP), after 30 min of ischemia (pP0´) and after 30 min of ischemia with 30 min of reperfusion (pP30´) with and without 600 mg LA 15 min prior to ischemia, values as means ± SD, $n$ = 12 each group, $^{a}P < 0.05$ *vs* NaCl.

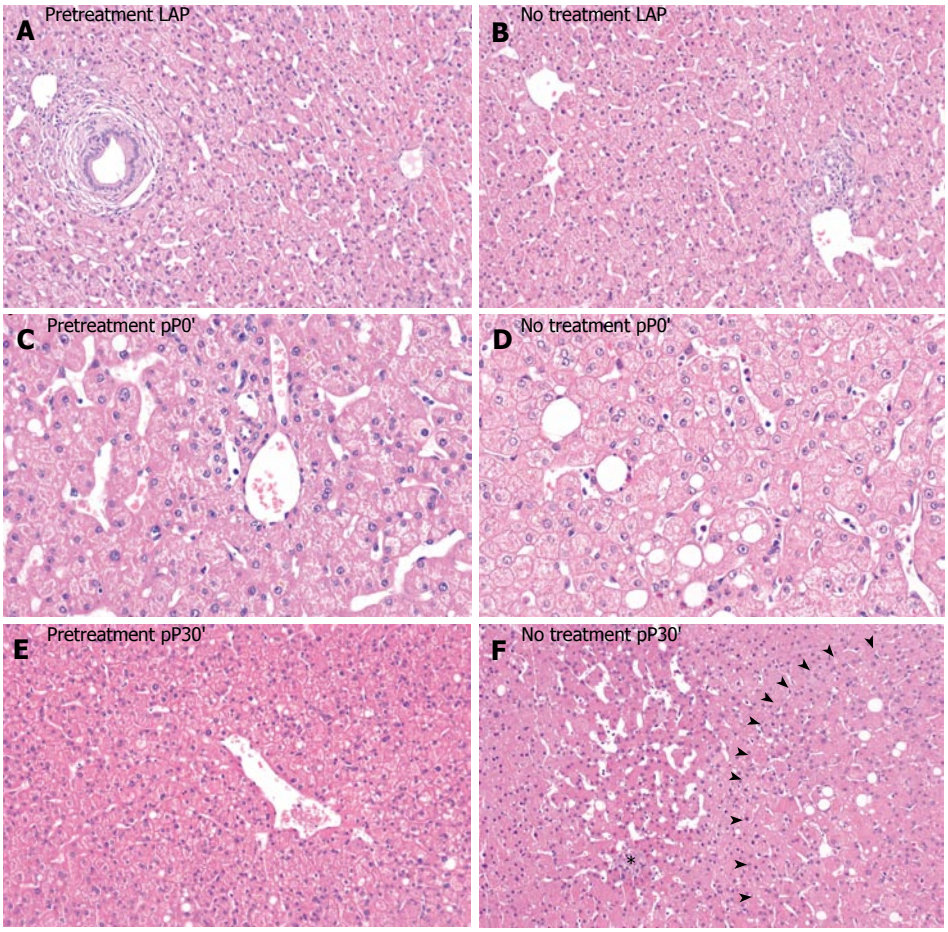


**Figure 4** Representative liver sections after laparotomy (LAP), after 30 min of ischemia (pP0') and after 30 min of ischemia with 30 min of reperfusion (pP30') with and without 600 mg LA 15 min prior to ischemia, (HE× 200-400). **A**, **B**: In both groups no features of cell injury after LAP; **C**, **D**: At pP0' there was a mild oncotic injury in the untreated and pretreated group such as hepatocytes swelling and vacuolization; **E**, **F**: At pP30' oncotic injury was increased especially in the untreated group (**F**): there were areas with focal necrosis (asterisk) and areas with eosinophilia and oncosis (arrows). In the LA pretreated group oncotic cell injury was rare (**E**).

untreated group as well as at pP30`. In the LA-pretreated group TUNEL-positive hepatocytes were significantly reduced (Figure 5).

## DISCUSSION

Our data strongly support the hypothesis that LA reduces hepatic IRI in humans after inflow occlusion and liver resection. The protection is facilitated by preservation of ATP levels in liver tissue and characterized by reduced apoptotic hepatocyte injury upon LA preconditioning.

LA is established in the therapy of diabetic polyneuropathy[14]. It is a safe substance without serious side effects. Although LA is known to increase blood sugar utilization[12] we did not observe any changes in blood sugar during our study after LA administration (data not shown). LA predominantly undergoes β-oxidation and methylation in the liver which is why it is subjected to a high hepatic extraction[15].

Until now there is a lack of clinical trials that describe pharmacological preconditioning of the liver to protect against IRI. Moreover, established strategies, such as IP, lose their potential in patients with age over 60 years or in cases of high volume liver resection[6]. Our current pilot study points to LA as a potential strategy to protect against hepatic IRI of the liver. We observed histomorphological increased features of oncosis in the non-treated group accompanied by significantly increased levels of enzyme

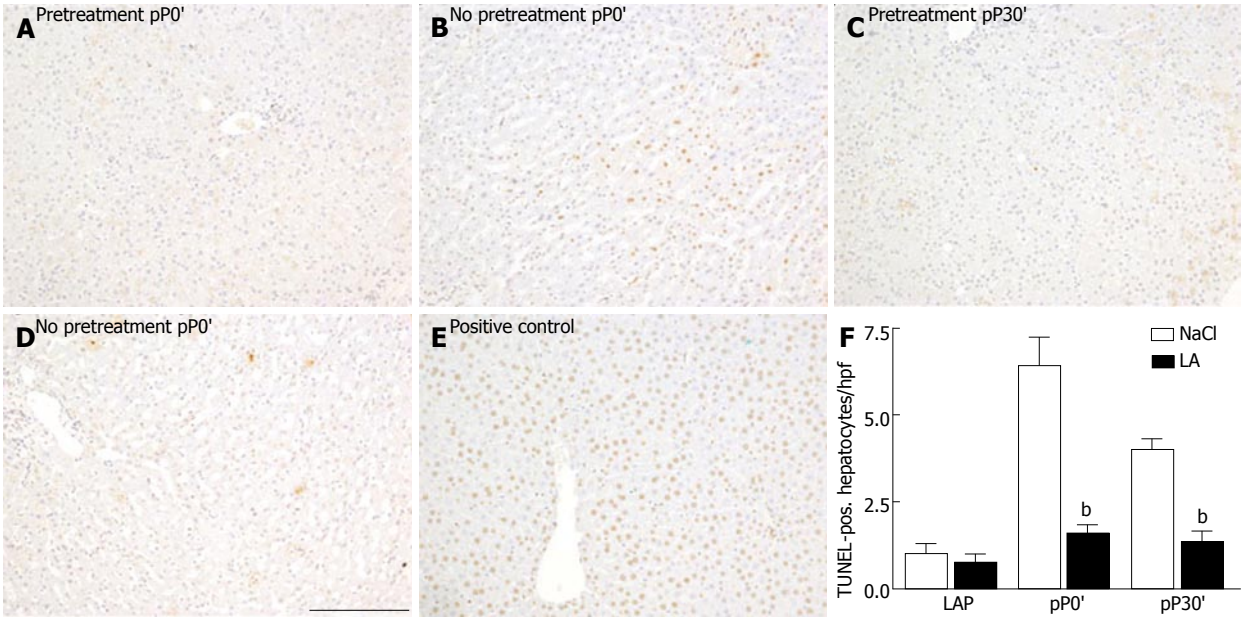


**Figure 5** Representative liver sections stained with TUNEL assay after 30 min of ischemia (pP0') and after 30 min of reperfusion (pP30') with and without 600 mg LA 15 min prior to ischemia. Positive control was prepared from liver-tissue samples by treating with DNase I in accordance with manufactures guidelines (**E**). After LAP TUNEL-positive hepatocytes were rare (data not shown). We observed TUNEL-positive hepatocytes in the untreated group at pP0' and at pP30' (**B**, **D**) while TUNEL staining was decreased in the LA pretreated group at the same time point (**A**, **C**); **F**: TUNEL-positive hepatocytes after laparotomy (LAP), after 30 min of ischemia (pP0') and after 30 min of reperfusion (pP30') with and without 600 mg LA 15 min prior to ischemia, values as means ± SD, $n = 5$ each group, $^{b}P < 0.01$ *vs* vehicle (NaCl) group.

release into plasma. Interestingly, hepatocyte injury clearly occurs in the course of ischemia as it has previously been shown in experimental animal studies[16]. This finding supports the necessity for interventions against IRI to begin *before* the onset of ischemia. Strategies that concentrate on reperfusion of the liver, such as glutathione, may not be sufficient because the initial damage has already started[17].

The clamping period was set exactly at 30 min. This period is the shortest ischemic time associated with elevated transaminase levels after surgery[11]. The lack of significant effects of pretreatment by LA on patient outcome and other parameters of hepatic injury may be related to this short period of ischemia. On the other hand we could demonstrate that LA improves cholinesterase activity in serum. Former studies have shown increased activity of cholinesterase and liver function in patients with compensated liver cirrhosis after LA treatment[18]. We therefore suggest that LA pretreatment also protects against IRI in patients with liver disease, such as cirrhosis or steatosis.

The detailed mechanism of action of LA remains speculative. We observed increased levels of ATP in liver tissue in the LA group accompanied by improved liver histology. One possible explanation for this result may be the known potential of LA to reform vicinal thiol groups in the ATP synthase. Dithiols have been shown to form disulfide bridges during ischemia/reperfusion. ATP synthesis occurs only if the dithiols between the $F_1$ and the $F_0$- part of the enzyme remain functional[19]. Since ATP depletion is known to induce cellular swelling and other features of oncotic and necrotic cell injury[20], LA might decrease hepatic injury by increased levels of ATP.

We observed that LA decreases apoptotic related cell death. The influence of apoptotic cell death on IRI of the liver in humans has been described[4]. The underlying mechanism of LA to reduce apoptotic related cell death was not within the scope of this study. However, results of our studies in rats showed that LA influences pro- and anti-apoptotic proteins towards the anti-apoptotic Bcl-2 (unpublished data). LA might therefore improve expression of anti-apoptotic proteins, such as Bcl-2 but there is a need for respective investigations in the clinical setting.

In summary, we report for the first time the protection of hepatic IRI after liver surgery and hepatic inflow occlusion by a single infusion of LA 15 min prior to ischemia. This strategy might represent a cost effective, safe and efficient strategy to attenuate ischemia reperfusion injury of the liver in the clinical setting.

## ACKNOWLEDGMENTS

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*BASIC RESEARCH*

# Promoted differentiation of cynomolgus monkey ES cells into hepatocyte-like cells by co-culture with mouse fetal liver-derived cells

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## Abstract

**AIM:** To explore whether a co-culture of cynomolgus monkey embryonic stem (cES) cells with embryonic liver cells could promote their differentiation into hepatocytes.

**METHODS:** Mouse fetal liver-derived cells (MFLCs) were prepared as adherent cells from mouse embryos on embryonic d (ED) 14, after which undifferentiated cES cells were co-cultured with MFLCs. The induction of cES cells along a hepatic lineage was examined in MFLC-assisted differentiation, spontaneous differentiation, and growth factors (GF) and chemicals-induced differentiations (GF-induced differentiation) using retinoic acid, leukemia inhibitory factor (LIF), FGF2, FGF4, hepatocyte growth factor (HGF), oncostatin M (OSM), and dexamethasone.

**RESULTS:** The mRNA expression of α-fetoprotein, albumin (ALB), α-1-antitrypsin, and hepatocyte nuclear factor 4α was observed earlier in the differentiating cES cells co-cultured with MFLCs, as compared to cES cells undergoing spontaneous differentiation and those subjected to GF-induced differentiation. The expression of cytochrome P450 7a1, a possible marker for embryonic endoderm-derived mature hepatocytes, was only observed in cES cells that had differentiated in a co-culture with MFLCs. Further, the disappearance of Oct3/4, a representative marker of an undifferentiated state, was noted in cells co-cultured with MFLCs, but not in those undergoing spontaneous or GF-induced differentiation. Immunocytochemical analysis revealed an increased ratio of ALB-immunopositive cells among cES cells co-cultured with MFLCs, while glycogen storage and urea synthesis were also demonstrated.

**CONCLUSION:** MFLCs showed an ability to induce cES cells to differentiate toward hepatocytes. The co-culture system with MFLCs is a useful method for induction of hepatocyte-like cells from undifferentiated cES cells.



**Key words:** Primate embryonic stem cells; Fetal liver; Hepatic differentiation; Co-culture

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## INTRODUCTION

Embryonic stem (ES) cells are self-renewing, pluripotent cells derived from the inner cell masses of preimplantation blastocysts[1,2]. They can be expanded without limit and retain a potential to differentiate into various somatic cell types of the three germ layers. We previously reported the differentiation of mouse ES (mES) cells into insulin-producing cells[3,4], intestinal tract-related cells[5], dopamine-producing cells[6], photoreceptor-like cells[7], and hepatocytes[8-10].

Several of the characteristics of mES and primate ES cells are different[11-13]. To better understand the differentiation ability and therapeutic potential of human ES (hES) cells, the use of primate ES cells is indispensable. Although the differentiation of hepatocyte-like cells from hES cells has also been reported by some researchers, the use of hES cells for basic and clinical research is regulated in many countries, because of bio-ethical issues. Thus, monkey ES cells might be useful as a substitute model for basic and preclinical research using hES cells.

Herein, we report promoted differentiation of cynomolgus monkey ES (cES) cells into hepatocyte-like cells by use of a co-culture system with mouse

fetal liver-derived cells (MFLCs). The expression of α-fetoprotein (AFP) mRNA expression was not observed until d 21 and 14 in spontaneous and growth factor (GF)-induced differentiations, respectively, and that of cytochrome P450 7a1 (CYP7A1), a possible marker of embryonic endoderm-derived mature hepatocytes[14], was undetectable even on d 28 in both cultures. Further, Oct3/4, a marker of an undifferentiated state, never disappeared throughout the experimental period. In contrast, in the present study of cES cells that were co-cultured with MFLCs on a membrane with 0.4-μm sized pores, the expressions of AFP, albumin (ALB), hepatocyte nuclear factor 4α (HNF4α), and CYP7A1 were detected as early as d 10, while the expression of Oct3/4 was detected only faintly on d 14 and not at all by d 21. Immunocytochemical analysis revealed an increased ratio of ALB-immunopositive cells among cES cells co-cultured with MFLCs, while glycogen storage and urea synthesis were also demonstrated.

These results show that MFLCs provide a conductive environment for the differentiation of cES cells toward hepatocyte-like cells, and suggest that the present co-culture system of cES cells with MFLCs may be useful for preparation of a cell replacement source that is rich with hepatocyte-like cells and does not retain undifferentiated cES cells.

## MATERIALS AND METHODS

### *Cynomolgus monkey ES cells*

A cynomolgus monkey ES (cES) cell line (CMK6) was obtained from Asahi Techno Glass Corp., Chiba, Japan[15]. Undifferentiated cES cells were maintained on a feeder layer of 40 Gy-irradiated mouse embryonic fibroblasts (MEF) in DMEM/F-12 (Asahi Techno Glass Corp.), supplemented with 20% Knockout Serum Replacement (KSR; GIBCO-Invitrogen, Carlsbad, CA, USA), 0.1 mmol/L 2-mercaptoethanol, 1 mmol/L sodium pyruvate, 2 mmol/L L-glutamine, 0.1 mmol/L nonessential amino acids, and a mixture of penicillin (25 U/mL) and streptomycin (25 μg/mL). The medium was changed daily. Cell colonies composed of closely packed cells were split every 3-4 d by incubation in a 0.25% trypsin-EDTA solution for 5 min at 37℃ before transfer by pipetting onto the 40-Gy-irradiated MEF cells.

### *Preparation of mouse fetal liver-derived cells (MFLCs)*

Embryonic liver tissues were collected from C57BL/6CrSlc mice (E14.0) and minced, then dissociated in 0.1% trypsin-EDTA solution for 20 min at 37℃ followed by hemolysis with hypotonic buffer. Dissociated cells were suspended in culture media composed of DMEM supplemented with 10% fetal calf serum (FCS), 2 mmol/L L-glutamine, 1x non-essential amino acid solution, and a mixture of penicillin (25 U/mL) and streptomycin (25 μg/mL), after which $1 \times 10^7$ cells were plated onto a 0.1% gelatin-coated tissue culture dish sized 100 mm in diameter. After 24 h, contaminating hematopoietic cells and cell debris were removed by extensive washing with culture media, and the adherent cells were then cultured for 48 h to reach the confluent growth stage.

### *Spontaneous and GF-induced differentiation of cES cells*

Spontaneous differentiation of undifferentiated cES cells was carried out as follows. Undifferentiated cES colonies were incubated with a 0.25% trypsin-EDTA solution for 5 min at 37℃, which obtained clusters of closely packed cells. Approximately $1 \times 10^6$ of these cells were then cultured in a 60-mm dish in DMEM supplemented with 10% FCS, 2 mmol/L L-glutamine, and 1 × non-essential amino acid solution (DMEM basic medium) for 28 d without any defined chemical factors (Figure 1A). The medium was exchanged with fresh medium every 2-3 d.

For GF-induced differentiation toward hepatocyte-like cells, undifferentiated cES cells were treated stepwise with combinations of defined chemicals and growth factors, according to a method reported previously[16], with some modifications. Approximately $1 \times 10^6$ undifferentiated cES cells, prepared as cellular clusters by enzymatic digestion of undifferentiated cES colonies, were seeded in a 60-mm dish and cultured in DMEM basic medium containing leukemia inhibitory factor (LIF, 100 units/mL; GIBCO BRL, Rockville, MD, USA) and $10^{-8}$ mol/L all-trans retinoic acid (RA; Sigma) for 3 d. The cells were passaged in DMEM basic medium containing fibroblast growth factor 2 (FGF2, 10 mg/L; Genzyme/Techne, Minneapolis, MN, USA), FGF4 (20 mg/L, Genzyme/Techne), hepatocyte growth factor (HGF, 25 mg/L; Genzyme/Techne), and Oncostatin M (OSM, 10 mg/L; Genzyme/Techne). After 5 d, the cells were further cultured in DMEM basic medium containing $10^{-7}$ mol/L dexamethasone (Dex; Sigma), 0.2 mmol/L L-ascorbic-2-phosphate (AP; Wako, Osaka, Japan), and 10 mmol/L nicotinamide (NA; Sigma) until d 28 (Figure 1B).

### *Co-culture of cES cells with MFLCs*

MFLCs showing confluent growth were digested with a 0.25% trypsin-EDTA solution for 5 min at 37℃. One million undifferentiated cES cells, prepared as cell clusters by enzymatic digestion of undifferentiated cES colonies, were co-cultured in a 60-mm dish with the same number ($1 \times 10^6$) of MFLCs in a 35-mm culture insert across a 0.4-μm Millicell CM membrane (Millipore Corp., Bedford, MA USA) for 28 d (Figure 1C). A half volume of medium in the 60-mm dish was discarded with floating cells and replaced with fresh medium every 2 d.

### *RT-PCR*

For RNA extraction and RT-PCR analysis, total RNA was purified using Trizol (Invitrogen) following the protocol of the manufacturer. One microgram of DNase-treated total RNA was used for the first-strand cDNA reaction, which was performed using a random primer (Invitrogen) and M-MLV reverse transcriptase (Promega, Madison, WI, USA). cDNA samples were subjected to PCR amplification with specific primers. The cycling parameters were as follows; denaturation at 94℃ for 1 min, annealing at 55℃-60℃ for 1 min (depending on the primer), and elongation at 72℃ for 1 min (40 cycles). The PCR primers and length of the amplified products are shown in Table 1.

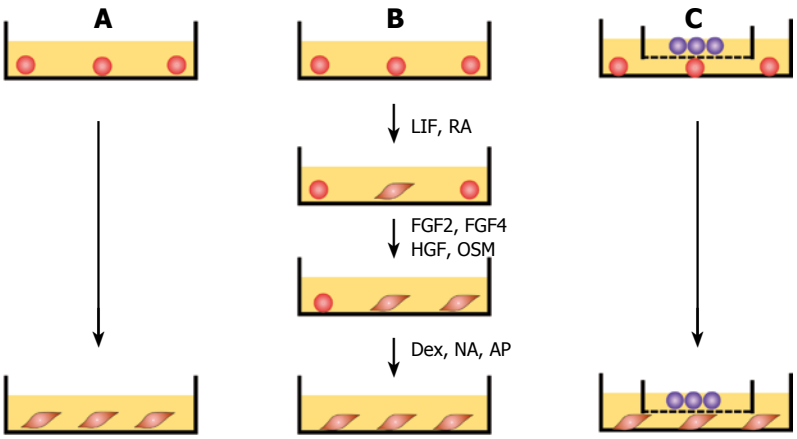


**Figure 1** Protocol for differentiation of cES cells. **A:** Spontaneous differentiation. Undifferentiated cES cells were cultured in gelatin-coated dishes in DMEM basic medium for 28 d without additional feeders or growth factors; **B:** GF-induced differentiation. Undifferentiated cES cells were cultured in DMEM basic medium containing LIF (100 units/mL) and RA ($10^{-8}$ mol/L) for 3 d, followed by a 5-d culture in DMEM basic medium containing FGF2 (10 mg/L), FGF4 (20 mg/L), HGF (25 mg/L), and OSM (10 mg/L). Thereafter, the cells were cultured for 20 d in DMEM basic medium containing $10^{-7}$ mol/L dexamethasone (Dex), 0.2 mmol/L, L-ascorbic-2-phosphate (AP), and 10 mmol/L nicotinamide (NA); **C:** Promoted differentiation by co-culture with MFLCs. Undifferentiated cES cells were plated onto 60-mm dishes and co-cultured with MFLCs in a 35-mm culture insert across a 0.4-μm Millicell CM membrane for 28 d.

**Table 1 The PCR primers and length of the amplified products**

| For detection | Gene | Primer sequence | Products [bp] Annealing temp.[℃] | GenBank accession No. |
|---|---|---|---|---|
| *Cynomolgus* | OCT3/4 | Sense: 5′-ACCACAGTCCATGCCATCAC-3′ | 660 | Z11898 |
| | | Antisense: 5′-TCCACCACCCTGTTGCTGTA-3′ | 60 | |
| | Albumin | Sense: 5′-GCATCCTGATTACTCTGACATG-3′ | 229 | AB158629 |
| | | Antisense: 5′-CTTGGTGTAACGAACTAATTGC-3′ | 60 | |
| | AFP | Sense: 5′-TGCAGCCAAAGTGAAGAGGGAAGA-3′ | 217 | NM_001134 |
| | | Antisense: 5′-CATAGCGAGCAGCCCAAAGAAGAA-3′ | 60 | |
| | HNF4α | Sense: 5′-CCGGATCAGCACTCGAA-3′ | 411 | NM_178849 |
| | | Antisense: 5′-AGCTCGTCAAGGATGCGTATG-3′ | 60 | |
| | CYP7A1 | Sense: 5′-ATTTGGTGCCAATCCTCTTG-3′ | 312 | NM_000780 |
| | | Antisense: 5′-CGTTGGAGGTTTTCCATCAT-3′ | 60 | |
| *Cynomolgus, mouse* | GAPDH | Sense: 5′-ACCACAGTCCATGCCATCAC-3′ | 452 | NM_002046 |
| | | Antisense: 5′-TCCACCACCCTGTTGCTGTA-3′ | 60 | |
| *mouse* | OCT3/4 | Sense: 5′-CGCCCGCATACGAGTTCTG-3′ | 678 | X52437 |
| | | Antisense: 5′-GGTGTCCCTGTAGCCTCAT-3′ | 60 | |
| | AFP | Sense: 5′-CTTTGGACCCTCTTCTGTGA-3′ | 909 | NM_007423 |
| | | Antisense: 5′-CACTGCTGCAACTCTTCGTA-3′ | 55 | |
| | Albumin | Sense: 5′-TGAACTGGCTGACTGCTGTG-3′ | 718 | AJ457860 |
| | | Antisense: 5′-CATCCTTGGCCTCAGCATAG-3′ | 60 | |
| | α1AT | Sense: 5′-TGGGGTCTACTGCTTCTGG-3′ | 693 | M25529 |
| | | Antisense: 5′-TCATGGGCACCTTCACCGT-3′ | 60 | |
| | HNF4α | Sense: 5′-CTAAGCTGTCCCCACAAGGCTATGCA-3′ | 864 | NM_008261 |
| | | Antisense 5′-CAGAGCTCCACCTGGAAAGGTGTTTG-3′ | 60 | |
| | TDO | Sense: 5′-AGAGCCAGCAAAGGAGGAC-3′ | 500 | BC018390 |
| | | Antisense: 5′-CTGTCTGCTCCTGCTCTGAT-3′ | 60 | |
| | PEPCK | Sense: 5′-TCTGCCAAGGTCATCCAGG-3′ | 290 | AF009605 |
| | | Antisense: 5′-GTTTTGGGGATGGGCACTG-3′ | 60 | |
| | CYP7A1 | Sense: 5′-AGGACTTCACTCTACACC-3′ | 453 | AK050260 |
| | | Antisense: 5′-GCAGTCGTTACATCATCC-3′ | 56 | |
| | Desmin | Sense: 5′-ATGACCGCTTCGCCAACTA-3′ | 461 | NM_010043 |
| | | Antisense: 5′-CATACTGAGCCCGGATGTC-3′ | 60 | |
| | Vimentin | Sense: 5′-TCAAGAACACCCGCACCAACGA-3′ | 463 | NM_011701 |
| | | Antisense: 5′-GTTTGACACCTGCTTGGCCTGG-3′ | 60 | |

AFP: Alpha-fetoprtein; HNF4α: Hepatic nuclear factor 4α; CYP7a1: Cytochrome P450 7A1; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; α1AT: Alpha-1 antitrypsin; TDO: Tryptophan 2, 3-dioxygenase; PEPCK: phosphoenolpyruvate carboxykinase.

### *In vitro immunofluorescence analysis*

Immunofluorescence analysis was carried out using standard protocols. Briefly, the cells were fixed in 4% paraformaldehyde and incubated with cell specific marker antibodies in blocking serum at 4℃ overnight. After incubation in species-specific IgG conjugated with Alexa Fluor 488 (donkey anti-sheep IgG; Invitrogen) or RITC (goat anti-mouse IgG; Biomeda Foster City, CA, USA), the cells were washed with PBS and examined under a microscope. All nuclei were stained with DAPI (Dojindo, Kumamoto, Japan). The primary antibodies and dilutions used were as follows: sheep polyclonal anti-human ALB (Biomeda), 1:100; mouse monoclonal anti-human AFP (Biomeda), 1:100; rabbit polyclonal anti-human HNF4α (Santa Cruz Biotechnology, Santa Cruz, CA, USA), 1:100; and mouse monoclonal anti-human alpha-1 antitrypsin (Biomeda), 1:100.

To examine the immunological similarities between

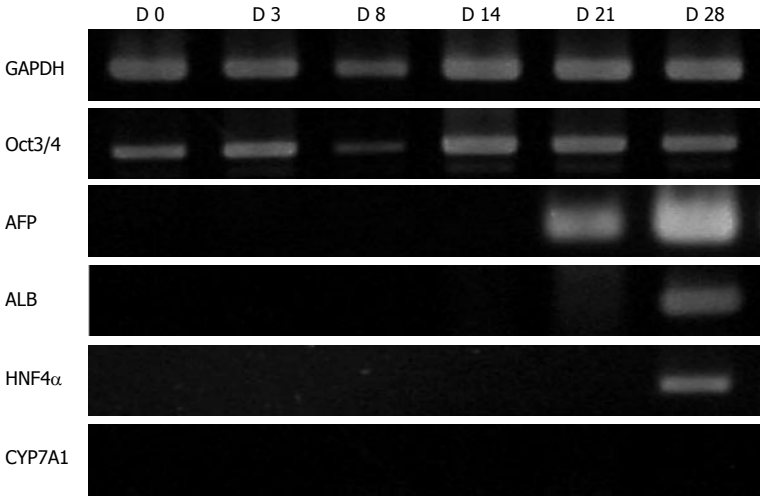


**Figure 2** RT-PCR analysis of spontaneous differentiation of cES cells. AFP mRNA expression was not observed prior to d 14, while ALB was detected on d 21. On d 28, ALB and HNF4α expression became detectable. CYP7A1 was not detected throughout the experimental period, while Oct3/4 was distinctly detected at all time points.

cES-derived hepatocyte-like cells and human hepatocytes, a monoclonal antibody mouse anti-human hepatocyte clone OCH1E5 (HepPar1; DAKO) was used[17,18]. HepPar1 reacts with both normal and neoplastic hepatocytes, but not with cholangiocytes.

In some experiments, cultured cES cells were trypsinized into single cells in suspension. After reattachment to the culture dish by an overnight culture, the cells were subjected to immunofluorescence analysis to determine the ratio of ALB-immunopositive cells. The numbers of total cells and ALB-immunopositive cells in 3 different microscopic fields were then counted.

### *Periodic acid Schiff (PAS) staining*

Staining of glycogen was performed using a PAS reaction. For negative controls, fixed cells in 4% paraformaldehyde were treated with 1 mg/mL of α-amylase (3000 U/mg protein, Sigma) in 0.1 mol/L sodium phosphate buffer (pH 6.2) at 37℃ for 30 min before PAS staining.

### *Measurement of urea*

To examine urea synthesis, cES cells were subjected to spontaneous, GF-induced, or MFLC-co-cultured differentiation for 14 and 28 d. Then they were incubated in serum-free α-MEM medium in the presence of ammonium chloride (20 mmol/L) for 60 min. The level of urea nitrogen in the incubation medium was determined using a colorimetric assay (Determiner LUN kit, Kyowa Medix, Tokyo), after removal of endogenous ammonium by treatment with glutamate dehydrogenase.

### *Analysis*

For qualitative analysis, all cES differentiation experiments were performed in duplicate and repeated. $P < 0.05$ was taken as significant.

## RESULTS

### *Spontaneous differentiation of cES cells*

Undifferentiated cES cell clusters were cultured in basic DMEM for 28 d and differentiation toward hepatocyte-like cells was analyzed by RT-PCR (Figure 2). AFP mRNA expression was not observed until d 14, while ALB remained undetectable on d 21. On d 28, ALB and HNF4α were both detected, whereas CYP7A1 was never detected throughout the experimental period. Further, immunocytochemical results demonstrated that ALB-immunopositive cells on d 28 comprised fewer than 1% of the total cultured cells. The expression of Oct3/4, a marker of an undifferentiated state, was distinctly detected throughout the experimental period.

### *GF-induced differentiation toward hepatocyte-like cells*

Efficient induction of mouse ES cells into hepatocyte-like cells by sequential treatments with defined chemicals and without the use of embryoid bodies (EBs) has been reported[16]. Therefore, we applied that method to the present cES cells with some modifications. Undifferentiated cES cells were first treated with RA and LIF for 3 d, followed by 5 d of treatment with FGF2, FGF4, and HGF, and finally subjected to culture medium containing OSM and Dex.

AFP mRNA expression was not detected following the 3-d treatment with RA and LIF (Figure 3, lane D3), and was faintly detected on d 8 at the end of the 5-d treatment with FGF2, FGF4, and HGF (Figure 3, lane D8). Further, the expression of ALB and HNF4α became detectable after the differentiating cES cells were exposed to OSM and Dex (Figure 3, lanes D14, D21 and D28). Although ALB and HNF4α were clearly detected on d 21 and 28, no expression of CYP7A1 was observed. The immunocytochemical results demonstrated that about 7.2% ± 0.7% of the cultured cells were ALB-immunopositive on d 28. As for the presence of undifferentiated cES cells, Oct3/4 mRNA was still detected in the differentiating cES cells on d 28 of the induction culture oriented for hepatocyte-like cells.

### *Characterization of mouse fetal liver-derived cells (MFLCs)*

Although the cES cells that underwent GF-induced differentiation into hepatocyte-like cells showed an accelerated gene expression pattern as compared to those that underwent spontaneous differentiation, the prolonged detection of Oct3/4 mRNA and lack of CYP7A1 expression in both spontaneous and GF-induced differentiations suggested the presence of undifferentiated

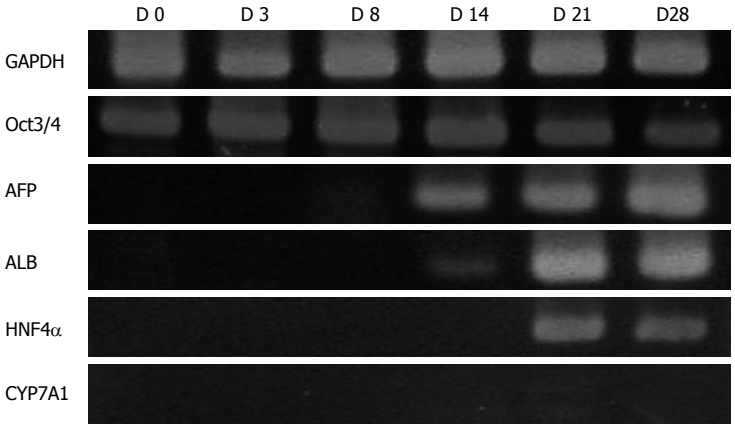


**Figure 3** RT-PCR analysis of GF-induced differentiation of cES cells. AFP mRNA expression was not detected following a 3-d treatment with RA and LIF, though it was detected on d 8, and at the end of 5 d of treatment with FGF2, FGF4, and HGF. ALB and HNF4α were detected after the differentiating cES cells were exposed to OSM and Dex. Although ALB and HNF4α were strongly detected on d 21 and 28, no expression of CYP7A1 was observed. Oct3/4 mRNA expression remained up to d 28.

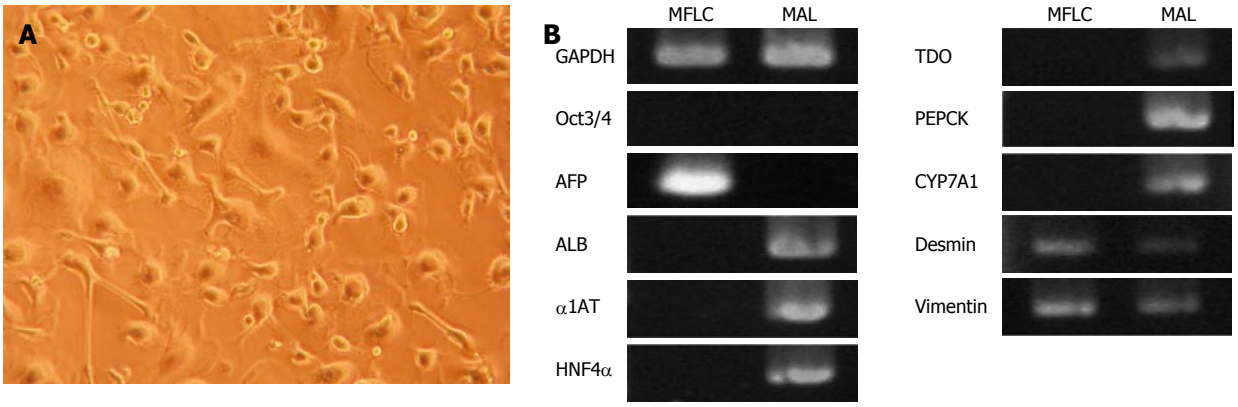


**Figure 4** Characterization of MFLCs. **A:** Optical microscope image of MFLCs (× 100). MFLCs were prepared from E14.0 mouse livers and cultured for 48 h. After the floating cell fraction was discarded from the culture, resting adherent cells were further cultured until semi-confluent. MFLCs showed various morphologies, including cuboidal and stellate-shaped cells; **B:** RT-PCR analysis of MFLCs. AFP, desmin, and vimentin were expressed, whereas ALB was not. MAL: Mouse adult liver cells.

cES cells and insufficient differentiation into mature hepatocyte-like cells, respectively. We performed a co-culture of cES cells with MFLCs to search for epigenetic cues for the *in vitro* differentiation of cES cells toward hepatocyte-like cells. However, before performing that co-culture experiment, we examined the characteristics of MFLCs.

MFLCs were derived from E14.0 mouse livers and prepared as adherent cells by removal of the floating cell fraction from the culture of dissociated liver cells. They showed various morphologies, including cuboidal and stellate-shaped cells (Figure 4A). RT-PCR analysis demonstrated a distinctly different expression pattern as compared to that of adult liver tissues (Figure 4B). The MFLCs expressed AFP mRNA, but not ALB or other markers for differentiated hepatocytes, suggesting the presence of fetal hepatocytes. Further, the expressions of desmin and vimentin suggested the co-existence of cell types different from fetal hepatocytes, such as stellate cell-like cells, in accordance with the presence of cells with various shapes.

### *Promoted differentiation by co-culture with MFLCs*

Undifferentiated cES cells were co-cultured with MFLCs across a membrane with 0.4-μm sized pores for 28 d and the gene expression of the differentiating cES cells were analyzed (Figure 5). There was no expression of AFP, ALB, or HNF4α detected in the differentiating cES cells on d 7, however, each was detectable within the next few days and all by d 10, which remained throughout the experimental period. Further, CYP7A1 mRNA expression, which was undetectable in the spontaneous and GF-induced differentiated cES cells, was observed on d 10. Immunocytochemical results demonstrated a high ratio of ALB-immunopositive cells (28.6% ± 4.2% as early as d 14), though the ratio did not greatly increase throughout the culture period (32.8% ± 5.6% on d 28). Despite the prompt induction of ALB mRNA, AFP mRNA was detected throughout the experimental period, suggesting an incapability of full maturation by the entire fraction of cES-derived hepatocyte-like cells by MFLCs alone. The expression pattern of Oct3/4 in cES cells co-cultured with MFLCs was quite different from that of cES cells subjected to spontaneous or GF-induced differentiation, as Oct3/4 mRNA, which never disappeared in the spontaneous and GF-induced differentiation experiments, was detected only faintly on d 14 and became undetectable by d 21. Without MFLCs on the Millicell CM membrane, no promoted differentiation of cES cells was observed.

### *Immunocytochemical analysis*

cES cells differentiated in the co-culture with MFLCs were examined immunocytochemically on d 28 (Figure 6). Immunopositive reactions to AFP, ALB, and α1-AT

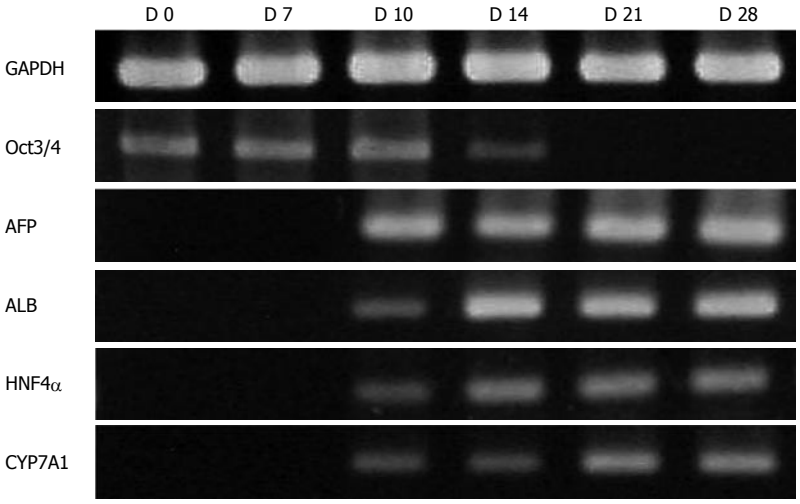


**Figure 5** RT-PCR analysis of cES cells co-cultured with MFLCs. cES cells co-cultured with MFLCs across a membrane with 0.4-μm sized pores for 28 d were analyzed. AFP, ALB, HNF4α, and CYP7A1 mRNA expression was detected on d 10. Oct3/4 expression was faint on d 14 and disappeared by d 21.

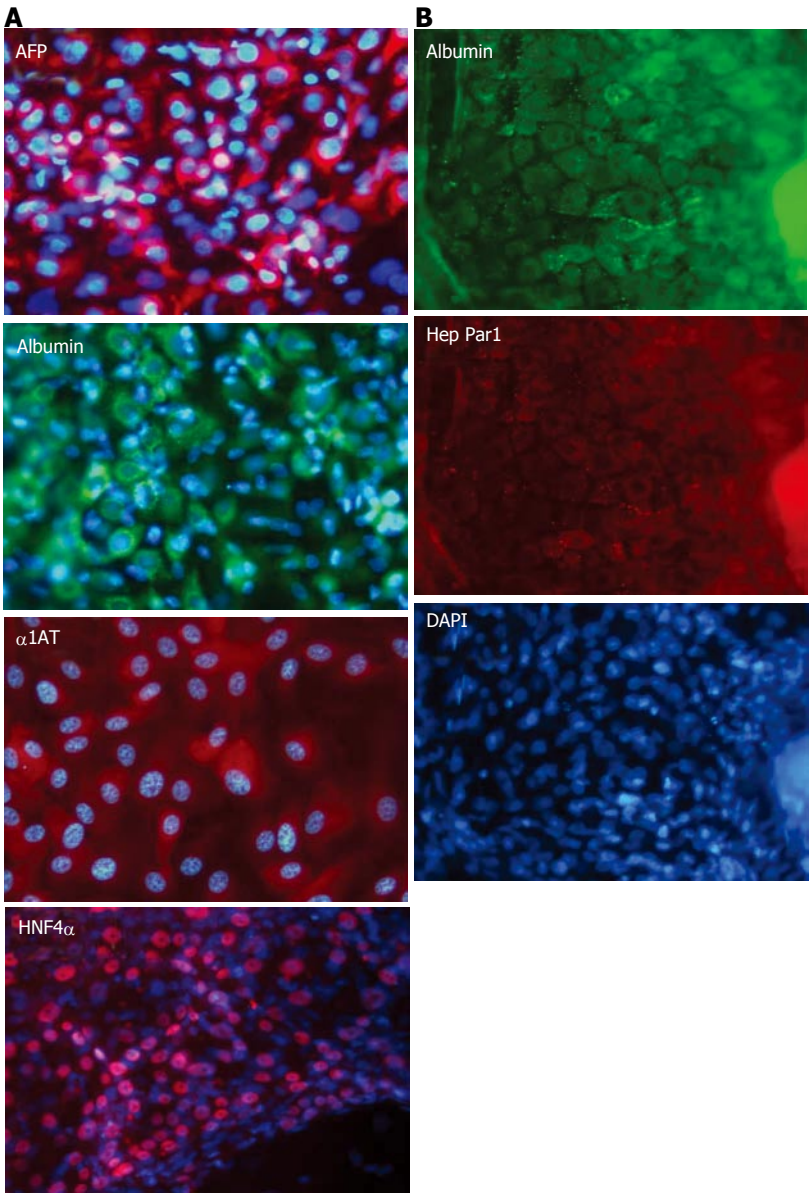


**Figure 6** Immunocytochemical analysis of cES cells co-cultured with MFLCs. **A:** After being co-cultured with MFLCs for 28 d, cES cells were immunostained with anti-AFP, anti-ALB, anti-alpha-1-antitrypsin, and anti-HNF4α antibodies; **B:** Immunoreactivity to the anti-human hepatocyte antibody Hep Par1 was shown by the albumin-positive cells. Original magnification × 100.

were demonstrated as scattered colonized foci among the adherent cells. Representative areas of immunopositive distribution are shown in Figure 6A. In addition, HNF4α, an important transcription factor for mature hepatocytes, was also clearly detected in the cES cells co-cultured with MFLCs. We also examined immunoreactivity to the anti-human hepatocyte antibody Hep Par1. Cells immunopositive for human-albumin were also

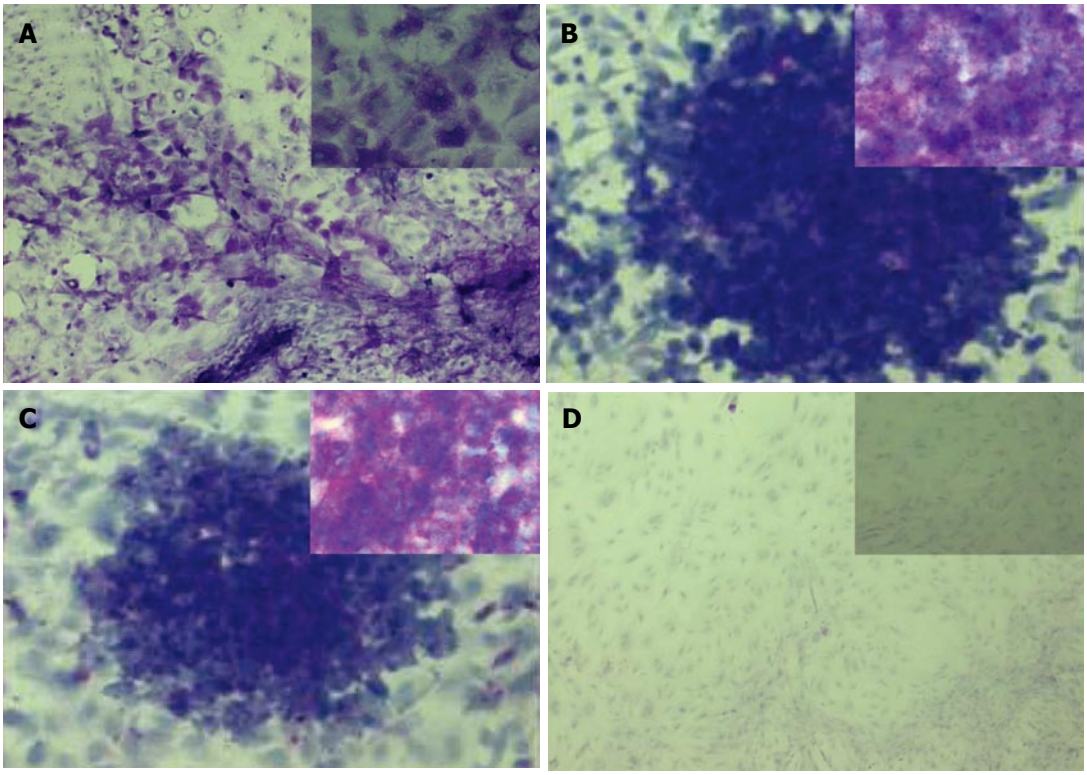


**Figure 7** Histochemical staining of glycogen. Periodic acid Schiff (PAS) staining was performed on cES cells co-cultured with MFLCs for 28 d (**A**) and undifferentiated cES cells (**B**), as well as those following treatment with α-amylase (**C** and **D**, respectively). Both the undifferentiated cES cells and those co-cultured with MFLCs showed violet staining (**A, B**). Violet staining was apparent in the undifferentiated cES cells (**C**), while such staining did not appear in the differentiated cES cells pretreated with α-amylase(**D**). Original magnification ×100 (**A-D**), × 400 (insets in **A-D**).

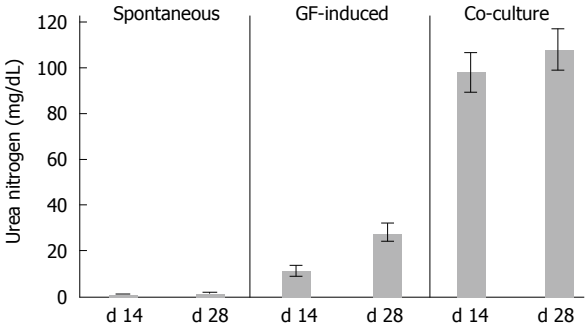


**Figure 8** Urea synthesis by differentiated cES cells. The level of urea nitrogen in the culture medium of cES cells cultured with MFLCs was greater than that in the medium of cES cells subjected to GF-induction, though amounts of urea synthesized by cES cells co-cultured with MFLCs scarcely increased from d 14 to 28. Spontaneously differentiated cES cells showed nearly no ability to synthesize urea.

immunopositive for Hep Par1 (Figure 6B).

### *Presence of glycogen in differentiated cES cells co-cultured with MFLCs*

We performed PAS staining of undifferentiated cES cells and cES cells co-cultured with MFLCs to examine whether glycogen was stored, and both showed violet colored stains (Figure 7A and 7B). Similar staining results were obtained with undifferentiated cES cells pretreated with α-amylase (Figure 7C), while such staining did not appear following pretreatment with α-amylase in the differentiated cES cells co-cultured with MFLCs (Figure 7D). These results suggest that undifferentiated cES cells have an α-amylase-insensitive carbohydrate, while cES cells differentiated by MFLCs have an α-amylase-sensitive carbohydrate, which is probably glycogen.

### *Urea synthesis in differentiated cES cells co-cultured with MFLCs*

We also examined ammonia metabolization in cES cells subjected to spontaneous, GF-induced, and MFLC-assisted differentiation after 14 and 28 d (Figure 8). The urea nitrogen level in the medium from cES cells co-cultured with MFLCs showed a high level as compared to that from cES cells under GF-induction. In a comparison between the levels on d 14 and 28, the amount of synthesized urea in cES cells co-cultured with MFLCs did not increase significantly. Further, spontaneously differentiated cES cells showed nearly no synthesized urea.

## DISCUSSION

A number of studies have investigated the differentiation of mouse and human ES cells into hepatocyte-like cells[8-10,14,16,19-32]. In general, the methods used in those studies can be divided into spontaneous and directed differentiation. For spontaneous differentiation, the formation of EBs has been mostly utilized[8,14,19-23]. As for directed differentiation, a process of enrichment of a specific differentiated cell type that uses elements to promote the differentiation of ES cells into an endodermal lineage, such as the addition of growth factors (GFs) and hormones[16,24-31], and the constitutive expression of hepatic transcription factors[9,10,32], has been utilized.

In the present study, we used MFLCs to promote the differentiation of cES cells into hepatocyte-like cells. We co-cultured undifferentiated cES cells with MFLCs across a membrane with 0.4-μm sized pores and found early expression of AFP, ALB, and HNF4α mRNA, as compared to cES cells subjected to non-EB-mediated spontaneous or GF-induced differentiation. Further, the

expression of CYP7A1 was detected in MFLC-assisted differentiated cES cells, but not in those undergoing non-EB-mediated spontaneous or GF-induced differentiation. In mice, CYP7A1 has been reported to be expressed in the liver, but not in yolk sac tissues[14], and suggested to be a definitive marker for endoderm-derived mature hepatocytes. The detection of CYP7A1 in the present cES cells co-cultured with MFLCs seems to indicate the usefulness of MFLCs in induction toward hepatocytes, though it has not been concluded whether CYP7A1 is a suitable marker for hepatocytes in primate cells.

We confirmed the promoted differentiation of cES cells along a hepatic lineage by immunocytochemistry. The ratio of ALB-immunopositive cells on d 28 was significantly higher with MFLC-assisted differentiation (32.8%) than non-EB-mediated spontaneous (less than 1%) or GF-induced (7.2%) differentiation. Similarly, cells immunopositive for alpha 1 antitrypsin and HNF4α were more abundant among those subjected to MFLC-assisted differentiation (data not shown). We further examined an immunoreactivity of differentiated cES cells to HepPar1. Although the target antigen recognized by HepPar1 has not been identified, HepPar1 is known to react in a restricted manner to adult and fetal human hepatocytes[17,18]. We found that the cES cells immunopositive to human-albumin were also immunopositive to Hep Par1, suggesting that cES-derived hepatocyte-like cells are immunologically very similar to human hepatocytes.

Both glycogen storage and ammonia metabolization are representative functions of hepatocytes. Periodic acid-Shiff (PAS) staining detected glycogen as α-amylase-sensitive carbohydrate in the cES cells differentiated by MFLCs. Although the undifferentiated cES cells were stained violet by PAS staining, they seemed to produce α-amylase-insensitive carbohydrate, not glycogen, which was in accordance with our previous observation of PAS staining of mES cells and mES-derived hepatocyte-like cells[10]. As for the ability to metabolize ammonia, the cES cells co-cultured with MFLCs produced urea more abundantly than those subjected to GF-induced differentiation.

We also evaluated the presence of undifferentiated cES cells among the differentiating cES cells by examining the mRNA expression of Oct3/4. In contrast to the continuous and stable detection of Oct3/4 mRNA throughout the entire 28-d experimental periods in the spontaneous and GF-induced differentiation experiments, the expression of Oct3/4 became faint on d 14 and was undetectable by d 21 in MFLC-assisted differentiation, indicating the absence of undifferentiated cES cells in cES cells co-cultured with MFLCs for more than 21 d. Since contamination by undifferentiated cES cells in transplants is critical for the development of tumors, cES cells co-cultured with MFLCs may be useful as a source rich in hepatocyte-like cells that is less risky for the development of cES-derived tumors.

The precise mechanisms by which MFLCs promote the differentiation of undifferentiated cES cells along a hepatic lineage were not investigated in the present study. However, it is known that non-parenchymal cells are required for differentiation of primitive hepatic endodermal cells and hepatoblasts into mature hepatocytes[33-38]. The MFLCs used in the present study were composed of heterogeneous cells, based on their various morphologies that showed cuboidal and stellate shapes, and on the results of PCR analysis demonstrating the expression of AFP, a hepatocyte-related marker, and desmin and vimentin, which are stellate cell-related markers. It is conceivable that the non-parenchymal cells among the MFLCs were involved in the differentiation of cES cells toward a hepatic lineage.

In the present study, MFLCs were prepared as adherent cells from E14.0 mouse fetal livers. The fetal liver is the major organ of hematopoiesis, and stromal cells there, like those in adult bone marrow, create a hematopoietic microenvironment and promote embryonic hematopoiesis[39]. Fetal liver-derived cells may also induce hematopoietic differentiation of ES cells, as in the case of stromal cells from bone marrow[40-45]. Indeed, a previous report documented the efficient induction of human ES cells into hematopoietic cells by fetal liver-derived cells[46]. In our experiments, MFLC-assisted differentiation of cES cells might have generated a number of hematopoietic cells in the culture as non-adherent floating cells, though they were removed from the culture by repeated media exchanges.

As the process of gestation proceeds from mid-gestation to birth, the fetal liver increases dramatically in size and switches to a metabolic organ from a hematopoietic organ at around the time of birth. Therefore, we anticipated that MFLCs might provide a conductive environment for cES cells to differentiate along a hepatic lineage. However, prior to starting the present experiments, we speculated that any promotion effect by MFLCs toward the hepatic differentiation of cES cells may be not distinguished or exerted only minimally. First, the species from which the cES cells and MFLCs originated were different. Further, the cES cells that were subjected to a co-culture with MFLCs were used in an undifferentiated state and they had not been previously induced toward an endoderm or hepatic lineage. Also, the undifferentiated cES cells were cultured separately from MFLCs by use of a 0.4-μm Millicell CM membrane, which did not allow direct cellular contact between the cES cells and MFLCs, and only permitted the transfer of diffusible factors. However, in contrast to our expectation, the co-culture of undifferentiated cES cells with MFLCs led to the promoted differentiation of cES cells toward a hepatic lineage. These results suggest that diffusible factors from MFLCs worked effectively in a cross-species manner on undifferentiated cES cells and seemed to be sufficient to stimulate the induction of hepatic differentiation of cES cells, though complete maturation of all cES-derived hepatocytes was not achieved, as shown by the prolonged detection of AFP mRNA throughout the experimental period.

In conclusion, the present results demonstrate that cynomolgus monkey ES cells can be induced into hepatocyte-like cells using a co-culture method with mouse fetal liver-derived cells. This co-culture system has been found to be an efficient method to obtain hepatocyte-like cells and may be useful for preparation of cellular grafts

that do not contain undifferentiated ES cells, which would provide a limited risk for the development of tumors.

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*BASIC RESEARCH*

# Hepatic lipid metabolism changes in short- and long-term prehepatic portal hypertensive rats

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## Abstract

**AIM:** To verify the impairment of the hepatic lipid metabolism in prehepatic portal hypertension.

**METHODS:** The concentrations of free fatty acids, diacylglycerol, triglycerides, and phospholipids were assayed by using D-[U-$^{14}$C] glucose incorporation in the different lipid fractions and thin-layer chromatography and cholesterol was measured by spectrophotometry, in liver samples of Wistar rats with partial portal vein ligation at short- (1 mo) and long-term (1 year) (i.e. portal hypertensive rats) and the control rats.

**RESULTS:** In the portal hypertensive rats, liver phospholipid synthesis significantly decreased (7.42 ± 0.50 *vs* 4.70 ± 0.44 nCi/g protein; $P < 0.01$) and was associated with an increased synthesis of free fatty acids (2.08 ± 0.14 *vs* 3.36 ± 0.33 nCi/g protein; $P < 0.05$), diacylglycerol (1.93 ± 0.2 *vs* 2.26 ± 0.28 nCi/g protein), triglycerides (2.40 ± 0.30 *vs* 4.49 ± 0.15 nCi/g protein) and cholesterol (24.28 ± 2.12 *vs* 57.66 ± 3.26 mg/g protein; $P < 0.01$).

**CONCLUSION:** Prehepatic portal hypertension in rats impairs the liver lipid metabolism. This impairment consists in an increase in lipid deposits (triglycerides, diacylglycerol and cholesterol) in the liver, accompanied by a decrease in phospholipid synthesis.



**Key words**: Portal hypertension; Free fatty acids; Diacylglycerides; Triglycerides; Phospholipids; Cholesterol

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## INTRODUCTION

The most used experimental model to study prehepatic portal hypertension is that obtained by calibrated stenosis of the portal vein in the rat[1,2]. It has been suggested that this experimental model has a homogeneous evolution, with a narrow range of portal hypertension, degree of porto-systemic shunts and hepatic atrophy[2-4]. However, prehepatic portal hypertensive rats have a far from uniform evolution, since they can present wide variability in both the type and degree of portosystemic collateral circulation which is developed[5] as well as in the degree of liver atrophy[5,6].

The different evolution of hepatic weight in rats with prehepatic portal hypertension is an interesting finding, since it demonstrates the existence of a heterogeneous hepatic response in this experimental model. A previous histological study of the liver was performed in order to verify whether the existence of a liver disease could justify this wide spectrum of liver weight. This study demonstrated that prehepatic portal hypertension in the rat produces fatty infiltration of the liver. Moreover, this fat accumulation in hepatocytes progresses from a short-term (1 mo) to a long-term (1 year) evolutive stage of portal hypertension[7].

In order to study hepatic lipid metabolism in these evolutive stages of experimental prehepatic portal hypertension, we determined the concentrations of free fatty acids (FFA), diacylglycerides (DG), triglycerides (TG),

phospholipids (PL) and cholesterol (Ch) in the liver of the rats with calibrated stenosis of the portal vein, both after short-term (1 mo) and long-term (1 year) evolution.

## MATERIALS AND METHODS

Male Wistar rats, weighing 250-300 g, were obtained from the *Vivarium* of the Complutense University of Madrid. The animals were anesthetized by im injection of ketamine (100 mg/kg) and xylacine (12 mg/kg). The animals were fed standard laboratory rodents' diet (rat/mouse AO4 maintenance diet: 17.6% proteins, 43.3% starch 2.5% lipids, Panlab, Spain) and water *ad libitum*. They were housed in a temperature- (22 ± 2℃), humidity- (65%-70%) and light-controlled room (light, 7:30 am-7:30 pm) in groups of three to four animals.

The experimental procedures employed in this study were in accordance with the principles and practices of the 1986 European Guide for the Care and Use of Laboratory Animals, published in Spain in Royal Decree 1201/2005.

### Experimental design

The animals were randomly divided into four groups: group I ($n$ = 5) consisted of control rats at 1 mo of evolution; group II ($n$ = 11) consisted of triple calibrated portal vein stenosis (TPVS) rats at 1 mo of postoperative evolution; group III ($n$ = 4) consisted of control rats at 1 year of evolution; and group IV ($n$ = 6) consisted of TPVS rats at 1 year of postoperative evolution. The control rats (group I and III) did not undergo any operative intervention. All the animals were sacrificed by exsanguination and body (BW), liver (LW) and spleen (SW) weights were determined.

### Establishment of the rat model of portal hypertension

The surgical procedure used to establish portal hypertension by triple partial ligation of the portal vein (TPVS) has been described previously[8]. In brief, the portal vein was isolated and three partial ligatures were performed in its superior, medial and inferior portions. The stenoses were calibrated by a simultaneous ligature (4-0 silk) around the portal vein with a 20-gauge needle. The midline abdominal incision was closed in two layers with catgut and 3-0 silk.

### Portosystemic collateral circulation study method

Portosystemic collateral circulation was studied as follows: first, a midline abdominal incision with a large bilateral subcostal extension was performed and then the areas in which the collateral venous circulation was developed (i.e. the splenorenal, gastroesophageal, colorectal and hepatic hilum) were carefully studied for the presence of increased collateral veins[9].

### Portal vein pressure measurement

Splenic pulp pressure, an indirect measurement of portal pressure (PP), was measured by inserting a fluid-filled 20-gauge needle into the splenic parenchyma[10]. The needle was joined to a PE-50 tube and then connected to a pressure recorder (PowerLab 200 ML 201) and to a transducer (Sensonor SN-844) with a Chart V 4.0 computer program (ADI Instruments) and was calibrated before each experiment. The pressure reading was considered satisfactory when a stable recording was produced and respiratory variations were observed. Previous studies have demonstrated the excellent correlation between splenic pulp pressure and PP[11].

### Lipid synthesis

**Chemicals:** D-[U-$^{14}$C] glucose was purchased from the Radiochemical Centre (Amersham, Buckinghamshire, UK). Standard lipids and 2', 7'-dichlorofluorescein were purchased from Sigma Chemical Co. (Saint Louis, MO).

**Lipid synthesis:** "*De novo*" lipid synthesis was measured by the incorporation of either D-[U-$^{14}$C] glucose (12.5 Ci/mol) into TG, PL, DG and FFA. Labeled glucose was injected after dissolving in saline (5 mL/kg). All the rats were sacrificed 24 h after treatment, and the livers were excised and washed. After specific extraction, lipids were determined. Briefly, 50 mg of tissue was rapidly frozen in acetone chilled with dry ice. After addition of 0.7 mL of acid methanol (methanol/HCl, 100:1) to the tubes, the content was homogenized, centrifuged and sonicated in an MSE ultrasonic disintegrator (Branson, Danburry, CT). The lipids were then extracted with 1.3 mL of chloroform and 0.4 mL of salt solution[12] for 1 h at room temperature. The organic phase was then washed three times with 1.0 mL of the aqueous phase of a system composed of chloroform/methanol/salt solution/concentrated HCL (266:133:100:1). The organic phase was dried under $N_2$ and re-dissolved in 40 μL of chloroform/methanol (2:1). A sample (30 μL) was then applied to precoated plates of silica gel 60 (20 cm × 20 cm; Merck) previously activated for 1 h at 110℃. Lipid separation was performed by unidimensional chromatography. Then, the plates were sprayed with 2' 7'-dichlorofluorescein, and the following spots were detected under UV light and identified with suitable markers: PL, TG, DG and FFA. Each spot was scraped off into a scintillation vial, and its radioactivity was measured.

### Cholesterol determination

Cholesterol determination was performed with a specific commercial kit (Spinreact, Richmond, 1972)[13] based on the action of the cholesterol enzyme stearase which hydrolyzed the esters present in the sample, giving free cholesterol and fatty acids. A subsequent enzymatic oxidation using the cholesterol enzyme oxidase formed hydrogen peroxide and cholesterine. The peroxide was evaluated by the Trinder reaction by a chromogene, in the presence of peroxidase, forming a quinonimine with a red coloring. The intensity of this color was proportional to the cholesterol concentration in the sample. Finally, colorimetric determination was performed in a spectrophotometer (Eppendorf, model Biophotometer) at a wavelength of 500 nm.

### Determination of liver protein concentrations

This was done by the colorimetric method described by Bradford[14]. This method is based on the binding

of Coomassie Blue to proteins. This binding causes a displacement of the peak absorption of the dye from 465 to 595 nm. Absorbance is measured in the samples at the latter wavelength against a known reference curve. The protein complex dye has a high coefficient of extinction, which gives it a high sensitivity at measuring the protein.

Protein determination was carried out because the concentration of lipids and cholesterol is expressed in relation to the concentration of liver proteins.

### Statistical analysis

Results were expressed as means ± SD. ANOVA and Duncan tests were used for the statistical comparison of the variables between the four groups. The results were considered statistically significant when $P < 0.05$.

## RESULTS

### Body and organ weights

The animals with portal hypertension (groups II and IV) showed a markedly less body weight increase than that found in the control animals (groups I and III): 82.49 ± 29.08 g *vs* 64.72 ± 29.32 g and 268.02 ± 78.03 g *vs* 361.02 ± 58.32 g, $P < 0.05$). The animals with TPVS showed a decrease in liver weight (9.98 ± 1.55 g in group II *vs* 12.01 ± 2.64 g in group I , $P < 0.05$; 15.84 ± 2.02 g in group IV *vs* 16.77 ± 2.87 g in group III).

All animals with TPVS showed splenomegaly: 1.00 ± 0.16 g in group II *vs* 0.49 ± 0.06 g in group I ($P < 0.05$) and 1.43 ± 0.84 in group IV *vs* 0.91 ± 0.10 g in group III ($P < 0.05$) (Table 1).

### Portal venous pressure

All the TPVS rats showed characteristic features of splanchnic venous congestion with dilation of mesenteric venous circulation. The portal pressure in groups II and IV was obviously higher compared to groups I and III (14.63 ± 2.84 mmHg *vs* 7.32 ± 1.15 mmHg; and 9.05 ± 2.84 mmHg *vs* 6.72 ± 1.52 mmHg, respectively; $P < 0.05$) (Table 1).

### Portosystemic collateral circulation

In the rats with TPVS (groups II and IV), splenorenal portosystemic collateral circulation was the commonest type (group II: $n = 11$, 100%; group IV: $n = 4$, 66%).

### Hepatic lipid metabolism

The hepatic concentration of phospholipids underwent a significant increase ($P < 0.005$) in the TPVS animals after one year (group IV) compared to the control group (group III) (Figure 1).

The increase in diacylglyceride in the both groups II (TPVS 1 evolutive month) and IV (TPVS 1 evolutive year) was not statistically significant compared to their corresponding controls. Moreover, although the hepatic concentrations of FFA increased in the animal series with portal hypertension (groups II and IV), this increase was not significant compared to their corresponding controls (Figure 2).

A statistically significant increase in hepatic concentrations of TG (Figure 2) and Ch (Figure 1) in the animals with portal hypertension (groups II and IV) compared to their corresponding control groups (group I and III, respectively) was observed.

**Table 1 Body weight increase (BWI), liver weight (LW), spleen weight (SW) and portal pressure (PP) in the control (C) groups of 1 mo and 1 year of evolution and in the rats with triple portal vein stenoses (TPVS) at 1 mo and 1 year of evolution (mean ± SD)**

| Group | BWI (g) | LW (g) | SW (g) | PP (mmHg) |
|---|---|---|---|---|
| C (1 mo) ($n = 5$) | 82.49 ± 29.08 | 12.01 ± 2.64 | 0.49 ± 0.06 | 7.32 ± 1.15 |
| TPVS (1 mo) ($n = 11$) | 64.72 ± 29.32[a] | 9.98 ± 1.55[a] | 1 ± 0.16[a] | 14.63 ± 2.84[a] |
| C (1 yr) ($n = 4$) | 361.02 ± 58.32 | 16.77 ± 2.87 | 0.91 ± 0.10 | 6.72 ± 1.52 |
| TPVS (1 yr) ($n = 6$) | 268.02 ± 78.03[c] | 15.84 ± 2.02 | 1.43 ± 0.84[c] | 9.05 ± 2.84[c] |

[a]$P < 0.05$, [c]$P < 0.05$ *vs* control group.

## DISCUSSION

Our study demonstrated that prehepatic portal hypertension in the rats, for both in the short-term (1 mo) and long-term (1 year) period, produced hepatic accumulation of triglycerides and cholesterol. Our results provided further evidence to support a long-term evolution (1 year) in the production of a severe drop in hepatic phospholipids (Figures 1 and 2).

Liver steatosis could be the cause of the hepatomegaly described in the evolution of prehepatic hypertension in the rat[5,6]. If so, portal stenosis ligation could be used to make a model of steatosis as well as to obtain an experimental model of portal hypertension[7].

Hepatic steatosis alone is thought to be the most common form of non-alcoholic fatty liver disease (NAFLD) and is considered to be "benign", but not quiescent[15]. The NAFLD spectrum is wide and ranges from simple fat accumulation in hepatocytes (fatty liver), without biochemical or histological evidence of inflammation or fibrosis, to fat accumulation plus necro-inflammatory activity with or without fibrosis (non-alcoholic steatohepatitis, NASH) and the development of advanced liver fibrosis or cirrhosis (cirrhotic stage)[15-19].

However, although a progressive hepatocytic fatty infiltration occurs during chronic evolution in TPVS rats, this is not associated with histological signs of inflammation or fibrosis. Therefore, hepatic steatosis could be considered as a "benign" type of the broad spectrum of NAFLD occurring in these rats with prehepatic portal hypertension[7].

The lipid content of hepatocytes is regulated by the integrated activities of cellular enzymes that catalyze lipid uptake, synthesis, oxidation and export. Fat accumulates within the hepatocytes when the "input" (either uptake or synthesis) of fatty acids to hepatocytes exceeds their "output" (oxidation and export)[20].

Different factors, extrahepatic and intrahepatic, can

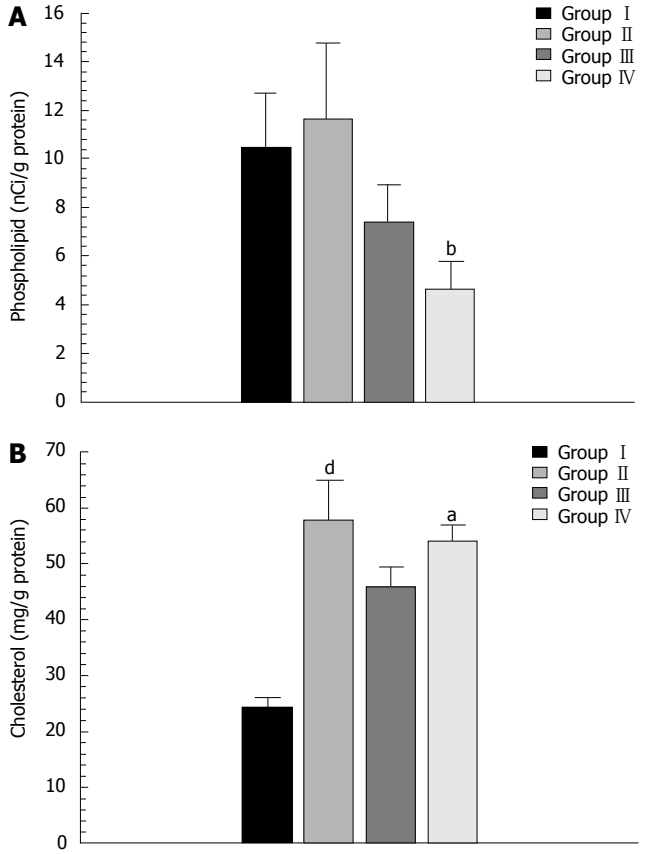


**Figure 1** Liver synthesis of phospholipids and cholesterol liver content in group Ⅰ (control rats) and group Ⅱ (portal hypertensive rats) at 1 mo of postoperative evolution and in group Ⅲ (control rats) and group Ⅳ (portal hypertensive rats) at 1 year of postoperative evolution. [a]*P* < 0.05, [b]*P* < 0.01 *vs* group Ⅲ; [d]*P* < 0.01 *vs* group Ⅰ.

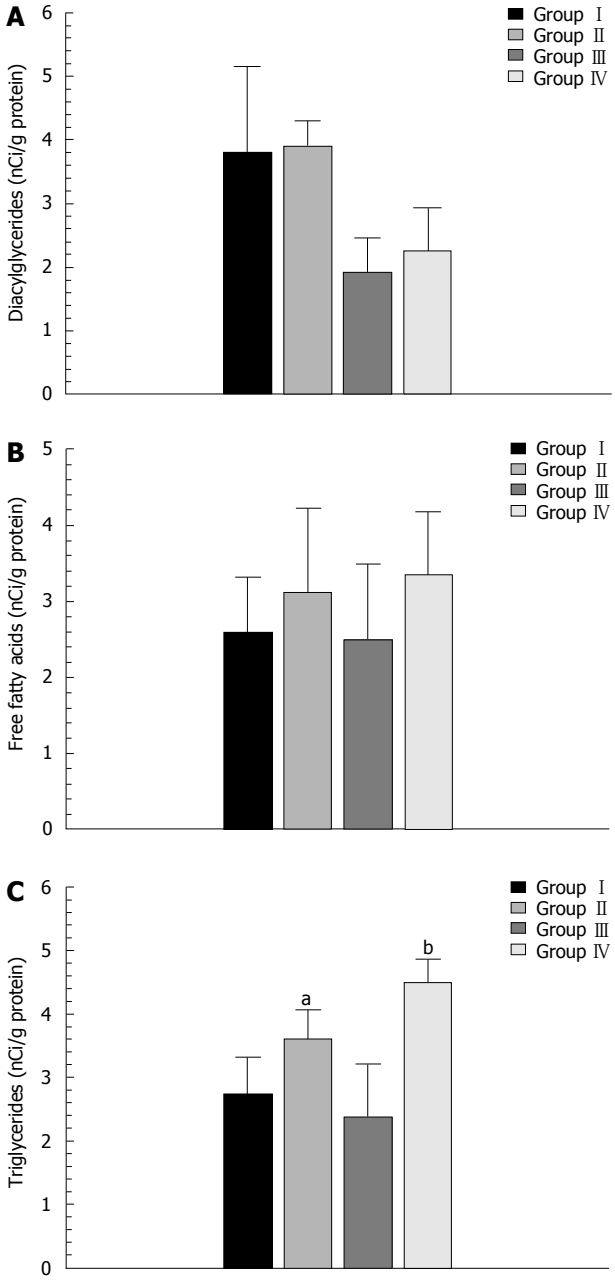


**Figure 2** Liver synthesis of diacylglycerides, free fatty acids and triglycerides in group Ⅰ (control rats) and group Ⅱ (portal hypertensive rats) at 1 mo of postoperative evolution and in group Ⅲ (control rats) and group Ⅳ (portal hypertensive rats) at 1 year of postoperative evolution. [a]*P* < 0.05 *vs* group Ⅰ; [b]*P* < 0.01 *vs* group Ⅲ.

impair both regulator mechanisms and, therefore, promote triglyceride and cholesterol accumulation in the liver of rats with short-term (1 mo) and long-term (1 year) prehepatic portal hypertension. These factors could be divided into neuroendocrine and immunologic for their etiopathogenic study. The possible neuroendocrine factors involved in fat accumulation within the hepatocytes include an increase in the components of the neuroendocrine response to TPVS stress: plasma corticoids[8], catecholamines[21,22] and glucagon[23] levels in portal hypertensive rats. In the neuroendocrine response to stress, a promotion of lipolysis in adipose tissues is also produced with increased plasma free fatty acid concentrations and subsequently, an excess "input" of fatty acids to the liver in short-term prehepatic portal hypertensive rats. In turn, the expanded pool of NAFLD would induce accumulation of triglycerides within the cytoplasm of hepatocytes as fat microvesicles[24-26]. Insulin resistance causes an insufficient suppression of hormone-sensitive lipase activity and reduced glucose uptake in adipose tissue. This results in enhanced lipolysis and increased fatty acid flux into the plasma non-sterified fatty acid pool[26]. It has been demonstrated that approximately 60% of triacylglicerides accumulated in the liver are derived from the plasma non-sterified fatty acids pool. The enhanced adipocyte lipolysis, in turn, is associated with an increased activity of lipogenic enzymes, contributing to hepatic steatosis[26]. Insulin resistance induced by glucocorticoids could also contribute to hepatic steatosis by favouring peripheral lipolysis and hepatic uptake of fatty acids[25,27].

Therefore, in the presence of insulin resistance, increased lipolysis results in the continuous release of long-chain fatty acids (LCFA) from adipose tissue, leading to a characteristic increase in the circulating LCFA concentration[26,28]. When this process occurs in fat-swollen omental and mesenteric fat cells, as it occurs in portal hypertension in the rat, this results in an especially large increase in the mass of LCFAs entering the portal circulation and being delivered directly to the liver[28]. The increase of FFA delivery to the liver, in turn, causes hepatic insulin resistance, as well as tumor necrosis

factor alpha (TNF-α) production[29]. When TNF-α is overproduced relative to adiponectin, adiponectin activity declines. Reduced adiponectin activity also favors hepatic insulin resistance and fat accumulation[29,30]. In addition, low adiponectin activity permits TNF-α activity, which induces insulin resistance[29,30].

The cytokine TNF-α is among the most important immunologic factors potentially involved in the production of hepatic steatosis[17,31,32]. The increase in TNF-α activity, which increases nitric oxide (NO) and carbon monoxide (CO) levels, two potent vasodilators, is at least partially responsible for development of the hyperdynamic systemic and splanchnic state that contributes to maintaining an elevated portal pressure in early evolutive periods of experimental prehepatic portal hypertension[33-37]. However, TNF-α and TNF-regulated cytokines are also considered as effector molecules in liver damage in the animals and patients with non-alcoholic liver diseases, ranging from steatosis to cirrhosis[17,32]. It has been accepted that liver injury requires at least two "hits": One that increases exposure of the hepatocytes to TNF-α and another one that interferes with the normal ability of fat, but renders the liver more vulnerable to a second insult because the hepatocytes became sensitized to the TNF-mediated cell death[32,38].

Although Kupffer cells are a major source of liver-derived pro-inflammatory cytokines, other liver cells can also synthesize cytokines. Particularly, fat accumulation within hepatocytes triggers intracellular signals, activates nuclear factor kappa B (NF-κB) and induces excessive hepatocyte production and insulin resistance[39].

On the other hand, TNF-α has been proposed as causing the hyperlipidemia that occurs in different pathological situations. It has also been shown that TNF-α decreases the level of lipase activity against lipoproteins in adipose tissue[40]. In addition, TNF-α-induced increase in lipid synthesis is accompanied by both an increase in the activity of acetyl coenzyme A carboxylase (a rate-limiting enzyme for fatty acid synthesis) and a rise in its allosteric activator, citrate. This suggests that a relative increase in the activity of this key regulatory enzyme may be one of the mechanisms responsible for. There is also evidence that the rate of triacylglycerol synthesis is controlled by regular coordination of the activities of phosphatidate phosphatase and diacylglycerol acyl transferase[41] .The activities of both enzymes are increased in many conditions of stress, including metabolic stress and exposure to ethanol and glucocorticoids[41].

Accumulated evidence suggests a major role for mitochondrial dysfunction in steatosis[24]. Also, oxidative stress with formation of reactive oxygen species (ROS) increases in fatty livers[42,43]. This abundant ROS formation may oxidize the unsaturated lipids of fat deposits to cause lipid peroxidation[34]. Both lipid peroxidation products and TNF-α may damage mitochondria and directly attack and inactivate respiratory chain components[15,24,25,44,45]. ROS and lipid peroxidation can also lead to ultrastructural mitochondrial alterations and megamitochondria[24,46] and hepatocyte necrosis or apoptosis[44]. The pro-inflammatory cytokines, particularly TNF-α, induce iNOS hyperactivity with an increased NO synthesis. ROS, on binding to NO, form peroxynitrite and this reactive nitrogen radical could bind proteins of the mitochondrial respiratory chain, resulting in its functional impairment with a decrease in oxidative phosphorylation and, consequently, of ATP[47].

ROS, in turn, can cause NF-κB activation, which induces the synthesis of TNF-α, and this cytokine damages mitochondria and increases mitochondrial ROS formation[24]. Mitochondrial dysfunction can, therefore, reduce oxidative phosphorylation and the ATP production that occurs in hepatic steatosis[25,46] and would explain the low resistance of steatotic livers to a second aggressive stimulus or "second hit"[17].

Since the accumulation of triglycerides and cholesterol in the hepatocytes persisted in the long-term (1 year) evolutive stage of prehepatic portal hypertension, possibly, the etiopathogenic mechanisms involved in its production could also persist. Moreover, in this evolutive stage of experimental prehepatic portal hypertension, the increase in lipid deposits associated with a decline in the synthesis of lipid complexes would be associated with the decreased synthesis of lipid complexes, such as phospholipids (PL) (Figure 1), which would worsen the sensitivity to liver injury. Phospholipids are a critical part of the structure of eukaryotic cell membranes. When PL synthesis was examined, we found a significant decrease after one year. Since PC is the main PL in liver membranes, the observed decrease in PL could reflect a decrease in PC synthesis. PC synthesis in the liver mainly occurs through two pathways, the cytidine diphosphate (CDP) choline pathway and the methylation of phosphat-idylethanolamine. In the CDP-choline pathway, the most important pathway, cytidine diphosphate phosphocholine cytidyl transferase catalyses the rate-limiting step. A possible mechanism for the induced PL decrease could be through the release of cytidyl transferase from the membranes, probably mediated by the release of inflammatory mediators, including glucocorticoids and/or cytokines[47].

The excess liver storage of TG and cholesterol results in steatosis and also provides substrates for lipid peroxidation[48]. The accumulation of cholesterol and the PL decrease have been associated with the metabolic syndrome[49]. This classical lipid changes may be envisioned as a highly conserved evolutionary response aimed at tissue repair[50].

An inflammatory hypothesis has been proposed in relation to the etiopathogeny of the portal hypertensive enteropathy. This consists in the involvement of the mast cells[51,52] and the inflammatory mediators released by them, of which histamine would be one of the most important. Histamine, as an effective metabolite, could affect the lipid metabolism in liver cells by facilitating the liver cells' accumulation of lipid droplets (fatty liver)[53]. Therefore, in this experimental model of prehepatic portal hypertension, histamine released by activated mast cells could also be involved in producing a fatty liver.

In summary, prehepatic portal hypertension in rats produces changes in liver lipid metabolism, which are similar to those associated with chronic inflammatory conditions and with sepsis[54].

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*BASIC RESEARCH*

# Antioxidants vitamin E and C attenuate hepatic fibrosis in biliary-obstructed rats

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## Abstract

**AIM:** To investigate whether antioxidants vitamin E and C can retard development of hepatic fibrosis in the biliary-obstructed rats.

**METHODS:** Fifty Wistar albino rats were randomly assigned to 5 groups (10 rats in each). Bile duct was ligated in 40 rats and they were treated as follows: group vitC, vitamin C 10 mg/kg sc daily; group vitE, vitamin E 15 mg/kg sc daily; group vitEC, both of the vitamins; bile duct-ligated (BDL, control) group, physiological saline sc. The fifth group was assigned to sham operation. At the end of fourth week, the rats were decapitated, and hepatic tissue biochemical collagen content and collagen surface area were measured. Hepatic tissue specimens were histopathologically evaluated according to Scheuer system. Serum hyaluronate levels were measured by ELISA method.

**RESULTS:** Despite being higher than sham group, hepatic collagen level was significantly decreased in each of the vitC, vitE and vitEC groups (32.7 ± 1.2, 33.8 ± 2.9, 36.7 ± 0.5 μg collagen/mg protein, respectively) compared to BDL (48.3 ± 0.6 mg collagen/g protein) ($P$ < 0.001 for each vitamin group). Each isolated vitamin C, isolated vitamin E and combined vitamin E/C supplementation prevented the increase in hepatic collagen surface density (7.0% ± 1.1%, 6.2% ± 1.7%, 12.3% ± 2.0%, respectively) compared to BDL (17.4% ± 5.6%) ($P$ < 0.05 for each). The same beneficial effect of vitamin C, vitamin E and combined vitamin E/C treatment was also observed on the decrease of serum hyaluronate levels compared to BDL group ($P$ < 0.001). The relative liver and spleen weights, serum transaminases, cholestatic enzymes, bilirubins and histopathological inflammation scores were not different between the antioxidant treatment groups and the control. However, fibrosis staging scores were obviously reduced only in the vitamin E/C combination group (vit EC: 2.4 ± 0.8 *vs* BDL: 3.1 ± 0.7; $P$ < 0.05).

**CONCLUSION:** Each antioxidant vitamin E, vitamin C and their combination retard hepatic fibrosis in biliary-obstructed rats. Oxidative stress may play a role in the pathogenesis of hepatic fibrosis in secondary biliary cirrhosis.



**Key words:** Hepatic fibrosis; Biliary obstruction; Collagen; Vitamin E; Vitamin C

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## INTRODUCTION

Hepatic fibrosis is a highly integrated cellular response to tissue injury[1]. It is essentially characterized by activation of hepatic stellate cells, secretion and accumulation of extracellular matrix proteins[2]. Various causes of cholestasis such as primary biliary cirrhosis, primary sclerosing cholangitis, biliary strictures, atresia, cysts or neoplasia may induce hepatic fibrosis that may potentially culminate in biliary cirrhosis. However, mechanisms underlying the development of hepatic fibrosis in cholestatic liver disease are not thoroughly elucidated.

It is suggested that free radicals might contribute to the development of hepatic fibrosis in biliary obstruction[3-5]. Cholestasis *per se* reduces antioxidative capacities in liver

mitochondria in bile duct-ligated rats[6,7]. Accumulation of hydrophobic bile acids and inflammatory cells in the liver tissue may cause increased production of free radicals in biliary obstruction[8-10]. Bile acids especially, enhance reactive oxygen species released by polymorphonuclear leukocytes[11]. In addition, intraluminal bile salt deficiency in extrahepatic biliary obstruction results in vitamin E malabsorption[12]. Serum and hepatic tissue levels of vitamin E are reduced threefold in biliary-obstructed rats[13]. Accordingly, hepatic tissue levels of lipid peroxides were increased in bile duct-ligated rats[13]. Although the presence of oxidative stress in the course of biliary cirrhosis has been reported, it is still unclear whether it is the cause or consequence of tissue injury or contributes any to hepatic fibrosis. This may partly stem from inadequate number of studies performed to test the efficiency of antioxidants in the prevention of hepatic fibrosis in chronic cholestatic liver diseases.

Type I collagen is the major extracellular matrix protein deposited in the liver during hepatic fibrosis[1,2]. It was reported that lipid peroxides stimulated hepatic deposition of type I collagen in cultured human hepatic stellate cells[14,15]. This suggestion established a possible link between the lipid peroxidation (i.e. oxidative stress) and hepatic fibrosis. Studies demonstrating the beneficial effect of vitamin E supplementation in prevention of both enhancement of lipid peroxidation and synthesis of type I collagen might support this suggestion[16-18]. However, it has been reported that short-term treatment (seven days) of bile duct-ligated rats with vitamin E completely prevented the increase in lipid peroxidation in liver and plasma, but failed to prevent tissue injury histologically[19]. Thus, the current controversies between the few studies performed to evaluate the role of oxidative stress in tissue injury induced by bile duct ligation make newer studies to be performed necessary.

In the liver, vitamin E is the primary fat-soluble chain-breaking antioxidant protecting lipid bilayers whereas hydrophilic vitamin C is the scavenger particularly in plasma, cytosol and other aqueous compartments[20]. In the present study, we aimed to investigate the effect of antioxidants vitamin E and C on hepatic fibrosis in bile duct-ligated rats. Vitamin C was combined to obtain the benefit from synergism of these drugs as the vitamin C reacts with oxidised vitamin E, thereby regenerating vitamin E[21]. Serum hyaluronate levels were used as a marker of liver fibrosis and to predict the response to therapy.

## MATERIALS AND METHODS

Weanling 50 female Wistar albino rats weighing 198 ± 15 g were housed in cages with stainless steel wire tops and with 12-h light-dark cycles under standard animal laboratory conditions at room temperature. The rats had free access to standard rat chow and water. Under aseptic conditions, extrahepatic cholestasis was induced by double ligation with 5/0 polipropilene sutures and transection of the common bile duct in between. In sham-operated rats, the operation was performed in the same way but without bile duct ligation or transection.

The rats were randomly assigned to 5 groups, 10 in each. The 40 bile duct-ligated rats were treated as follows: vitC group, given subcutaneous vitamin C at a daily dose of 10 mg/kg (ascorbic acid; Redoxon®; Roche, Istanbul, Turkey); vitE group, given subcutaneous vitamin E at a daily dose of 15 mg/kg (D-α-tocopherol acetate; Evigen®; Aksu Farma, Istanbul, Turkey) as previously described[22]; vitEC group, given both vitamins by the same route and dose; BDL (bile duct-ligated) (control) group, given subcutaneous physiologic saline. The sham group rats were sham-operated. Administration of vitamin C and E was started immediately after bile duct ligation. At the end of the study period (fourth week), the rats were weighed and decapitated and trunk blood was obtained. Serum samples were collected for liver biochemical tests and serum hyaluronate determinations and then stored at -86℃. The total body weights, liver and spleen weights of each rat were also recorded. Hepatomegaly was defined as liver wet weight of more than 3 g per 100 g rat body weight[23].

This study was approved by the Local Ethical Committee, School of Medicine, Trakya University.

### *Liver tissue sampling*

The left, middle and right lobes of each liver were explored. By cutting six different 5 mm × 5 mm × 5 mm slices, each liver was randomly sampled in 0.75 $cm^3$ tissue. The slices sampled were fixed in 10% buffered formalin, routinely processed and blocked into paraffin for detecting collagen content by biochemical methods and image analysis.

### *Biochemical collagen content determination*

The collagen content of the liver was assayed by the colorimetric method described by Lopez de Leon and Rojkind[24]. The principle is the coloring of collagenous protein by Sirius red (36554-8, 2610-10-8; Aldrich Chemical, Deisenhofen, Germany) and non-collagenous proteins by fast green (14280; MERCK KGaA, Darmstadt, Germany). Fifteen micrometer-thick liver slices taken from each paraffin block were layered on glass slides. Slices were deparaffinized and assayed as originally described. Collagen content was calculated using the formula described by the authors[24] as microgram collagen per milligram protein.

Slices at 5-, 10- and 15- μm thickness were tested beforehand and the optimum depth was determined to be 15 μm, which gave higher absorbency.

### *Histological image analysis of liver fibrosis*

Five-micrometer liver sections of each paraffin block were stained with trichrome for collagen. SAMBA 4000-image analysis system (Unilog, France) was used to determine liver collagen content. Only lobular areas of the liver sections were randomly sampled with × 20 objective magnification. Portal areas were excluded from the analysis by a meticulous area selection. Each representative image frame was 1.278 mm × 0.958 mm (= 1.224 324 $mm^2$). One hundred frames were selected for analysis to reach about 1 $cm^2$ of liver tissue sample. A homemade algorithm generated with SAMBA-IPS software was run. The program automatically outlined and measured the total liver area and the blue-stained collagen area within each frame. Me-

**Table 1 Liver biochemical tests and relative liver and spleen weights of rats at the end of 4th wk of bile duct obstruction (mean ± SE)**

| parameter | Vit C | Vit E | Vit EC | Sham | Control |
|---|---|---|---|---|---|
| AST (IU/L) | 548 ± 82 | 446 ± 75[b] | 505 ± 58[a] | 223 ± 26 | 473 ± 64[a] |
| ALT (IU/L) | 156 ± 34 | 148 ± 16 | 156 ± 23 | 98 ± 9 | 165 ± 29 |
| ALP (IU/L) | 446 ± 95 | 403 ± 41 | 396 ± 27 | 296 ± 35 | 477 ± 59 |
| GGT (IU/L) | 64 ± 13 | 86 ± 5 | 74 ± 8 | 64 ± 4 | 101 ± 17 |
| Total bilirubin (mg/dL) | 5.9 ± 1.2[d] | 7.2 ± 0.9[d] | 7.1 ± 1.3[d] | 0.5 ± 0.2 | 8.4 ± 1.4[d] |
| Conjugated bilirubin (mg/dL) | 3.9 ± 0.6[d] | 3.7 ± 1.0[d] | 3.5 ± 0.6[d] | 0.2 ± 0.1 | 4.2 ± 0.7[d] |
| Albumin (g/dL) | 3.2 ± 0.1[b] | 3.0 ± 0.1[a] | 3.3 ± 0.2 | 3.8 ± 0.0 | 2.9 ± 0.1[d] |
| Relative liver weight (%) | 5.9 ± 0.4[a] | 5.6 ± 0.6[a] | 5.9 ± 0.5[b] | 3.4 ± 0.6 | 5.9 ± 0.1[d] |
| Relative spleen weight (%) | 0.40 ± 0.07 | 0.39 ± 0.08 | 0.42 ± 0.08 | 0.34 ± 0.04 | 0.44 ± 0.05 |

AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; ALP: Alkaline phosphatase; GGT: Gamma-glutamyl transferase; T. bilirubin: Total bilirubin; C. Bilirubin: Conjugated bilirubin. [a]$P < 0.05$ , [b]$P < 0.01$, [d]$P < 0.001$ *vs* sham.

**Table 2 Serum hyaluronate levels, liver collagen content by biochemical method and collagen surface density by image analysis of groups at the end of 4th wk of study (mean ± SE)**

| parameter | Vit C | Vit E | Vit EC | Sham | Control |
|---|---|---|---|---|---|
| Liver collagen content microgram collagen/mg protein | 32.7 ± 1.2[b,d] | 33.8 ± 2.9[b,d] | 36.7 ± 0.5[a,d] | 23.8 ± 1.3[d] | 48.3 ± 0.6[a] |
| Collagen surface density (%) | 7.0 ± 1.1[c] | 6.2 ± 1.7[c] | 12.3 ± 2.0[c] | 2.6 ± 0.7[c] | 17.4 ± 5.6 |
| Hyaluronate (ng/mL) | 63 ± 25[d] | 87 ± 28[d] | 47 ± 25[d] | 56 ± 16[d] | 783 ± 111 |

[a]$P < 0.001$ , [b]$P < 0.01$ *vs* sham; [c]$P < 0.05$ , [d]$P < 0.001$ *vs* control.

asurements of each frame were added to each other consecutively. The total liver lobular area, total blue spectrum-stained collagen area, and the ratio of the collagen within the liver tissue were summed. Histological image analysis was performed at the Pathology Department of Haydarpasa Hospital.

### Histopathological investigations

Five-micrometer liver sections were stained by HE and trichrome. Necroinflammatory activity grading and fibrosis staging were performed with Scheuer system[25].

### Biochemical assays and serum hyaluronate

Aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), gamma-glutamyl transferase (GGT), bilirubins (total and conjugated) and albumin were assayed in an Olympus AU 800 biochemical automated analyzer.

Hyaluronate quantitative test kit was used to measure serum hyaluronate levels (Corgenix Westminster, CO, USA) by ELISA method.

### Statistical analysis

Data are expressed as mean ± standard error of the mean (SE). The Shapiro-Wilks test was used to assess the normality of continuous data. For all variables, the statistical differences between groups were tested using one-way analysis of variance for normally distributed data. The nonparametric Kruskal-Wallis test was used for nonnormally distributed data. Multiple comparisons were made using the Tukey post hoc multiple comparisons test for normally distributed data, and the non parametric Tukey post hoc multiple comparisons test for nonnormally distributed data. $P < 0.05$ was considered statistically significant. Correlation was calculated using Pearson's test. SPSS/PC+ version 11.0 statistical analysis program (SPSS Inc., Chicago, Illionis) was used for the statistical analysis.

## RESULTS

Forty percent of rats in each vitC, vitE, vitEC and control groups were died, whereas there was no mortality in the sham group. The causes of deaths were biliary peritonitis and various infectious complications. There were no statistically significant differences among the animals in vitamin treated groups and controls in terms of mortality rates and causes of deaths. The numbers of rats that were alive at the end of study period and taken into account were 6, 6, 7, 6 and 10 for vitC, vitE, vitEC, BDL and sham groups, respectively. Body weights of the rats included in the study were not significantly changed at the end of the study period in any of the groups. However, relative liver weights, as expressed in grams per 100 g of body weight, were significantly increased in all BDL groups compared to sham ($P < 0.05$ for both vitC and vitE groups; $P < 0.01$ for vitEC; $P < 0.001$ for control group) (Table 1). No significant difference was observed among the alternative antioxidant regimens or controls. The relative spleen weights were the same among the groups including the sham-operated (Table 1).

### Biochemical collagen content measurement and histological image analysis for fibrosis

The hepatic collagen contents in control group were significantly increased compared with the sham-operated group by biochemical method ($P < 0.001$) (Table 2). However, the antioxidant vitamin groups had significantly less total liver collagen compared to control (physiologic

**Table 3 Histologic fibrosis staging and grading of necroinflammatory activity scores according to Scheuer system at the end of the study (mean ± SE)**

| parameter | Vit C | Vit E | Vit EC | Sham | Control |
|---|---|---|---|---|---|
| Stage | 3.2 ± 0.4[b] | 2.5 ± 0.5[b] | 2.4 ± 0.8[a,b] | 0.5 ± 0.5 | 3.1 ± 0.7[b] |
| Grade | 1.5 ± 0.5[d] | 1.5 ± 0.5[d] | 1.4 ± 0.5[d] | 0.2 ± 0.4 | 1.9 ± 0.7[f] |

[a]$P < 0.05$ *vs* control; [b]$P < 0.001$, [d]$P < 0.01$, [f]$P < 0.001$ *vs* sham.

**Table 4 Correlation of serum hyaluronate with histologic fibrosis staging, collagen surface density and biochemical collagen content**

| | | Fibrosis stage | Collagen density | Collagen content |
|---|---|---|---|---|
| Serum | *r* | + 0.38 | + 0.56 | + 0.71 |
| Hyaluronate | *P* < | 0.025 | 0.005 | 0.0001 |

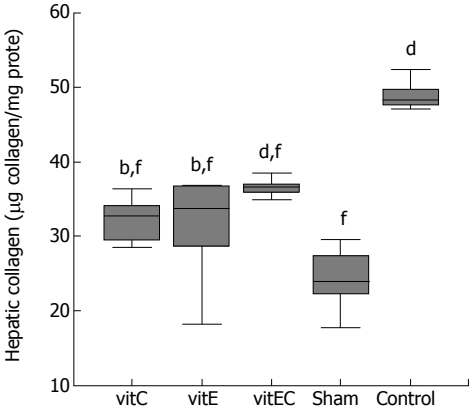


**Figure 1** Effect of treatment on liver collagen content by biochemical method. [b]$P < 0.01$, [d]$P < 0.001$ *vs* sham; [f]$P < 0.001$ *vs* control.

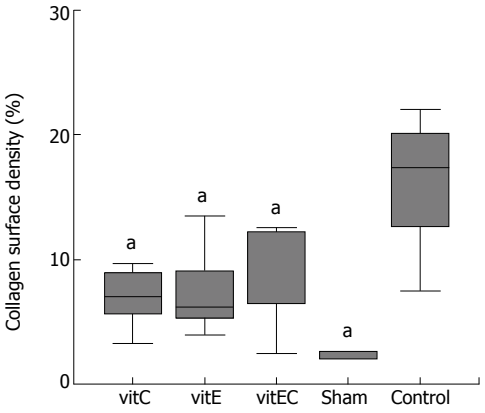


**Figure 2** Effects of vitamin E, C and combination treatment on hepatic fibrosis as determined by computerized image analysis. [a]$P < 0.05$ *vs* control.

saline) group ($P < 0.001$ for all vitamin groups *vs* control group) (Figure 1). The total hepatic collagen contents in antioxidant groups were about 23% less than the BDL (control) group by biochemical method at the end of fourth week. Collagen surface areas were also found to be significantly better in rats treated with antioxidants by computerized image analysis compared to controls ($P < 0.05$) (Table 2, Figure 2). There were no statistically significant differences among the antioxidant treatment groups in regard to the total collagen contents biochemically or collagen surface densities by image analysis (Table 2). Although combination therapy was effective in reducing hepatic fibrosis, it was not statistically more effective than either drugs used alone ($P > 0.05$).

### *Biochemical findings*

The biochemical hepatic profile, including AST, ALT, ALP, GGT, total and conjugated bilirubin measurements, showed no statistically significant difference among the treatment groups and controls ($P > 0.05$) (Table 1). In contrast, serum concentrations of the AST, albumin and bilirubins among the aforementioned biochemical parameters were significantly changed in all of the BDL groups compared to sham-operated group (at least, $P < 0.05$), and without difference in any of the antioxidant regimens that could suggest an improvement in hepatic functions ($P > 0.05$).

### *Histopathological findings and serum hyaluronate levels*

Examination of the liver sections demonstrated inflammation (Figure 3A), bile duct proliferation (Figure 3B) around the portal tracts and fibrosis (Figures 3C and D) in all the BDL groups. Using Scheuer system, the histological necroinflammatory activity observed in the BDL groups were not different compared with any of the treatment regimens (Table 3). However, fibrosis staging was statistically significantly better in the vitamin E and C combination group ($P < 0.05$). The serum hyaluronate levels were significantly elevated in the control group compared to vitamin and sham groups ($P < 0.001$) (Table 2, Figure 4). There was significant correlation between the serum hyaluronate levels and fibrosis staging ($r = 0.38$, $P < 0.05$), biochemical collagen content ($r = 0.71$, $P < 0.0001$) and collagen surface density by image analysis ($r = 0.56$, $P < 0.005$) (Table 4).

## DISCUSSION

Our study showed that antioxidants vitamin E and C retarded the development of hepatic fibrosis without effecting necroinflammation in biliary-obstructed rats. Antioxidants vitamin E and C individually had anti-fibrotic properties. They were equally effective.

In order to demonstrate the efficiency of vitamin E and C in preventing hepatic fibrosis, we started vitamin treatments on the first day of the experiment. As a limitation to our study, we could not measure and compare the serum or hepatic tissue vitamin E levels. However, it has recently been shown that subcutaneous administration of emulsified vitamin E to rats results in substantially elevated serum and liver concentrations of vitamin E comparable with levels achievable by dietary supplementation for conditions in which hepatic oxidative stress is present[26]. Therefore, we preferred to give our

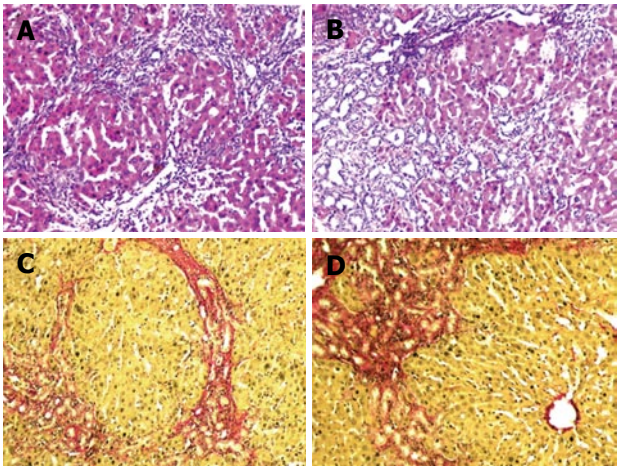


**Figure 3** **A**: Necroinflammation disperses the lobular parenchyma from the portal areas (HE x 100); **B**: Prominent bile duct proliferation; **C**, **D**: Severe portal fibrosis with porto-portal bridging by sirius red collagen stain (x 100).

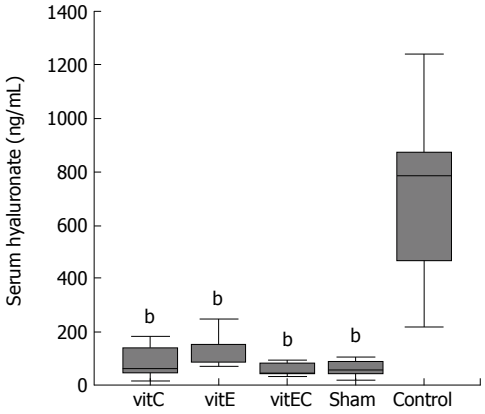


**Figure 4** Effects of vitamin E, C and combination treatment on serum hyaluronate levels. $^{b}P < 0.001$ *vs* control.

rats the vitamins by parenteral route. In contrast, it was reported that oral supplementation with vitamin E and C fails to show any benefit in rats with biliary obstruction[27].

Mild to moderate degrees of hepatic inflammation is usually present especially early in biliary obstruction[28]. Neutrophils infiltrate periductal areas. In our study, we observed this low-grade inflammatory activity in all bile duct-ligated rats. However, antioxidant treatments did not improve the histologic necroinflammation and its surrogate markers serum transaminases and cholestatic enzymes. The reduction in hepatic fibrosis without improvement in necroinflammation needs to be clarified. One possible explanation may be the effect of vitamin E on rat hepatic stellate cell in inducing apoptosis and thus reducing extracellular collagen accumulation[29]. Therefore, in our study, the decrease in hepatic fibrosis in rats taking vitamin E may result from reduced hepatic stellate cell numbers. As another explanation, it is suggested that free radicals may induce apoptosis of hepatocytes in low dose but necrosis in high dose[3]. In biliary obstruction such as in our study, the levels of hepatic tissue free radicals may not be high enough to contribute to the hepatic necroinflammation and disturb liver biochemical enzymes. It would be better if we had preferred to use higher doses of vitamin E or C in order to overcome the necroinflammation and to improve the liver biochemical tests. In our recently published study, the combination of vitamins E and C was associated with decreased ethanol-induced hepatic glutathione peroxidase activity and hepatic fibrosis in protein-deficient rats fed with a high-fat liquid diet[30]. The improvement in hepatic fibrosis was accompanied by reduced necroinflammation and hepatomegaly. However, not all studies indicate that the reduction in fibrosis was due to the reduction in necroinflammation. Similar to the findings of our current study, it has been reported that vitamin E and C combination therapy, although retarded hepatic fibrosis, did not improve hepatic inflammation in patients with nonalcoholic steatohepatitis (NASH) where oxidative stress has a prime role in tissue injury[31]. Antifibrogenic effect of antioxidants vitamin E and C probably interfered with fibrogenesis by mechanisms other than prevention of inflammation or necrosis in both patients with NASH and in our biliary-obstructed rats. In concert with these two studies, the reduction of hepatic collagen by antioxidant sylimarin treatment in biliary-obliterated rats[32] was not accompanied by improvement in clinical findings or biochemical liver enzymes. Meanwhile, antifibrotic effect of sylimarin was mediated by downregulation of type I collagen synthesis[33]. In fact, quiescent stellate cells in tissue culture have the ability to respond to free radicals to express collagen independent of necrosis, inflammation or any other confounding variables[18]. Hepatic stellate cells have been shown to respond to antioxidant vitamin E by downregulating collagen secretion[16-18]. This response was supposed to occur by reduced transforming growth factor-β secretion from hepatic stellate cells in the liver[34].

Bile duct ligation model of rats mimics best the clinical and histopathological aspects of hepatic fibrosis secondary to extrahepatic biliary obstruction in humans[35]. This model allows researchers to study in detail all the developmental stages of hepatic fibrosis, ultimately, terminating in cirrhosis within about 4 wk. In our study the beneficial effect of antioxidants did not reach the levels of improving clinical findings. Neither the liver sizes nor the spleen weights were affected by the antioxidant treatment. Mortality was not improved, either. Maybe, more prolonged study such as 5-6 wk' period would be necessary to observe the expected difference in liver and spleen weights and to judge the effect of treatment on the outcome. The high mortality in our rats was related to postoperative complications. However, these increased complications might also result from the liver damage or from dosage of vitamins which was borderline high for such a long-term treatment. Therefore, by using lower vitamin dosages further studies may also be designed for future experiments. In our study, although the number of rats taken into consideration was not too much, we used four different methods to quantify the extent of hepatic fibrosis. All of the results were in very good correlation and in concordance with each other.

Serum hyaluronate has been suggested to be used in prediction of hepatic fibrosis and the evaluation of treatment response of various antifibrotic drugs in human

studies[36] and in rat[37,38] models of hepatic fibrosis. Several influencing factors such as hepatic inflammatory activity and the indicators of this activity (i.e. liver chemical tests) may reduce diagnostic value of hyaluronic acid and other fibrosis indexes[39]. In our study the presence of low inflammatory activity in the livers of the rats and the absence of difference in serum biochemistry test results between the groups probably attributed to the good correlation between the hyaluronic acid serum levels and the hepatic tissue fibrosis measurements with all methods used. The serum levels of hyaluronic acid in our rats treated with antioxidants were as low as in sham group and correlated well with hepatic collagen quantity. Antioxidant treatment was very effective in preventing the elevation of serum hyaluronic acid levels. However, we could not explain such a significant reduction of the hyaluronic acid level in the treated rats, compared to the negative control with only slight changes in hepatic inflammation and liver biochemistry. It may be appropriate to investigate, if the presumed mechanism, how the vitamins may protect the liver, takes place, or if the hyaluronate serum levels are reduced by another pathway. Nevertheless, our study supported that serum hyaluronic acid levels may be used as an index of liver fibrosis and to evaluate the antifibrotic effects of antioxidant vitamins in biliary-obstructed rats.

In conclusion, oxidative stress might contribute to the pathogenesis of secondary biliary cirrhosis. Antioxidant vitamins E and C or combination of both attenuate the development of hepatic fibrosis without improving necroinflammatory activity in bile duct-ligated rats. Long-term, prospective studies in humans with chronic cholestatic liver diseases may be helpful to evaluate the beneficial effects of these vitamins.

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*BASIC RESEARCH*

# Expression patterns and action analysis of genes associated with blood coagulation responses during rat liver regeneration

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## Abstract

**AIM:** To study the blood coagulation response after partial hepatectomy (PH) at transcriptional level.

**METHODS:** After PH of rats, the associated genes with blood coagulation were obtained through reference to the databases, and the gene expression changes in rat regenerating liver were analyzed by the Rat Genome 230 2.0 array.

**RESULTS:** It was found that 107 genes were associated with liver regeneration. The initially and totally expressing gene numbers occurring in initiation phase of liver regeneration (0.5-4 h after PH), $G_0/G_1$ transition (4-6 h after PH), cell proliferation (6-66 h after PH), cell differentiation and structure-function reconstruction (66-168 h after PH) were 44, 11, 58, 7 and 44, 33, 100, 71 respectively, showing that the associated genes were mainly triggered in the forepart and prophase, and worked at different phases. According to their expression similarity, these genes were classified into 5 groups: only up-, predominantly up-, only down-, predominantly down-, up- and down-regulation, involving 44, 8, 36, 13 and 6 genes, respectively, and the total times of their up- and down-regulation expression were 342 and 253, respectively, demonstrating that the number of the up-regulated genes was more than that of the down-regulated genes. Their time relevance was classified into 15 groups, showing that the cellular physiological and biochemical activities were staggered during liver regeneration. According to gene expression patterns, they were classified into 29 types, suggesting that their protein activities were diverse and complex during liver regeneration.

**CONCLUSION:** The blood coagulation response is enhanced mainly in the forepart, prophase and anaphase of liver regeneration, in which the response in the forepart, prophase of liver regeneration can prevent the bleeding caused by partial hepatectomy, whereas that in the anaphase contributes to the structure-function reorganization of regenerating liver. In the process, 107 genes associated with liver regeneration play an important role.



**Key words:** Partial hepatectomy; Rat Genome 230 2.0 array; Blood coagulation response; Genes associated with liver regeneration

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## INTRODUCTION

The liver is the main site where coagulation factors are synthesized[1]. Tissue damage is often companied with angiorrhexis, bleeding and blood coagulation. Blood coagulation is a complex hemostatic process in which zymogens convert into coagulation factors, and promote blood coagulation at the site of wound[2]. This process is classified into extrinsic pathway and intrinsic pathway[3]. A proportion of coagulation factors of the former originate from blood, while that of the latter all from blood. Blood coagulation abnormity can lead to hereditary prothrombin deficiency, vitamin K deficiency, haemophilia and hepatic coagulation defects[4,5].

Partial hepatectomy (PH)[6] causes blood vessel injury and bleeding. Meanwhile, the remnant hepatocytes activated by it enter into cell cycle to compensate for the lost liver tissue, which process is called liver regeneration (LR)[7,8]. Generally, based on the physiological activities of the cells, the regeneration process is classified into 4 phases: the initiation (0.5-4 h after PH), the transition from $G_0$ to $G_1$ (4-6 h after PH), the cell proliferation (6-66 h after PH), the cell differentiation and structure-function reorganization (66-168 h after PH)[9]. According to time course, it is also divided into 4 phases including

forepart (0.5-4 h after PH), prophase (6-12 h after PH), metaphase (16-66 h after PH), and anaphase (72-168 h after PH)[10], which are regulated by many factors including blood coagulation response[11]. To study the relationship between the blood coagulation response after PH and liver regeneration at transcriptional level[10,12], we examined the expression changes of genes in regenerating liver after partial hepatectomy by Rat Genome 230 2.0 array[13] containing 174 blood coagulation-associated genes. One hundred and seven genes were identified which were associated with liver regeneration[14], and we primarily studied the expression changes, patterns and functions of these genes.

## MATERIALS AND METHODS

### Regenerating liver preparation

Healthy SD rats weighing 200-250 g were obtained from the Animal Center of Henan Normal University. The rats were separated into groups at random and each group included 6 rats (male:female = 1:1). PH was performed according to Higgins and Anderson[6]: the left and middle lobes of the liver were removed. Rats were sacrificed by cervical vertebra dislocation at 0.5, 1, 2, 4, 6, 8, 12, 24, 36, 54, 66, 72, 120, 144 and 168 h after PH and the regenerating livers were examined at corresponding time points. The livers were rinsed three times in PBS at 4℃. Then 100-200 mg liver tissues from middle parts of the right lobe, six samples of each group were gathered and mixed together to 1-2 g (0.1-0.2 g × 6) of total liver tissue, then stored at -80℃. The sham-operation (SO) groups were treated the same with partial hepatectomy ones except that the liver lobes were not removed. The guidelines of animal protection of China were followed strictly.

### RNA isolation and purification

Total RNA was isolated from the frozen livers according to the manual of Trizol kit (Invitrogen)[15] and then purified based on the guide of RNeasy mini kit (Qiagen)[16]. In brief, total liver tissues frozen at -80℃ were homogenized liquid nitrogen, and homogenates were split in TRIzol reagent, followed by chloroform extraction and isopentyl alcohol precipitation. The total RNA integrity was confirmed by agarose electrophoresis and checked by the ratio of 28S to 18S. Total RNA concentration and purity were estimated by optical density measurements at 260/280 nm[17].

### cDNA and cRNA synthesis and purification

As template, 0.4 ng total RNA was used for cDNA synthesis. cDNA purification was proceeded based on the methods established by Affymetrix[18]. cRNA labeled with biotin was synthesized using cDNA as the template and then purified[18]. Measurement of cDNA, cRNA concentration and purity were the same as above.

### cRNA fragmentation and microarray detection

Fifteen microliter (1 μg/μL) cRNA incubated with 5 × fragmentation buffer at 94℃ for 35 min was digested into 35-200 bp fragments. The hybridization buffer was added to the prehybridized Rat Genome 230 2.0 microarray produced by Affymetrix, then hybridization was carried out for 16 h at 45℃ on a rotary mixer at 60 rotation/min. The microarray was washed and stained by GeneChip fluidics station 450 (Affymetrix Inc., USA). The chips were scanned by GeneChip Scan 3000 (Affymetrix Inc., USA), and the signal values of gene expression were observed[13].

### Microarray data analysis

Signal values were quantified and normalized with GCOS1.2 software. Quantified signal intensities were obtained by deducting foreground signal values. Signal intensities were replaced by 200 when they were < 200. When experiment/control (Ri) was between 0.1 and 10, Ri was taken as natural logarithms to generate lnRi, and the normalize coefficient factor (ND) was taken by averaged Ri. The modified signal values were generated by ND multiplying control, and were replaced by 200 when it was < 200[13].

### Normalization of microarray data

To minimize error from the microarray analysis, each analysis was performed three times. Results whose total ratio was maximal ($R^m$) and that whose average value of three housekeeping genes (β-actin, hexokinase and glyseraldehyde-3-phosphate dehydrogenase) approached 1.0 ($R^h$) were taken as a reference. The modified data were generated using a correction factor ($R^m/R^h$) multiplying the ratio of every gene in $R^h$ at each time point. To remove spurious gene expression changes resulting from errors in the microarray analysis, the gene expression profiles at 0-4 h, 6-12 h and 12-24 h after PH were reorganized with NAP software (normalization analysis program) according to the cell cycle progression of the regenerating hepatocytes. Data statistics and cluster analysis were done using GeneMath, GeneSpring, and Microsoft Excel software[13,19,20].

### Identification of genes associated with liver regeneration

Firstly, the nomenclature of blood coagulation was adopted from the GENEONTOLOGY database (www.geneontology.org), and input into blood coagulation at NCBI (www.ncbi.nlm.nih.gov) and RGD (rgd.mcw.edu) to identify the rat, mouse and human genes associated with the blood coagulation. According to maps of biological pathways embodied by GENMAPP (www.genmapp.org), KEGG (www. genome.jp/kegg/pathway.html#amino) and BIOCARTA (www.biocarta.com/genes/index.asp), the genes associated with blood coagulation were collated. The results of the analysis were codified, and compared with the results from human and mouse studies in order to identify human and mouse genes which are different from those of rat. In comparison of these genes with the analysis output of the Rat Genome 230 2.0 array, those genes which showed a greater than twofold change in expression level, regarded as meaningful[14] rat homologous genes, were referred to as rat specific genes associated with blood coagulation response under evaluation. Genes, which displayed reproducible results on three independent analyses with the chip and which showed a greater than

**Table 1 Expression abundance of 107 blood coagulation response-associated genes during rat liver regeneration**

| Gene Abbr. | Associated with | Fold difference | Gene Abbr. | Associated with | Fold difference | Gene Abbr. | Associated with | Fold difference | Gene Abbr. | Associated with | Fold difference |
|---|---|---|---|---|---|---|---|---|---|---|---|
| C2 | 1 | 2.1 | Anxa8 | 1, 2 | 0.4 | Fbn1 | 1, 2 | 2.8 | Rab27a | 1, 2 | 3.4 |
| Cd59 | 1 | 0.5 | Anxa9 | 1, 2 | 4.6 | Fgb | 1, 2 | 4.3 | Scube1 | 1, 2 | 3.2 |
| Coch | 1 | 0.2, 2.3 | App | 1, 2 | 6.4 | Fgl2 | 1, 2 | 3.1 | Tbxa2r | 1, 2 | 0.2, 2.0 |
| Daf1 | 1 | 0.2 | B4galt1 | 1, 2 | 3.1 | Fli1 | 1, 2 | 4.9 | Tm4sf3 | 1, 2 | 3.6 |
| Hrg | 1 | 0.5, 2.8 | Bdkrb2 | 1, 2 | 0.4 | G7c | 1, 2 | 3.9 | [3]Tnf | 1, 2 | 3.2 |
| Il1b | 1 | 0.4 | C1qA | 1, 2 | 0.3 | Ggcx | 1, 2 | 0.3, 2.6 | Trove2 | 1, 2 | 0.4 |
| [3]Il6 | 1 | 0.3, 6.1 | C1qr1 | 1, 2 | 5.5 | Gna12 | 1, 2 | 2.5 | [3]A2m | 2 | 0.4, 46.2 |
| Lman1 | 1 | 2.0 | C3 | 1, 2 | 0.2 | Gnaq | 1, 2 | 2.5 | Adora2a | 2 | 0.5, 2.0 |
| Masp1 | 1 | 3.0 | C3ar1 | 1, 2 | 2.3 | [3]Hgf | 1, 2 | 0.4 | C8g | 2 | 0.4, 2.0 |
| Mbl2 | 1 | 0.2 | C4a | 1, 2 | 0.5 | [3]Hnf4a | 1, 2 | 0.1, 4.5 | Cfh | 2 | 2.5 |
| Nfe2 | 1 | 0.1 | C4bpa | 1, 2 | 2.0 | Hs6st2 | 1, 2 | 0.1, 2.6 | Crp | 2 | 0.5, 2.0 |
| Ptgdr | 1 | 0.3, 2.0 | C5r1 | 1, 2 | 0.4, 2.6 | Itgb3 | 1, 2 | 0.2 | Cspg2 | 2 | 0.4, 6.8 |
| Ptger2 | 1 | 0.5 | Cd36 | 1, 2 | 0.1 | Klkb1 | 1, 2 | 0.4 | Ctrl | 2 | 0.2, 4.6 |
| Ptgs1 | 1 | 3.4 | Cfi | 1, 2 | 6.4 | Kng1 | 1, 2 | 2.1 | Dcbld2 | 2 | 0.5, 4.3 |
| Ptgs2 | 1 | 0.1, 2.1 | Clca2 | 1, 2 | 0.5 | Kptn | 1, 2 | 0.2 | Gc | 2 | 3.4 |
| Prss1 | 1 | 0.1 | Clca3 | 1, 2 | 0.2 | Mmrn1 | 1, 2 | 0.1 | Lrp1 | 2 | 0.1, 2.0 |
| Prss2 | 1 | 0.4, 39.4 | Clca4 | 1, 2 | 0.3, 5.7 | P2rx1 | 1, 2 | 9.7 | Phyh | 2 | 2.3 |
| Serpind1 | 1 | 0.1 | Cr2 | 1, 2 | 6.0 | P2ry12 | 1, 2 | 0.1 | Proc | 2 | 0.3, 2.0 |
| Tf | 1 | 2.7 | Crry | 1, 2 | 2.4 | P2y12 | 1, 2 | 0.2 | Procr | 2 | 6.5 |
| Tfpi | 1 | 4.4 | Edn1 | 1, 2 | 0.4, 2.6 | Pabpc4 | 1, 2 | 0.3, 2.1 | Pros1 | 2 | 2.1 |
| Tfpi2 | 1 | 0.4 | Efemp2 | 1, 2 | 0.5, 2.4 | Plat | 1, 2 | 0.4, 4.9 | Serpina5 | 2 | 0.1, 7.8 |
| Tp53 | 1 | 2.9 | Entpd2 | 1, 2 | 0.4 | Plau | 1, 2 | 0.4, 3.0 | Serpinb9 | 2 | 5.3 |
| Anxa2 | 1, 2 | 4.5 | F10 | 1, 2 | 0.5 | Plaur | 1, 2 | 13.9 | Serpine1 | 2 | 16.7 |
| Anxa3 | 1, 2 | 3.9 | F2 | 1, 2 | 0.3 | Plg | 1, 2 | 2.1 | Serpinf2 | 2 | 0.2, 2.0 |
| Anxa4 | 1, 2 | 2.0 | F2rl2 | 1, 2 | 0.2 | Plscr1 | 1, 2 | 7.5 | Serpini2 | 2 | 0.4, 7.0 |
| Anxa5 | 1, 2 | 2.3 | F3 | 1, 2 | 0.1, 2.0 | Ppbp | 1, 2 | 0.1, 2.1 | [3]Thbd | 2 | 9.6 |
| Anxa7 | 1, 2 | 6.8 | F5 | 1, 2 | 0.5 | Rab11fip1 | 1, 2 | 0.3, 8.6 | | | |

1: Extrinsic blood coagulation pathway; 2: Intrinsic blood coagulation pathway; [3]Reported genes associated with liver regeneration.

twofold change in expression level at least at one time point during liver regeneration with significant difference ($0.01 \leqslant P < 0.05$) or extremely significant difference ($P \leqslant 0.01$) between PH and SO, were referred to as associated with liver regeneration.

## RESULTS

### *Expression changes associated with blood coagulation response during liver regeneration*

According to the databases at NCBI, GENMAPP, KEGG, BIOCARTA and RGD, 357 genes were involved in blood coagulation, in which, 174 genes were contained in the Rat Genome 230 2.0 array. Among them, the expression of 107 genes displayed meaningful changes at least at one time point after PH, showed significant or extremely significant differences in expression between PH and SO, and displayed reproducible results at three detections with Rat Genome 230 2.0 array, suggesting that the genes were associated with LR. Fold changes in up-regulation range were more than 2 to 46 fold compared with control, and down-regulation more than 2 to 10 fold (Table 1). The analysis indicated that 44 genes were up-regulated, 36 genes down-, and 27 genes up/down- during liver regeneration. The total up- and down-regulation times were 342 and 253, respectively (Figure 1A). At the initiation phase (0.5-4 h after PH), 24 genes displayed up-regulation, 14 genes down, and 2 genes up/down; at the transition phase from $G_0$ to $G_1$ (4-6 h after PH), 24 genes up, and 8 genes down; at the cell proliferation phase (6-66 h after PH), 45 genes up, 42 genes down, and 11 genes up/down; at cell differentiation and structure-function reorganization phase (66-168 h after PH), 38 genes up, and 27 genes down, and 5 genes up/down (Figure 1B).

### *Initial expression time points associated with blood coagulation response during liver regeneration*

At each time point of liver regeneration, the numbers of initial up-, down-regulated and total up-, down-regulated genes are as follows in sequence: both 9 and 6 at 0.5 h; 6, 10 and 12, 14 at 1 h; 8, 0 and 16, 3 at 2 h; 4, 1 and 17, 4 at 4 h; 4, 2 and 21, 7 at 6 h; 1, 0 and 13, 5 at 8 h; 0, 5 and 12, 10 at 12 h; 7, 7 and 15, 13 at 16 h; 5, 8 and 17, 24 at 18 h; 3, 0 and 18, 16 at 24 h; 2, 3 and 11, 11 at 30 h; 0, 4 and 23, 18 at 36 h; 1, 1 and 9, 9 at 42 h; 1, 2 and 22, 24 at 48 h; 0, 0 and 12, 13 at 54 h; 0, 0 and 22, 11 at 60 h; 2, 0 and 21, 9 at 66 h; 0, 1 and 15, 11 at 72 h; 0, 0 and 11, 10 at 96 h; 4, 0 and 13, 9 at 120 h; 0, 0 and 15, 9 at 144 h; 0, 0 and 12, 13 at 168 h (Figure 2). Generally, gene expression changes occurred during the whole liver regeneration, with the up- and down-regulation times of 342 and 253, respectively. The initially up-regulated genes were predominantly expressed in the forepart, and the down-regulated genes in the prophase and metaphase, whereas there was little initial expression in the anaphase.

### *Expression similarity and time relevance associated with blood coagulation response during liver regeneration*

Totally 107 genes could be characterized based on their similarity in expression as following: only up-,

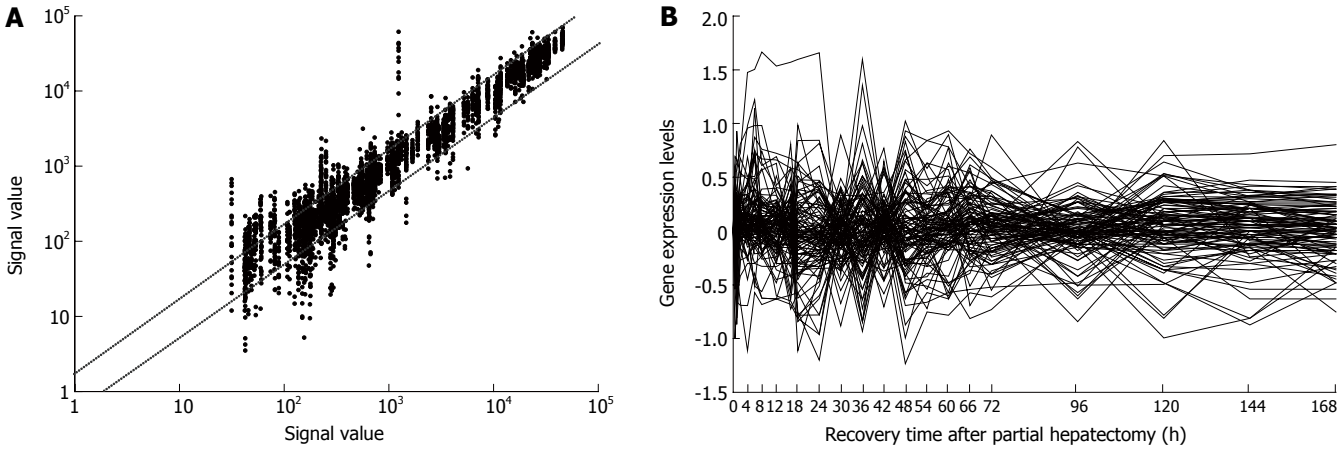


**Figure 1** Expression frequency, abundance and changes of 107 blood coagulation response-associated genes during rat liver regeneration. Detection data of Rat Genome 230 2.0 array were analyzed and graphed with Microsoft Excel. **A**: Gene expression frequency. The dots above bias represent the genes up-regulated more than two fold, and total times of up-regulation were 342; those below bias down-regulated more than two fold, and times of down-regulation were 253; and the ones between biases no-sense alteration; **B**: Gene expression abundance and changes. Seventy-six genes were 2-46 fold up-regulated, and 62 genes 2-10 fold down- regulated.

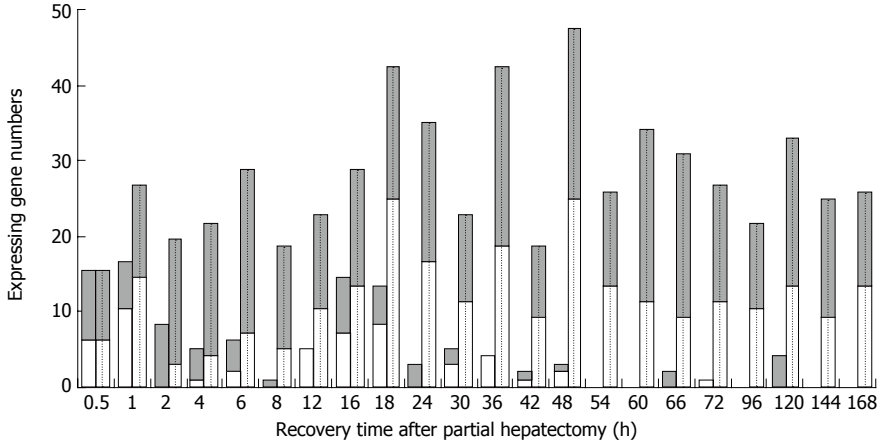


**Figure 2** The initial and total expression profiles of 107 blood coagulation response-associated genes at each time point of liver regeneration. Grey bars: Up-regulated gene; White bars: Down-regulated. Blank bars represent initial expressing genes, in which up-regulated genes were predominant in the forepart, and the down-regulated genes in the prophase and metaphase, whereas there was little initial expression in the anaphase. Dotted bars represent the total expressing genes, in which some genes were up-regulated, and the others down regulated during the whole LR.

predominantly up-, only down-, predominantly down-, up-/down-regulated, involving 44, 8, 36, 13 and 6 genes, respectively (Figure 3). According to time relevance, they were classified into 15 groups, including 0.5 h, 1 h, 2 h, 4 and 6 h, 8 and 12 h, 16 h, 18 and 24 h, 30 and 42 h, 36 h, 48 h, 54 and 60 h, 66 and 72 h, 96 h, 120 h, 144 and 168 h, and the up- and down-regulation times were 9 and 6, 12 and 14, 16 and 3, 38 and 11, 25 and 15, 15 and 13, 35 and 40, 20 and 20, 23 and 18, 22 and 24, 34 and 24, 35 and 20, 11 and 10, 19 and 13, 27 and 22, respectively (Figure 3). The up-regulated expression genes were chiefly associated with promoting blood coagulation, and the down-regulated expression genes mostly associated with inhibiting blood coagulation.

### *Expression patterns of the genes associated with blood coagulation response during liver regeneration*

The 107 genes were categorized into 29 patterns, according to the changes in their expression. (1) up-regulation at one time point, i.e. 6, 16, 30, 66, 120 h after partial hepatectomy (Figure 4A), 8 genes involved; (2) up at two time points, i.e. 6 and 42 h, 30 and 42 h (Figure 4B), 2 genes involved; (3) up at three time points (Figure 4B), 2 genes involved; (4) up at multiple time points (Figure 4C), 5 genes involved; (5) up at one phase, i.e. 1-48, 4-6, 120-168 h (Figure 4D), 3 genes involved; (6) up at one time point/phase, i.e. 0.5 and 4-6 h, 24 and 66-72 h, 42 and 120-168 h, 66 and 120-168 h (Figure 4E), 4 genes involved; (7) up at one time point/two phases (Figure 4F), 2 genes involved; (8) up at one time point/three phases (Figure 4F), 1 gene involved; (9) up at two time points/one phase (Figure 4G), 5 genes involved; (10) up at two time points/phases (Figure 4H), 2 genes involved; (11) up at two time points/three phases (Figure 4H), 3 genes involved; (12) up at three time points/one phase (Figure 4I), 3 genes involved; (13) up at three time points/two phases (Figure 4I), 1 gene involved; (14) up at multiple time points/phases (Figure 4J), 3 genes involved; (15) down at one time point, i.e. 16, 30, 36, 42, 48, 72 h (Figure 4K), 8 genes involved; (16) down at two time points, i.e. 0.5 and 48 h, 1 and 72 h, 16 and 30 h, 16 and 96 h, 18 and 48 h, 18 and 54 h, 30 and 48 h, 30 and 96 h, 36 and 48 h (Figure 4L), 10 genes involved; (17) down at multiple time points (Figure 4M), 4 genes involved; (18) down at one phase, i.e. 6-12 h (Figure 4N), 1 gene involved; (19) down at two phases, i.e. 18-24 and 48-54 h (Figure 4N), 1 gene involved; (20) down at one time point/phase, i.e. 1 and 96-120 h, 18 and 120-144 h, 36 and 12-24 h, 96 and 16-24 h (Figure 4O), 4 genes

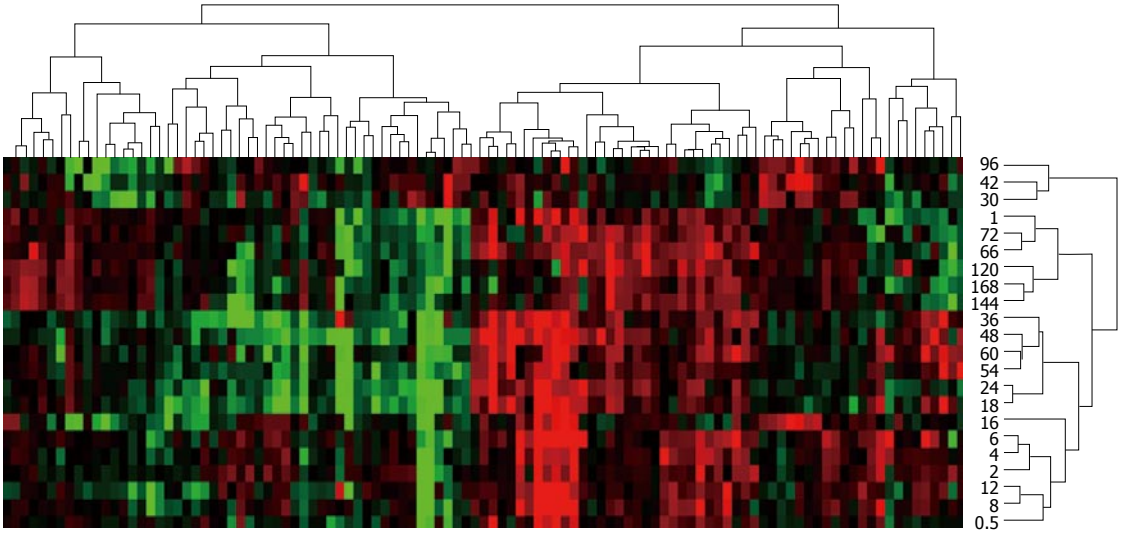


**Figure 3** Expression similarity and time relevance clusters of 107 blood coagulation response-associated genes during liver regeneration. Detection data of Rat Genome 230 2.0 array were analyzed by H-clustering. Red represents up-regulated genes chiefly associated with promoting blood coagulation; Green represents down-regulated ones mainly associated with inhibiting blood coagulation; Black: No-sense in expression change. The upper and right trees respectively show expression similarity and time series clusters, by which the above genes were classified into 5 and 15 groups separately.

involved; (21) down at one time point/two phases (Figure 4O), 1 gene involved; (22) down at one time point/three phases (Figure 4O), 1 gene involved; (23) down at two time points/one phase (Figure 4P), 3 genes involved; (24) down at two time points/phases (Figure 4P), 1 gene involved; (25) down at three time points/two phases (Figure 4Q), 1 gene involved; (26) down at multiple time points/phases (Figure 4Q), 1 gene involved; (27) predominantly up (Figure 4R), 8 genes involved; (28) predominantly down (Figure 4S), 13 genes involved; (29) similarly up/down (Figure 4T), 6 genes involved.

## DISCUSSION

Blood coagulation plays an important physiological role. PH induces blood coagulation, which results from direct or indirect interaction between various proteins. Tissue factor (TF), associated with extrinsic blood coagulation, initiates extrinsic blood coagulation by acting on coagulation factor 7 (F7)[21]. Interleukin 6 (IL6) accelerates the transformation from fibrinogen to fibrin[22]. Twelve proteins including protease serine 2 (PRSS2) inhibit blood coagulation by suppressing the activities of TF and coagulation factors[23-25]. Seven proteins including annexin A 7 (ANXA7) accelerate the transformation from plasminogen to plasmin by inhibiting the activities of factor 10 (F10) and thrombin, repress blood coagulation, and induce migration and tube repair of blood vessel endothelial cells as well[26-28]. The meaningful expression profiles of the proteins encoding the above genes were the same or similar at some points while different at others, indicating that they may co-regulate extrinsic blood coagulation. Among them, *il6* was up-regulated at 2-8, 18, 48, 60 and 96 h, and reached a peak at 96 h, which was 6.1 times higher than the control. This is generally in line with the result reported by Takatori *et al*[29]. *tf* was up-regulated at 16, 30 and 96 h during liver regeneration, and reached a peak at 16 h, which was 2.7 times higher than the control. *prss2* was up-regulated mainly at 18, 36 and 54-60 h, and reached a peak at 36 h, which was 39.4 times higher than the control. *anxa7* was up-regulated at 16, 30, 42 and 96 h, and reached a peak at 96 h, which was 6.8 times higher than the control. It suggests that these genes play a key role in blood coagulation during liver regeneration.

Moreover, six proteins including phospholipid scramblase 1 (PLSCR1), associated with intrinsic blood coagulation, promote blood coagulation by activating coagulation factors[30-33]. Amyloid beta precursor protein (APP) and beta-1,4-galactosyltransferase 1 (B4GALT1) accelerate blood coagulation[34,35]. Nine proteins including alpha-2-macroglobulin (A2M) inhibit blood coagulation by reducing thrombin and collagenase[36-39]. Five proteins including serine peptidase inhibitor clade E member 1 (SERPINE1) depress fibrinolysis by inhibiting the activities of plasma serine protease protein C and plasminogen activator[40,41]. Twelve proteins including plasminogen activator tissue (PLAT) inhibit blood coagulation by converting plasminogen to plasmin through cleaving the Arg-Val bond[42-44]. Thrombomodulin (THBD) restrains blood coagulation[45]. The meaningful expression profiles of the genes encoding the proteins mentioned above were the same -ness or similarity at some points while difference at others, suggesting that they may co-regulate intrinsic blood coagulation. Among them, *thbd* showed up-regulation during almost the whole LR, and had the highest abundance of 9.6 times higher than control at 6 h, which is consistent with the result reported by Takatori *et al*[46]. *a2m* was up-regulated at 0.5-24, 36 and 48-54 h, and reached a peak at 8 h, which was 46.2 times higher than the control. This is generally in conformity with the result reported by Scotte *et al*[47]. *plscr1* was up-regulated at 2-24, 36 and 48-120 h during liver regeneration, and reached a peak at 6 h, which was 2.7 times higher than the control. *app* was up-regulated at multiple phases after PH, and reached a peak at 168 h, which was 3.1 times higher than the control. *serpine1* was up-regulated at 1-48 h, and reached a peak at 6 h, which was 16.7 times

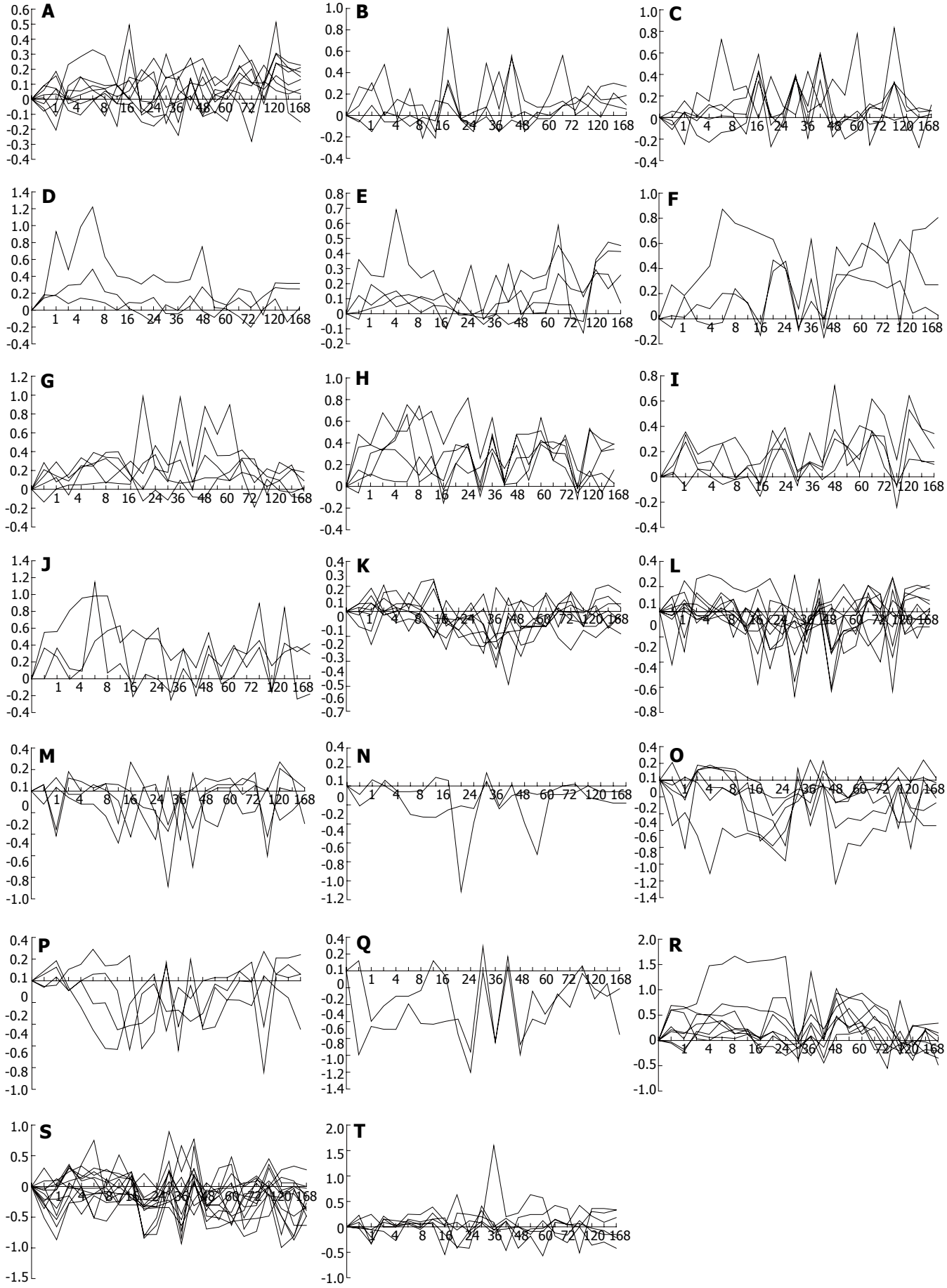


**Figure 4** Twenty-nine gene expression patterns of 107 blood coagulation response-associated genes during liver regeneration. Expression patterns were obtained by the analysis of detection data of Rat Genome 230 2.0 array with Microsoft Excel. **A**-**J**: 44 up-regulated genes; **K**-**Q**: 36 down-regulated genes; **R**-**T**: 27 up/down-regulated genes. X-axis represents recovery time after partial hepatectomy (h); Y-axis shows logarithm ratio of the signal values of genes at each time point to control.

higher than the control. *plat* was up-regulated at multiple phases after PH, and reached a peak at 66 h, which was 4.9 times higher than the control. It implies that these genes play a crucial role in blood coagulation during liver regeneration.

In summary, the expression changes of the genes associated with blood coagulation during liver regeneration have been investigated by high-throughput gene expression analysis and in long time range (0.5 h-7 d after PH) and at multiple time points (totally 23). It is preliminarily proved that PH can cause various physiological responses including blood coagulation, and that Rat Genome 230 2.0 array is a useful tool for analysis of the blood coagulation responses at gene transcriptional level. However, the processes of DNA→mRNA→protein are influenced by many factors including protein interactions. Therefore, our results need to be further analyzed using techniques, such as Northern blotting, protein chip, RNA interference, protein-interaction.

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*CLINICAL RESEARCH*

# Characterization of functional biliary pain and dyspeptic symptoms in patients with sphincter of Oddi dysfunction: Effect of papillotomy

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## Abstract

**AIM:** To characterize functional biliary pain and other gastrointestinal (GI) symptoms in postcholecystectomy syndrome (PCS) patients with and without sphincter of Oddi dysfunction (SOD) proved by endoscopic sphincter of Oddi manometry (ESOM), and to assess the post-endoscopic sphincterotomy (EST) outcome.

**METHODS:** We prospectively investigated 85 cholecystectomized patients referred for ERCP because of PCS and suspected SOD. On admission, all patients completed our questionnaire. Physical examination, laboratory tests, abdominal ultrasound, quantitative hepatobiliary scintigraphy (QHBS), and ERCP were performed in all patients. Based on clinical and ERCP findings 15 patients had unexpected bile duct stone disease and 15 patients had SOD biliary type Ⅰ. ESOM demonstrated an elevated basal pressure in 25 patients with SOD biliary-type Ⅲ. In the remaining 30 cholecystectomized patients without SOD, the liver function tests, ERCP, QHBS and ESOM were all normal. As a control group, 30 'asymptomatic' cholecystectomized volunteers (attended to our hospital for general cardiovascular screening) completed our questionnaire, which is consisted of 50 separate questions on GI symptoms and abdominal pain characteristics. Severity of the abdominal pain (frequency and intensity) was assessed with a visual analogue scale (VAS). In 40 of 80 patients having definite SOD (i.e. patients with SOD biliary type Ⅰ and those with elevated SO basal pressure on ESOM), an EST was performed just after ERCP. In these patients repeated questionnaires were filled at each follow-up visit (at 3 and 6 mo) and a second look QHBS was performed 3 mo after the EST to assess the functional response to EST.

**RESULTS:** The analysis of characteristics of the abdominal pain demonstrated that patients with common bile duct stone and definite SOD had a significantly higher score of symptomatic agreement with previously determined biliary-like pain features than patient groups of PCS without SOD and controls. In contrary, no significant differences were found when the pain severity scores were compared in different groups of PCS patients. In patients with definite SOD, EST induced a significant acceleration of the transpapillary bile flow; and based on the comparison of VASs obtained from the pre- and post-EST questionnaires, the severity scores of abdominal pain were significantly improved, however, only 15 of 35 (43%) patients became completely pain free. Post-EST severity of abdominal pain by VASs was significantly higher in patients with predominant dyspepsia at initial presentation as compared to those without dyspeptic symptoms.

**CONCLUSION:** Persistent GI symptoms and general patient dissatisfaction is a rather common finding after EST in patients with SOD, and correlated with the presence of predominant dyspeptic symptoms at the initial presentation, but does not depend on the technical and functional success of EST.



**Key words:** Postcholecystectomy pain; Sphincter of Oddi dysfunction; Functional biliary-pain; Dyspeptic symptoms; Endoscopic sphincterotomy; Follow-up

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## INTRODUCTION

Sphincter of Oddi (SO) dysfunction is thought to

responsible for postcholecystectomy abdominal pain in up to 13% of patients[1]. Endoscopic sphincter of Oddi manometry (ESOM) is considered the gold standard for the diagnosis of sphincter of Oddi dysfunction (SOD)[2], and in patients with definite SOD (patients with elevated SO basal pressure and those with SOD biliary type Ⅰ, i.e. clinical signs of biliary obstruction due to SO stenosis), endoscopic sphincterotomy (EST) is regarded as the most definitive treatment. Initially, several controlled landmark studies have supported this therapeutical approach[3-5]; however in the majority of these studies the primary end point was the general symptomatic improvement after EST, and no detailed analysis of the global spectrum of the GI symptoms has been performed. Moreover, as we learned from a recent cohort study, although up to 69% of the postcholecystectomy patients with definite SOD exhibited an overall symptomatic relief after EST as compared with only 31% relief in the sham control group, only 18% of the responder patients became completely asymptomatic after EST[6]. In other words, regardless of optimal diagnostic approach and therapy in patients with postcholecystectomy syndrome (PCS), a significant proportion of these patients will never be completely asymptomatic. One explanation might be that similar to those patients with poor outcome after cholecystectomy, the presence of dyspepsia and other GI symptoms before papillotomy may be continued after EST, and this might influence the overall outcome and patient satisfaction[7]. Another critical point could be that ESOM alone is an insufficient method for optimal patient selection for EST, and this might worsen the effectiveness. Therefore, the aims of the present prospective study were to precisely characterize functional biliary pain and other GI symptoms in patients having PCS with and without SO motility disorders documented by ESOM and/or quantitative hepatobiliary scintigraphy (QHBS), and to assess the post-EST outcome of these symptoms.

## MATERIALS AND METHODS

### *Patients*

During the period of year 2001-2005, we prospectively investigated 85 consecutive cholecystectomized patients referred to our endoscopy unit for ERCP because of suspected SOD (Figure 1). On admission, all patients completed our standardized and validated questionnaire on pain and GI symptoms. Liver function tests (LFTs) [ALAT, ASAT, alkaline phosphatase (AP), γGT, serum bilirubin], abdominal ultrasonography, upper GI endoscopy with antral biopsy to test *H pylori*, quantitative hepatobiliary scintigraphy (QHBS), ERCP and in the presence of clear indication ESOM were performed in each patient with suspected SOD. Based on clinical data and ERCP results our patients were categorised by the Geenen and Hogan classification as follows: SOD biliary group type Ⅰ, Ⅱ and Ⅲ. In order to prevent repeated ERCPs and reduce the post-procedure complication rate, we studied the presence of delayed transpapillary bile flow (longer than 45 min) by QHBS instead of measuring the ERCP contrast drainage time. In 15 of 85 patients an unexpected common bile duct (CBD) stone was detected on the ERCP, and therefore an EST and a stone extraction were completed immediately (CBD stone group). According to the elevated LFTs, abnormal QHBS and dilated bile duct on ERCP, 15 of 85 patients were categorised as SOD biliary type I (SOD I group). In these SOD type Ⅰ patients, an EST was performed just after the ERCP without ESOM. An EST was also performed in a further 25 patients with SOD biliary type Ⅱ or Ⅲ, accompanied with an elevated SO basal pressure (over 40 mm Hg) demonstrated on ESOM (ESOM positive SOD group). Finally, in 30 of 85 patients a PCS without SOD was diagnosed (all of them belonged to SOD biliary type Ⅱ and Ⅲ), based on normal LFTs, normal ERCP and negative QHBS and/or ESOM (SOD negative PCS group) (Figure 1). As a control group, 30 'asymptomatic' cholecystectomized volunteers (attended to our hospital for general cardiovascular screening and none of them had been investigated earlier for postcholecystectomy symptoms) were also included in our study, and they filled our questionnaire repeatedly (cholecystectomized control group). In the control group, no further investigation was performed to diagnose functional biliary diseases for lack of indication.

In 40 of 80 patients having definite SOD (i.e., patients with SOD biliary type Ⅰ and those with elevated SO basal pressure on ESOM) an EST was performed just after ERCP (Figure 1). The frequency of mild post-procedure pancreatitis was 14.8%, but no severe complication or procedure related death occurred. Thirty-five of these 40 patients were followed-up for up to 12 mo after the procedure. In these 35 patients repeated questionnaires were filled at each check-up (every 3-6 mo) and a second look QHBS was performed at the 3rd month after the procedure to assess the beneficial effect of EST. Unfortunately 5 patients were dropped out from this arm of the study, which was always due to poor patient co-operation.

Before entering the study all patients were informed about the study, and all gave written informed consent. The study protocol was approved by the Ethical Committee of University of Szeged and was in accordance with the Helsinki Ⅱ Declaration.

Our questionnaire consisted of 50 separate questions on personal data, symptom characteristics and severity of abdominal pain assessed with a visual analogue scale (VAS). The patients were asked to rate their pain frequency and intensity on two separate 100 mm scale with the margins of "no pain at all" on the left and "continuous pain" on the right, *vs* "no pain at all" on the left and "worst possible pain" on the right, respectively. Patients were requested to mark on the VAS how frequent and intensive of their abdominal pain. Severity scores of abdominal pain were calculated by simple addition of VASs of frequency and intensity. Symptomatic characteristics of abdominal pain were assessed further by six questions (four possible answers each), in which the localization, duration, type, connection with feeding, aggravating and relieving factors were precisely determined. According to the literature, biliary-type pains were initially defined as typically localized in the epigastrium or the right upper quadrant, with episodes of steady pain that lasts more than 15-30 min, maybe aggravated by food or fatty food and relieved

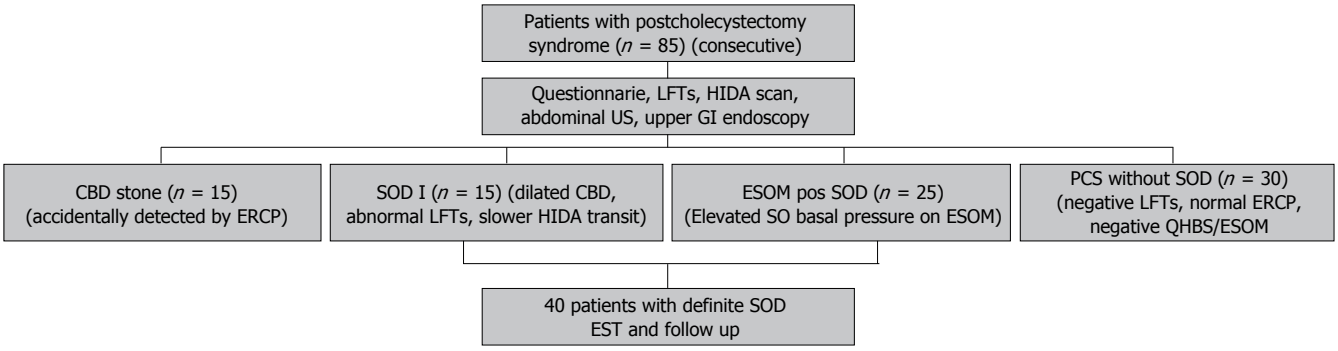


**Figure 1** Flow chart of our patient selection and diagnostic strategy.

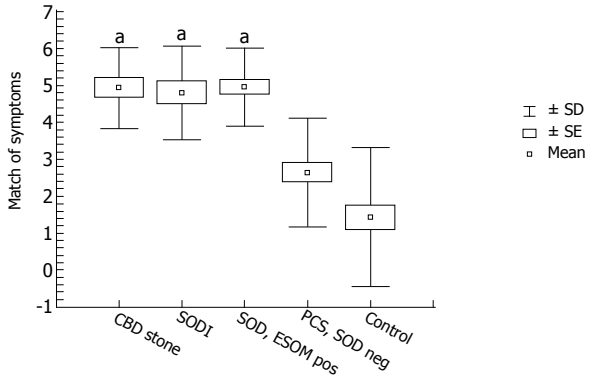


**Figure 2** Abdominal pain characteristics in different postcholecystectomy patient groups based on the results of our questionnaire. Note that significant match of symptom characteristics with the predefined biliary type pain observed predominantly in patients with common bile duct stone and in patients with definite SOD. $^{a}P < 0.05$ *vs* SOD negative PCS and controls.

after spasmolytics or due to low-fat diet. Other possible answers in this part of our questionnaire were focused to characterize possible symptoms of non-biliary diseases such as acid related disorders, dysmotility type dyspepsia, and radiating pain stemming from the thoracic or lumbar zygapophysial joints, i.e. facet syndrome. These abdominal pain characteristics were then scored by counting the cumulative number of the affirmative patient answers that precisely matched with previously described definitions of biliary-type pain.

There were further 28 questions on dyspeptic symptoms, and each of them was assessed by VAS of frequency and intensity in a similar manner as described above. In this part the patients were asked whether they were bothered by heartburn, acid regurgitation, early satiety, bloating and abdominal distension, nausea, vomiting, vomiting of bile, flatulence, borborygmus, diarrhea, constipation, intermittent constipation and diarrhea, anorexia and weight loss. The duration of symptoms and the relation to time of previous cholecystectomy were also determined. The next section aimed to detect psychological vulnerability and neurotic symptoms and consisted of separate questions on psychosomatic and neurotic symptoms, with yes/no category answers. Psychological vulnerability was also scored by counting the cumulative number of affirmative answers. Some previous points of medical history, such as pancreatitis and alcohol consumption were also noted.

### *Statistical analysis*

For statistical evaluation the Kruskal-Wallis followed by the Mann-Whitney *U* tests (quantitative variables) or the chi-square tests (qualitative variables) were applied. Significance was achieved at $P < 0.05$. All results are given as mean, standard error and standard deviation.

QHBS was performed with our standard technique as it was published and validated earlier[8] and was strictly performed in a few days before any invasive procedure. Time-to-peak (Tmax) and half-time-excretion (Thalf) parameters were calculated over the liver parenchyma (L), hepatic hilum (H) and common bile duct (C). The duodenal appearance time (DAT) was also measured. ESOM was performed with the application of the standard station pull through manometric technique[8]. ESOM recording was considered abnormal in cases demonstrating an elevated SO basal pressure (over 40 mm Hg, lasting more than 30 s).

## RESULTS

Analysis of the characteristics of abdominal pain demonstrated, that in patients with CBD stone, SOD I and ESOM positive SOD there was significantly higher score of symptomatic agreement with the previously determined biliary-type pain features than in patient groups with SOD negative PCS and controls (Figure 2). Interestingly, patients with definite SOD (i.e., SOD I and ESOM positive SOD) had exactly similar biliary pain characteristics compared with patients with CBD stone disease. However, most of our patients with SOD negative PCS had atypical pain and symptom presentation.

When the severity scores of abdominal pain at presentation were analysed, all patient groups (CBD stone, SOD I, ESOM positive SOD, and SOD negative PCS) had significantly higher pain severity than the cholecystectomized controls (Figure 3). In fact, the severity level of abdominal pain in SOD negative PCS patients was similar to that in patients having organic disorder such as CBD stone or SOD Ⅰ. Therefore, in contrast to symptomatic characteristics, the severity of abdominal pain had no diagnostic value in differentiating patients with and without SOD.

Based on the analysis of our questionnaire, in 40 patients with definite SOD (SOD Ⅰ patients and ESOM positive patients) a higher prevalence of dyspeptic

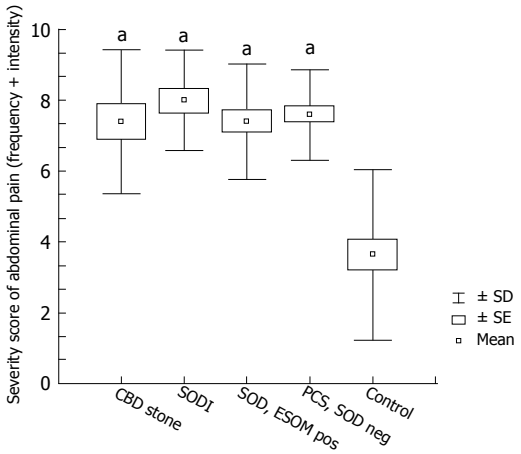


**Figure 3** Severity of abdominal pain (sum of frequency and intensity) determined by VAS in different postcholecystectomy patient groups. Note that patients with postcholecystectomy pain syndrome but without any objective signs of SOD (including negative ESOM/QHBS) have severity scores equivalent to those with common bile duct stone disease. [a]*P* < 0.05 *vs* control.

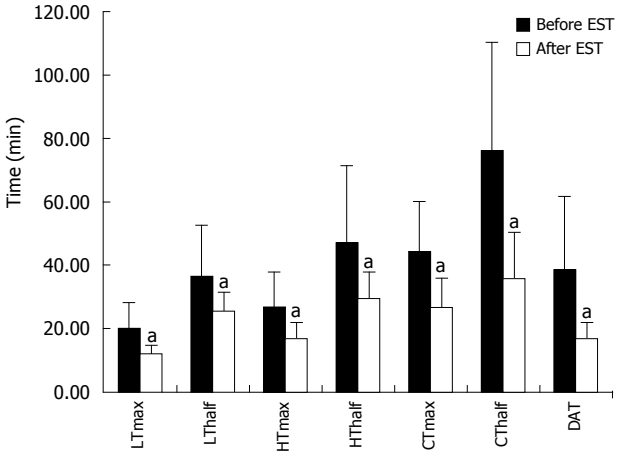


**Figure 5** Effect of endoscopic sphincterotomy on the quantitative parameters of hepatobiliary scintigraphy in patients with definite SOD. Note that all parameters depicts significant improvement of the transpapillary bile flow after papillotomy. [a]*P* < 0.05 *vs* before EST.

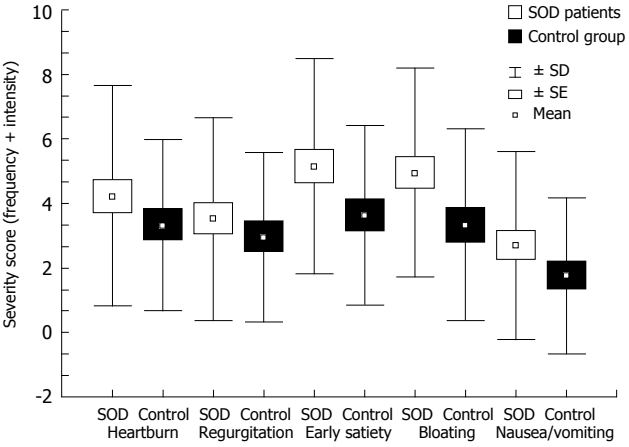


**Figure 4** Comparison of the severity of dyspeptic symptoms (sum of frequency and intensity) determined by VAS in patients with definite SOD and in postcholecystectomy controls. Note that in patients with definite SOD, dyspeptic symptoms were rather frequent coexisting symptoms accompanied with typical biliary pain, although no significant differences detected as compared to "asymptomatic" cholecystectomized controls.

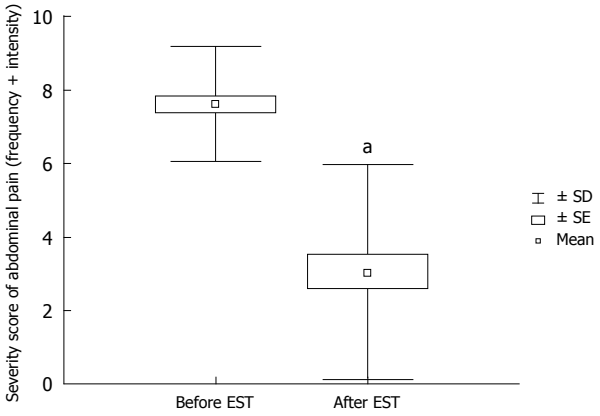


**Figure 6** Dramatic and significant symptomatic improvement of patients with definite SOD determined by the comparison of severity scores (sum of frequency and intensity) of abdominal pain before and after papillotomy documented on the questionnaires (VAS). [a]*P* < 0.05 *vs* before EST.

symptoms associated with biliary-pain was found as compared to controls (Figure 4). The most prevalent dyspeptic symptom was early satiety and bloating, followed by heartburn, regurgitation and nausea or vomiting. Although there was an obvious tendency of higher dyspeptic scores in definite SOD patients, none of the separate symptom scores reached the level of significance as compared to cholecystectomized controls. The prevalence of antral gastritis and *Helicobacter* infection was similar in patients with definite SOD versus in patients with SOD negative PCS: 77% and 27% *vs* 87% and 25%, respectively. No statistical difference was detected between these groups, indicating that dyspeptic symptoms had no relation to antral predominant gastritis or *H pylori* infection in these cholecystectomized patients.

Thirty-five out of 40 patients with definite SOD (SOD I and ESOM positive) were followed up after EST with QHBS and a repeated questionnaire. The median follow up time was 7.5 mo. When the post-EST results of QHBS were compared to the baseline QHBS study (before EST) we detected significant acceleration of all measured quantitative scintigraphic parameters corresponding to both hepatic secretion and transpapillary bile flow (Figure 5). As expected, the EST induced acceleration of the transpapillary bile flow caused a dramatic improvement in the parameters of CBD emptying (Tmax and Thalf), and also the DAT. Based on the comparison of VASs from the pre- and post-EST questionnaires, an impressive and highly significant improvement in the severity scores of abdominal pain was established (Figure 6), which was an obvious demonstration of the therapeutic benefit of EST in these patients with definite SOD. It must be stressed that despite homogenous improvement of biliary pain after endoscopic therapy, only 15 of 35 patients became completely free of pain after EST.

Finally, we categorized our patients based on initial presentation of dyspeptic symptoms into two groups: no or minimal dysmotility in 20 patients (less than 50%

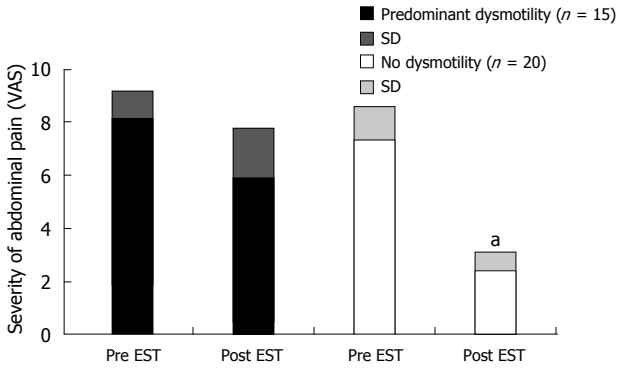


**Figure 7** Effect of the severity of the concomitant dyspeptic symptoms in patients with biliary-type pain and definite SOD on the post-papillotomy outcome. $^{a}P < 0.05$ *vs* those without dyspeptic symptoms.

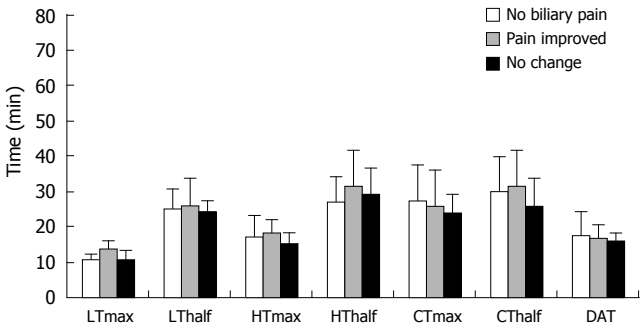


**Figure 8** Comparison of post-papillotomy quantitative hepatobiliary scintigraphic parameters in patient groups with different symptomatic outcome. Lack of statistically significant differences excluded endoscopic technical problems (incomplete papillotomy) in patients with poor symptomatic outcome and persisting biliary pain.

of total dysmotility scores according to the baseline questionnaire), and severe dysmotility in 15 patients (more than 50% of total dysmotility scores on the baseline questionnaire). Interestingly the severity of dyspeptic symptoms before EST was significantly correlated with the symptomatic failure of endoscopic therapy. In fact, we have demonstrated that post-EST severity of abdominal pain based on VASs was significantly higher in those patients with predominant dyspepsia at initial presentation as compared to those without dyspeptic symptoms (Figure 7). This difference in the post-EST outcome was not induced by possible endoscopic technical problems or patient-to-patient variation of the completeness of EST, since the analysis of post-EST QHBS parameters demonstrated no significant differences of these patient groups (Figure 8). No statistical differences were found in the prevalence of gastritis and *H pylori* infection between patients with no or minimal versus predominant dysmotility scores: 80% and 26% *versus* 64% and 27%, respectively. In fact, patients with predominant dysmotility scores had a lower frequency of antral gastritis detected by upper GI endoscopy.

Although there was a tendency for higher psychogenic vulnerability in those patients with definite SOD (SOD Ⅰ and ESOM positive SOD groups), no significant differences were detected when the results of patients were compared to asymptomatic cholecystectomized controls or those with CBD stone. Interestingly, the highest scores of symptom-stress association was detected in those patients belonged to the ESOM positive SOD group, but the patient population was not large enough to reach the level of significance.

## DISCUSSION

Historically, transduodenal biliary sphincteroplasty with transampullary septoplasty has been recommended for the therapy of SOD with a reported symptomatic benefit in 60%-70% of patients during a 1 to 10 years' follow-up[9]. Because of better patient tolerance, lower cost and less long-term re-stenosis endoscopic therapy has widely replaced surgical therapy. According to recent follow-up studies, EST produced a symptomatic relief in 55%-93% of patients with SOD and elevated SO basal pressure documented on ESOM[10-13]. This wide variation in therapeutic response after EST may reflect variations in patient groups treated, but more importantly, differences in criteria used to determine the benefit. Only a few studies evaluated the percentage of improvement after EST in patients with SOD[10,13], but most studies in the literature have applied only qualitative endpoints of symptomatic relief[3-5,11,12]. In the present study, we analysed the effect of EST on the severity scores of abdominal pain assessed by VAS, which demonstrated a homogenous and highly significant improvement in severity scores after EST in patients with definite SOD. In contrary, only 43% of these definite SOD patients became completely pain free after EST. Similar to the findings of Linder *et al*[6], our study clearly demonstrates that although EST does evoke a pain score reduction, complete pain relief is a relatively rare and fortunate outcome in these postcholecystectomy patients. This may be explained by the fact, that similar to patients with irritable bowel syndrome and other chronic functional pain syndromes of GI tract, the pathogenic mechanism of pain in SOD patients is more complex. Dysmotility of the SO is probably only one of the pathogenic components that are frequently accompanied by abnormalities of central nervous system, such as visceral hyperalgesia and hypersensitivity of the sensory receptors.

QHBS is a useful non-invasive method in the diagnosis of SOD by visualizing the consequent partial biliary obstruction, and according to some recent data, there is a highly significant correlation between SO basal pressure and quantitative parameters of transpapillary bile emptying[8,13]. Although the equality of QHBS and ESOM is not generally accepted in the literature[14] due to problems in reproducibility and lack of standardized methodology of QHBS, it is obvious that QHBS is an optimal non-invasive screening method to assess the presence of impaired transpapillary bile flow in SOD patients. Recently, some studies unexpectedly stated that in a certain postcholecystectomy patient population abnormal QHBS is a better predictor for favourable post-EST outcome than abnormal ESOM[13,15]. QHBS has obvious advantages as compared to the traditional ERCP contrast drainage time, as it is cheaper, non-invasive, less time-consuming and prevents unnecessary repeated ERCPs in that patient group, which has an inherent high

risk of post-ERCP complication[16]. Therefore, we suggest a new modified non-invasive SOD classification strategy, where patients must have an adequate biliary pain history, such as our validated questionnaire[17], some relevant LFTs, a QHBS to study bile flow, and an MRCP or endoscopic US to measure bile duct diameter. Based on these data one could be able to classify SOD patients similarly to the original Milwaukee system, but without ERCP and post-ERCP complication.

Although it is rarely mentioned and applied, QHBS is also applicable in the follow-up of SOD patients, to demonstrate the acceleration or normalization of the transpapillary bile flow after EST[13,18-20]. Similar to the study of Corazziari *et al*, in the present study we proved a significant acceleration of all quantitative parameters of QHBS after EST in definite SOD patients as compared to the initial study before ERCP. Interestingly, there was no correlation between the patient general symptomatic improvement or satisfaction and the normalization or improvement in QHBS parameters. Therefore, it seems that there is a patient subpopulation with definite SOD and having biliary pain accompanied with predominant dyspeptic symptoms, which has poor therapeutic response after EST and therefore a general dissatisfaction with endoscopic therapy, despite an effective and complete EST (normalization of transpapillary bile flow on QHBS) performed.

It has been demonstrated by surgical studies, that pain after cholecystectomy was significantly correlated with the concurrent presence of symptoms of dysmotility and dyspepsia[7]. Similarly, our current results demonstrated that post-EST intensity of persistent pain in patients with definite SOD was significantly more severe in the patients with predominant dyspepsia at the initial presentation. Interestingly, there was no correlation between dysmotility and dyspeptic symptoms and the prevalence of antral gastritis or *Helicobacter* co-infection in these patient groups. In comparison to the public data of the general population in Hungary, our patients had a higher prevalence of antral gastritis but a lower prevalence of *H pylori* infection. As a possible explanation, we may consider that duodenogastric bile reflux is a significant cause of gastritis in these postcholecystectomy patients, which might also partially prevent *H pylori* colonization[21]. Whether patients having postcholecystectomy pain are more sensitive to dysmotility and dyspeptic complaints, or they are dyspeptic in origin is unclear. Some studies suggested that the mechanism of dyspeptic symptoms after cholecystectomy might be related to the increased duodenogastric reflux[22]. However, the effect of EST on the volume of duodenogastric reflux is unclear.

In conclusion, EST evokes a significant improvement of pain severity scores in all patients with definite SOD (selected by ERCP or ESOM), but only 43% of these patients became pain free. Persistent gastrointestinal symptoms and general patient dissatisfaction are rather common findings after EST, and correlated with the presence of predominant dyspeptic symptoms at the initial presentation, however, they do not depend on the technical and functional success of EST. Application of a validated questionnaire at the patient selection for EST and the follow up can be extremely useful in all patients having postcholecystectomy syndrome.

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*RAPID COMMUNICATION*

# Medical malpractice litigation related to gastrointestinal endoscopy in Japan: A two-decade review of civil court cases

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## Abstract

**AIM:** To examine the allegations in malpractice litigations related to gastrointestinal endoscopy in Japan.

**METHODS:** A retrospective review of cases tried in the civil court system during the 21-year period from 1985 to 2005, identified in a computerized legal database, was undertaken.

**RESULTS:** Eighteen malpractice litigations and a total of 30 allegations were identified. Of the 18 (44%) malpractice litigations, 8 (44%) were related to esophagogastroduodenoscopy, 4 (22%) to colonoscopy, 4 (22%) to endoscopic sphincterotomy, and 2 (11%) to endoscopic retrograde cholangiopancreatography. Seventeen (94%) cases pertained to complications, and the remaining (6%) case pertained to misdiagnosis. In 10 cases, the patient died of the complications. Allegations were categorized as: (1) performance error during the endoscopic procedure ($n$ = 12, 40%); (2) lack of informed consent ($n$ = 9, 30%); (3) performance error during the treatment after the endoscopic procedure ($n$ = 4, 13%); (4) premedication error ($n$ = 3, 10%); (5) diagnostic error ($n$ = 1, 3%); and (6) indication error for the endoscopic procedure ($n$ = 1, 3%).

**CONCLUSION:** These data may aid in the design of risk prevention strategies to be used by gastrointestinal endoscopists.



**Key words:** Gastrointestinal endoscopy; Malpractice; Litigation; Risk management

## INTRODUCTION

The number of negligence litigations against physicians has been continuously increasing, not only in Western countries but also in Japan[1,2] where a 10-fold increase in malpractice litigations from 102 to 1019 cases per year, has been observed between 1960 and 2003. As the frequency of malpractice claims has risen, physicians have begun to focus on risk management activities. Gastrointestinal endoscopy plays an important role in medical practice. Not only is simple endoscopic examination performed, but also treatment procedures such as endoscopic polypectomy, endoscopic mucosal resection and endoscopic sphincterotomy (EST) are performed. However, various complications associated with these procedures have been reported[3,4]. Thus, gastrointestinal endoscopy is a field in which malpractice actions frequently occur[1,2,5-13].

Furthering the awareness of physicians may be one method of reducing the number of malpractice claims. However, little is known about the allegations underlying medical malpractice litigations in Japan or about those pertaining to gastrointestinal endoscopy in particular. To identify the allegations in malpractice litigations pertaining to gastrointestinal endoscopy, we undertook a review of all Japanese civil court trials from 1985 to 2005 that involved gastrointestinal endoscopy. Our goal was to extract medical information from published court reports to determine the types of gastrointestinal endoscopist errors in these cases.

## MATERIALS AND METHODS

A listing of Japanese civil court trials pertaining to gastrointestinal endoscopy from 1985 to 2005 was retrieved from a computerized legal database (MASTER Library, Shinnippon-hoki Publishing, Tokyo, Japan). Published reports of court decisions in these cases were then obtained from volumes of judicial precedents, and medical information was abstracted from these precedents.

**Table 1 Litigations related to gastrointestinal endoscopy in Japan, 1985-2005**

| | Procedure | Complication/diagnostic error | Allegation 1 | Decision | Allegation 2 | Decision | Allegation 3 | Decision |
|---|---|---|---|---|---|---|---|---|
| 1 | ERCP | Death due to peritonitis induced by duodenal perforation | Performance error during procedure | P | | | | |
| 2 | EGD | Massive hemorrhage | Performance error during procedure | L | | | | |
| 3 | EGD | Death due to premedication shock | Premedication error | P | | | | |
| 4 | Colonoscopic polypectomy | Colonic perforation | Indication error | L | Lack of informed consent | P | | |
| 5 | Colonoscopy | Colonic perforation | Performance error during procedure | P | | | | |
| 6 | EST | Death due to pancreatitis | Performance error during procedure | L | Performance error after procedure | P | | |
| 7 | EGD | Death due to premedication shock | Premedication error | L | Lack of informed consent | L | | |
| 8 | EGD | Massive hemorrhage | Performance error during procedure | P | | | | |
| 9 | EGD removal of foreign body | Unexpected emergent surgery due to fixation of foreign body | Lack of informed consent | P | Performance error during procedure | P | | |
| 10 | EGD | Traffic accident due to the effect of sedative agent | Lack of informed consent | P | | | | |
| 11 | EGD | Death due to premedication shock | Premedication error | L | Lack of informed consent | P | | |
| 12 | Colonoscopy | Colonic perforation | Performance error during procedure | L | Lack of informed consent | P | | |
| 13 | EGD | Misdiagnosis of gastric cancer | Diagnostic error | P | | | | |
| 14 | EST | Death due to pancreatitis | Performance error during procedure | L | Performance error after procedure | L | Lack of informed consent | L |
| 15 | EST | Death due to pancreatitis | Performance error during procedure | L | Performance error after procedure | L | Lack of informed consent | L |
| 16 | ERCP | Death due to pancreatitis | Lack of informed consent | L | Performance error during procedure | P | Performance error after procedure | P |
| 17 | Colonoscopy | Colonic perforation | Performance error during procedure | P | | | | |
| 18 | EST | Death due to peritonitis induced by duodenal perforation | Performance error during procedure | P | | | | |

ERCP: Endoscopic retrograde cholangiopancreatography; EGD: Esophagogastrodudenoscopy; EST: Endoscopic sphincterotomy. P: The claim prevailed; L: The claim was lost.

All allegations of negligence were assigned to 1 of the 6 categories: (1) performance error during the endoscopic procedure, (2) lack of informed consent, (3) performance error during treatment after the endoscopic procedure, (4) premedication error, (5) diagnostic error, and (6) indication error for the endoscopic procedure. Court decisions were also considered.

## RESULTS

Eighteen cases and 30 allegations related to gastrointestinal endoscopy were identified (Table 1). Of the 18 cases, 8 (44%) were related to esophagogastroduodenoscopy (EGD), 4 (22%) to colonoscopy, 4 (22%) to EST, and 2 (11%) to endoscopic retrograde cholangiopancreatography (ERCP). Seventeen (94%) cases pertained to complications, and the remaining (6%) case pertained to misdiagnosis. In 10 cases, the patient died of complications. Perforation was identified in 4 cases, massive hemorrhage in 2 cases, a traffic accident due to the effect of a sedative agent in 1 case, and emergency surgery in 1 case (Table 2). Allegations were as follows: (1) performance error during the endoscopic procedure, $n$ = 12 (40%); (2) lack of informed consent, $n$ = 9 (30%), (3) performance error during treatment after the endoscopic procedure, $n$ = 4 (13%); (4) premedication error, $n$ = 3 (10%); (5) diagnostic error, $n$ = 1 (3%), and (6) indication error for the endoscopic procedure, $n$ = 1 (3%).

In cases related to performance error during the endoscopic procedure, the most frequent procedure was EST (4 cases, 33%) followed by colonoscopy (3 cases, 25%). In all cases related to EST, the patients alleged performance error due to bad technique. Three of the 4 (75%) patients who underwent EST had severe acute pancreatitis after the procedure. The fourth patient died of panperitonitis due to duodenal perforation during the procedure. The 3 cases related to colonoscopy had colonic perforation during the procedure. In these 12 cases, allegations prevailed in 7 (58%) cases.

In cases related to lack of informed consent, 8 of the 9 (89%) allegations involved failure to warn the patient of risks of the procedure, such as possible pancreatitis after ERCP and EST, or the side effects of premedication. In the case related to the side effects of premedication, there were no documented instructions prohibiting driving after endoscopic sedation in the hospital. The remaining case had a foreign body in the patient's stomach, namely an artificial tooth that was 5 cm in length with sharp edges. Because both edges of the tooth were lodged in the esophageal wall during attempted endoscopic removal, the endoscopists abandoned the procedure and performed emergency surgery to remove the tooth. In this case, the

**Table 2 Complications referred to in the litigations ($n$ = 17)**

| Complication | $n$ (%) |
|---|---|
| Death | 9 (53) |
| Shock due to premedication of EGD | 3 (18) |
| Severe acute pancreatitis due to ERCP | 1 (6) |
| Panperitonitis due to duodenal perforation during ERCP | 1 (6) |
| Severe acute pancreatitis due to EST | 3 (18) |
| Panperitonitis due to duodenal perforation during EST | 1 (6) |
| Perforation | 4 (24) |
| Colonoscopy | 3 (18) |
| Colonoscopic polypectomy | 1 (6) |
| Massive hemorrhage after EGD | 2 (12) |
| Traffic accident due to the effect of a sedative used for EGD | 1 (6) |
| Unexpected emergency surgery due to lodging of a foreign body | 1 (6) |

EGD: Esophagogastroduodenoscopy; ERCP: Endoscopic retrograde cholangio-pancreatography; EST: Endoscopic sphincterotomy.

allegation was lack of information about the possible need for emergency surgery. The issue of informed consent never stands alone. In these 9 cases, allegations prevailed in 5 (44%) cases.

Three of the 4 (75%) cases of performance error during treatment after the endoscopic procedure were related to EST, and the remaining case was related to ERCP. All patients in these cases died of severe acute pancreatitis after the procedure. Diagnostic delay and inappropriate treatment to prevent pancreatitis were also alleged. The allegations prevailed in 2 of the 4 (50%) cases.

All of the premedication errors were related to EGD. All patients in these cases suffered shock just after administration of anesthesia or anti-cholinergic agent. Anaphylactic shock was diagnosed in the first 2 cases and acute poisoning due to overadministration of lidocaine was diagnosed in the third case. The allegations prevailed in 1 of the 3 (33%) cases.

The case of diagnostic error was related to misdiagnosis of scirrhous gastric cancer. The patient's stomach was not completely empty during EGD, thus complete gastric examination was not performed. However, the doctor diagnosed gastritis and did not re-examine the patient. She had scirrhous gastric cancer and died 6 mo after the examination. Her family alleged that the doctor overlooked the gastric cancer. The allegation prevailed.

The case of indication error was related to colonic polypectomy. In this case, colonic perforation occurred after colonoscopic polypectomy. The patient alleged that there was no indication for treatment, the allegation was lost.

## DISCUSSION

Medical malpractice has become a predominant concern of physicians in Japan as well as in Western countries. Clinical risk management is the process of collecting, evaluating, and applying data to reduce the frequency of preventable patient injuries[8]. Although general risk management information is abundant in the medical and lay literature, published material specific to gastrointestinal endoscopy is limited to editorials and case presentations[8]. The literature describes various adverse events associated with endoscopy. However, these reports focus on the frequency and nature of procedural complications, avoiding issues of negligence[3,4]. Endoscopists need an understanding of malpractice allegations to develop appropriate risk-reduction strategies for gastrointestinal endoscopy.

In the present study, the most frequent procedure was EGD (39%), followed by EST (22%), colonoscopy (17%), and ERCP (11%). However, according to a Japanese report[2], EGD has been performed 47 times as often as ERCP and 3.5 times as often as colonoscopy. The number of EST procedure is approximately one-tenth that of ERCP. Therefore, the relative frequency of litigation in Japan may be the highest in relation to EST, followed by ERCP and colonoscopy. Additionally, the mortality and complication rates of all endoscopies in Japan have been reported to be 0.00084% and 0.018%, respectively[2]. The mortality rate has been reported to be 0.00045% for EGD and 0.048% and 0.0063% for EST and ERCP, respectively[2]. Given these data showing the remarkable safety of endoscopy, serious complications naturally raise the issue of substandard care. However, an analysis in USA that was derived from an insurance industry database showed that the relative risks of malpractice claims arising from the performance of EGD, ERCP, and colonoscopy are similar[14]. Thus, the relative frequency of litigation may be higher in relation to EST and ERCP in Japan but not in USA. One possible explanation for this discrepancy may be the bias of claims filed against the insurance industry in USA. Another possibility is that strict informed consent may be obtained for EST and ERCP in USA. because of a high frequency of related adverse events. Sufficient information about the adverse events associated with these procedures may decrease litigations.

Performance error during the endoscopic procedure was the most frequent allegation in the malpractice litigations against endoscopists. The potential complications of endoscopy are known to be numerous and well documented. The significant risk of adverse events should be emphasized during the informed consent process[15-17]. Disclosure results in a sharing of risk between the endoscopist and patient may be protective for the endoscopist.

The second most frequent allegation was lack of informed consent. Studies have shown that increased communication reduces malpractice risk, thus the process of informed consent can be a useful tool for reducing this risk[7]. The process of disclosing the inherent risks of a procedure or a medical treatment decision essentially asks the patient to accept those risks as part of the medical plan and procedure. The risk shift does not apply to substandard care, but it would apply to many of the complications that can occur even when procedures are performed properly and medications are administered prudently.

The one diagnostic error in the present study pertained to misdiagnosis of gastric cancer. This case illustrates how an opportunistic diagnosis without complete examination can lead to charges of malpractice[18]. If a complete examination is not performed, doctor should recommend re-examination.

Knowledge of the factors leading to preventable patient injury is needed to develop optimal strategies for reducing malpractice risk related to gastrointestinal endoscopy. On the basis of our study results, we suggest the following risk prevention strategies to decrease the number of malpractice claims related to gastrointestinal endoscopy practice.

First, documenting possible complications during the informed consent process is essential. Complications during endoscopy frequently lead to litigation, especially if the possibility of such complications has not been discussed with the patient in advance.

Second, continued training in safe endoscopic techniques remains the principal method of preventing such complications. For example, participation in morbidity and mortality conferences may help physicians improve their diagnostic and therapeutic skills. Neale[7] examined malpractice claims against gastroenterologists and reported that 44% arose from adverse events related to endoscopy and that there was evidence of serious fault in 50% of the cases, confirming the importance of continued medical education and training in safe endoscopic techniques.

Third, it is important to establish practice guidelines, especially in areas vulnerable to litigation. Such guidelines offer the best method of decreasing endoscopist errors leading to malpractice litigation.

Fourth, endoscopists should recognize that delayed diagnosis of a disease, particularly malignancy, is the most common cause of litigation. Improving the diagnostic ability of endoscopists is essential. In some cases, other modalities such as radiologic and ultrasonographic methods, may aid in prompt and correct diagnosis. Use of other modalities in cases for which a definite diagnosis is not obtained may ensure proper treatment.

In Japan as in USA, most malpractice cases are settled out of court. It has been reported that approximately 90% of malpractice cases in Japan are settled out of court, and the remaining cases lead to litigations[19]. The court decisions are given in only approximately 30% of litigated cases. The compromised and withdrawn cases are not reported or available for review. Therefore, the denominator of the present study might have some bias. However, the aim of this study was to extract medical information from published court reports to determine the sources of endoscopy-related errors in cases requiring litigation. It is hoped that dissemination of the results of the present study among endoscopists can serve as a risk prevention strategy for reducing both complications and malpractice claims pertaining to gastrointestinal endoscopy.

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*RAPID COMMUNICATION*

# Different doses of consensus interferon plus ribavirin in patients with hepatitis C virus genotype 1 relapsed after interferon monotherapy: A randomized controlled trial

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## Abstract

**AIM:** To assess the efficacy of different schedules of consensus interferon (CIFN) plus ribavirin in retreating chronic hepatitis C patients who relapsed after recombinant interferon (rIFN) monotherapy.

**METHODS:** Forty-five patients (34 males and 11 females) with chronic hepatitis due to hepatitis C virus (HCV) genotype 1 who relapsed after a previous course of rIFN monotherapy were randomized to receive 9 μg CIFN three times per week for 52 wk (group A, *n* = 22) or 18 μg CIFN three times per week for 52 wk (group B, *n* = 23) in combination with ribavirin 800 to 1200 mg daily for 52 wk (according to body weight). Virological response was evaluated at week 24 (EVR), at the end of treatment (ETR) and at 76 wk (SVR).

**RESULTS:** By intention-to-treat analysis, subjects in group A had an EVR in 35% of cases, an ETR in 35% and a SVR in 27.3% of cases. Subjects in group B had an EVR in 32% of cases, an ETR in 35% and a SVR in 26.1% of cases. Treatment was stopped because of adverse effects (mostly intolerance) in 15 patients (6 in group A and 9 in group B). IFN dose reduction was needed in 2 patients (1 in group A and 1 in group B). Ribavirin dose was reduced in 2 patients in group A and 1 in group B respectively. Among the 15 subjects who received at least 80% of the intended schedule, the rate of SVR was 80% (6 in group A and 6 in group B).

**CONCLUSION:** CIFN in combination with ribavirin when given to HCV genotype 1 relapsers after rIFN monotherapy obtains an unsatisfactory rate of sustained viral clearance independently of dosage of the drug. This may be due to its scarce tolerability.



**Key words:** Interferon; Ribavirin; Hepatitis C virus; Hepatitis C; Relapser

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## INTRODUCTION

Retreatment of hepatitis C patients who relapsed after recombinant interferon (rIFN) monotherapy has a fair rate of success when ribavirin is added to the original protocol. About 30% of patients infected with hepatitis C virus (HCV) genotype 1 can clear their viral infection when retreated with rIFN plus ribavirin[1]. The efficacy of pegylated IFN (PEG-IFN) in this context has not been explored in large phase Ⅲ registration trials, but an increased effectiveness has been shown in investigator-driven smaller trials. Lawitz *et al*[2] have recently obtained a sustained viral response of 38% in HCV genotype 1 relapsers (24 out of 63 patients) upon retreatment with PEG-IFN plus ribavirin.

Consensus interferon (CIFN) is an engineered IFN α molecule containing the most frequently occurring amino acids among the non-allelic IFN α subtypes[3]. It was reported that CIFN can significantly decrease HCV-RNA in naïf patients with chronic hepatitis C as compared with IFN α -2b[4]. It is thus conceivable that this increased antiviral effect can lead to a better rate of HCV clearance in subjects with a former incomplete response to other types of IFN. Heathcote *et al*[5] found that CIFN monotherapy at a dose of 9 mg three times per week, can obtain a SVR in 39% (24 wk) and 52% (48 wk) of relapsers as compared to rIFN that can obtain a SVR in 12% (24 wk) and 17% (48 wk) of relapsers.

In order to further clarify the role of CIFN in retreatment, we explored its efficacy at different doses in combination with ribavirin in subjects with chronic hepatitis infected with HCV genotype 1 who relapsed after a course of rIFN monotherapy.

## MATERIALS AND METHODS

### Patients

Subjects enrolled between January and December 2001, were eligible if they had the following criteria: age 18-65 years, abnormal alanine aminotransferases (ALT), HCV-RNA positive by PCR (Amplicor® HCV, Roche Diagnostic Systems, Basel, Switzerland), genotype 1. All patients received monotherapy with rIFN (alfa-2a or alfa-2b) with an end-of-treatment virological response (HCV-RNA negative by PCR) and subsequent virological and biochemical relapse prior to this study.

Exclusion criteria were: decompensated cirrhosis, hepatocellular carcinoma, autoimmune hepatitis and other autoimmune diseases, metabolic liver diseases (Wilson's disease, haemochromatosis, α-1-antitripsin deficiency), active drug addiction or alcohol abuse, HBV and/or HIV infection, decompensated diabetes mellitus, haemoglobin concentration at baseline less than 120 g/L in women and 130 g/L in men, platelets below 100 000/mm$^3$ and white blood cells (WBC) below 3000/mm$^3$.

The study design was approved by the University's Ethical Committee. Patients provided their written informed consent before entering the study. All subjects after starting treatment were monitored as outpatients. All had a liver biopsy within 12 mo before starting treatment.

### Treatment schedule

All patients were randomized into two groups: group A ($n$ = 22) received 9 μg CIFN plus ribavirin while group B ($n$ = 23) received 18 μg CIFN plus ribavirin three times per week for 52 wk. Dose of ribavirin ranged according to body weight (800 mg < 65 kg, 1000 between 65 and 75 kg, 1200 > 75 kg). Treatment was stopped if HCV-RNA was still positive at 26 wk.

Patients were seen monthly up to the end of 24 wk post-treatment follow-up. At each visit symptoms and adverse event were recorded, physical examination, and biochemical tests were performed, and a serum sample was collected and stored at -80℃ for virology.

All blood tests were performed at our hospital laboratory. HCV-RNA was determined by qualitative and/or quantitative assays (Amplicor® HCV and Monitor® HCV, ver. 2.0, Roche Diagnostic Systems, Basel, Switzerland) with a detection limit of 100 (Amplicor®) and 1000 (Monitor®) genomes/mL.

The effects of treatment were evaluated in patients who obtained an ETR: early virological response (EVR): HCV-RNA negative after 24 wk of treatment; end-of-treatment response (ETR): HCV-RNA negative at the end of 52 wk of treatment; sustained virological response (SR): HCV-RNA negative 24 wk after stopping therapy.

### Statistical analysis

Statistical analysis was performed by using SPSS software version 11.0.1 for Windows. Means and standard deviation were calculated for continuous variables. The differences in mean values for each group were assessed by using the parametric Student's $t$ test for unpaired data. The statistical significance of differences between subgroups of patients was analyzed using the chi square test for categorical data. Statistical significance was set at $P < 0.05$. Positive predictive value (PPV) was defined as the percent of patients who were observed to have SVR out of the patients predicted to have SVR by a certain criterion (HCV-RNA suppression at the end of therapy).

**Table 1 HCV-RNA clearance at different time points in 2 groups after treatment $n$ (%)**

| | Group A ($n$ = 22) | Group B ($n$ = 23) | $P$ |
|---|---|---|---|
| At 26 wk (EVR) | 7 (31.8) | 8 (34.8) | NS |
| At 52 wk (ETR) | 7 (31.8) | 8 (34.8) | NS |
| At 76 wk (SVR) | 6 (27.3) | 6 (26.1) | NS |

EVR: Early virological response; ETR: End-of-treatment response; SVR: Sustained virological response; NS: Not significant.

## RESULTS

### Features of patients

Forty-five subjects were enrolled between January and December 2001. All patients were infected with HCV genotype 1, and had relapse after an EVR to rIFN monotherapy given 3 to 9 years before. Four subjects had blood transfusions and 4 had a history of i.v. drug abuse, but 37 subjects did not have major risk factors. Twenty-two patients were randomized into group A and twenty-three into group B. The mean age of patients was 41.9 ± 10.9 years. Liver biopsy showed that 3 patients had severe fibrosis (F3 by METAVIR) and 2 had cirrhosis (F4).

### Efficacy of treatment

At 24 wk 9 patients (4 in group A and 5 in group B) discontinued their therapy because they were still HCV-RNA positive. An ETR was observed at 52 wk in 15/45 patients (33.3%) and evolved into a SVR at 76 wk in 12/45 patients (26.6%). At all time points the highest dose of CIFN was not better in obtaining suppression of HCV-RNA replication (ETR: 31.8% in group A *vs* 34.8% in group B, $P$ = NS) and had ultimately a sustained viral clearance (SVR: 27.3% in group A *vs* 26.1% in group B, $P$ = NS, Table 1). The overall PPV value for SVR of negative HCV-RNA in patients with ETR was 80%.

ALT values (Table 2) were comparable between the two groups at different time points. ALT levels became normal under treatment in a number of patients who were still HCV-RNA positive. However, the concordance between ALT and HCV-RNA at 76 wk was 100% in both groups.

### Tolerability and safety

Treatment was stopped in 15 patients (6 in group A and 9 in group B). Five subjects in group A and 7 in group B stopped therapy between wk 1 and 12 (early withdrawal,

**Table 2 ALT normalization at different time points in 2 groups after treatment *n* (%)**

| | Group A (*n* = 22) | Group B (*n* = 23) | *P* |
|---|---|---|---|
| At 4 wk | 12 (54.5) | 10 (43.5) | NS |
| At 12 wk | 13 (59.1) | 13 (56.5) | NS |
| At 26 wk (EVR) | 13 (59.1) | 13 (56.5) | NS |
| At 52 wk (ETR) | 12 (54.5) | 10 (43.5) | NS |
| At 76 wk (SVR) | 6 (27.3) | 6 (26.2) | NS |

EVR: Early virological response; ETR: End-of-treatment response; SVR: Sustained virological response; NS: Not significant.

EW), 1 in group A and 2 in group B between wk 13 and 52 (late withdrawal LW). Reasons for EW and LW were severe flu-like symptoms and depression.

Reduction of the CIFN dose was needed in two subjects (1 in group A and 1 in group B). Reduction of the ribavirin dose was needed in 2 subjects of group A and 1 subject of group B. Overall, 7 subjects in group A and 8 in group B received at least 80% of the intended cIFN dose and at least 80% of the intended RBV dose. Among these ideally treated patients, the overall rate of SVR was 80% (6 in group A and 6 in group B).

## DISCUSSION

Our results do not support a major usefulness of cIFN in retreatment of patients who had a relapse after IFN monotherapy. ITT analysis was able to obtain a SVR only in 26.7% of all patients, without any clear dose. effect in favor of higher doses. Treatment was not particularly well tolerated, since one third of the patients discontinued their regimen, mostly in its early phase due to adverse reactions. When a reasonable compliance to the intended treatment schedule was obtained, the success rate was markedly higher. The main problem in our study was thus the scarce tolerability of combination therapy even at low CIFN doses.

CIFN was assessed in the patients who did not respond to previous courses of IFN. Lindsay[5], who reviewed the comparative virological efficacy of different interferons, stated that in patients who respond to an initial course of alpha interferon and then have a relapse, retreatment with cIFN for 48 wk obtains a high sustained virological response rate, which is similar to that with interferon alpha-2b combined with ribavirin for 24 wk[6]. An Italian group[7] later reported that cIFN given at a dose of 9 g, 5 times per week for 36 wk could obtain a sustained response in 5 out of 12 subjects (42%) who relapsed after combination therapy with interferon alpha-2b and ribavirin. Recently Moskovitz *et al*[8] found that high dose induction therapy with 15 g cIFN/day in prior non-responders to IFN -2b and ribavirin could lead to loss of detectable HCV-RNA in 50% of patients, but this response is only sustained in 8% of patients (2 out of 24) at the end of therapy. Successful retreatment of IFN/RBV non-responders has been reported by Kaiser *et al*[9], who assessed the efficacy of cIFN daily dosing and induction therapy followed by ribavirin combination treatment in 182 (92% genotype 1) non-responders to former combination therapies. cIFN was given at the dose s of 27, 18 or 9 μg for 4 wk, followed by a reduction to 9 μg for 8 wk in the two higher dosed arms. The sustained SVR was 38%-45% in standard interferon/ribavirin non-responders and 27%-31% in PEG-IFN/ribavirin non-responders. The cIFN dose was reduced in 16%-21% of patients and discontinued in 7%-9% of patients. A randomized controlled trial comparing cIFN plus RBV to standard alfa IFN plus RBV in naïf patients[10] has found a lower rate of treatment withdrawal (less than 10% withdrawals, and treatment within an 80/80/80 schedule achieved in ¾ of all patients). This study also showed that the efficacy of cIFN was higher than that of standard IFN-2b in combination therapy of naïf patients (SVR: 57% *vs* 40%).

The reason for the high rate of treatment interruption in our population is not clear. It was reported that when a formal comparison with IFN-2b was performed in the context or in relapsers after IFN monotherapy[11], the rate of SVR is 58% *vs* 29% in favor of cIFN. However, the overall treatment period was shorter (6 mo for both treatment arms) and 5% of the patients were treated with cIFN and 18% of those on IFN-2b were not infected with HCV genotype 1. Our study could not evaluate whether the low adherence is intrinsic to the drug regimens or attributable to patient-related factors. Since most withdrawals were decided early during therapy at a time when both the patient and the physician were not aware of any virological outcome, and some of these patients had normal or reduced ALT, it is unlikely that any perceived ineffectiveness of therapy may have influenced this choice. An issue that may potentially interfere with the treatment effectiveness is the concurrent diffusion of PEG-IFN based regimens, which are perceived as more tolerable and effective during the period of study. This fact might have oriented the decision of some patients or caregivers to stop therapy in order to receive the newest drug. Since the ultimate results with cIFN are not superior to those with PEG-IFN in the setting of combination therapy, we see no reason to further consider this drug in retreatment of HCV relapsers.

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*RAPID COMMUNICATION*

# Insulin resistance in *H pylori* infection and its association with oxidative stress

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## Abstract

**AIM:** To determine the insulin resistance (IR) and oxidative status in *H pylori* infection and to find out if there is any relationship between these parameters and insulin resistance.

**METHODS:** Fifty-five *H pylori* positive and 48 *H pylori* negative patients were enrolled. The homeostasis model assessment (HOMA) was used to assess insulin resistance. Serum total antioxidant capacity (TAC), total oxidant status (TOS) and oxidative stress index (OSI) were determined in all subjects.

**RESULTS:** The total antioxidant capacity was significantly lower in *H pylori* positive group than in *H pylori* negative group (1.36 ± 0.33 and 1.70 ± 0.50, respectively; $P < 0.001$), while the total oxidant status and oxidative stress index were significantly higher in H pylori positive group than in *H pylori* negative group (6.79 ± 3.40 and 5.08 ± 0.95, and 5.42 ± 3.40 and 3.10 ± 0.92, respectively; $P < 0.001$). Insulin resistance was significantly higher in *H pylori* positive group than in *H pylori* negative group (6.92 ± 3.86 and 3.61 ± 1.67, respectively; $P < 0.001$). Insulin resistance was found to be significantly correlated with total antioxidant capacity ($r = -0.251$, $P < 0.05$), total oxidant status ($r = 0.365$, $P < 0.05$), and oxidative stress index ($r = 0.267$, $P < 0.05$).

**CONCLUSION:** Insulin resistance seems to be associated with increased oxidative stress in *H pylori* infection. Further studies are needed to clarify the mechanisms underlying this association and elucidate the effect of adding antioxidant vitamins to *H pylori* eradication therapy on insulin resistance during *H pylori* infection.



**Key words:** *H pylori*; Insulin resistance; Total antioxidant capacity; Total oxidant status; Oxidative stress index

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## INTRODUCTION

*H pylori* is a noninvasive, microaerophile, nonspore-forming, and spiral-shaped microorganism. *H pylori* is associated with severe gastric pathologies, including chronic active gastritis, peptic ulcer, gastric adenocarcinoma and type B low-grade mucosa-associated lymphoid tissue lymphoma[1].

*H pylori* infection causes inflammation, accumulation of reactive oxygen species (ROS), and oxidative DNA damage in gastric mucosa[1]. *H pylori* induces infiltration and activation of neutrophils and macrophages[2]. One characteristic event in inflammation is the infiltration of the affected tissue by neutrophils, which produce large amounts of ROS in host defence reactions. Enhanced ROS levels due to neutrophil infiltration and increased oxidative DNA damage have been reported in *H pylori*-infected patients[3-6]. The increased level of pro-oxidative factors and decreased level of antioxidants in severe oxidative stress can modulate many processes in gastric epithelium[2].

Although *H pylori* seems to be a cause for gastric focal inflammation, it can invade and colonize human stomach, and directly interact with gastric epithelial cells. Moreover, it is associated with non-gastrointestinal tract conditions such as atherosclerosis, insulin resistance, diabetes mellitus and some autoimmune diseases[1,7-9]. The association of *H pylori* infection with insulin resistance has been reported[10-12]. However, to our knowledge, the association between insulin resistance and oxidative status has not been previously investigated in *H pylori* infection.

The present study was, therefore, to determine the insulin resistance, systemic parameters of oxidative stress

and antioxidative system in *H pylori* infection and to find out if there is any relationship between oxidative status and insulin resistance in *H pylori* infection.

## MATERIALS AND METHODS

### Subjects

One hundred and three patients who underwent upper gastrointestinal endoscopy for evaluation of dyspeptic complaints and diagnosed as non ulcer dyspepsia were included in the present study. The patients were divided into two groups according to the presence of *H pylori* infection. Fifty-five patients were *H pylori* positive and 48 patients were *H pylori* negative. The study protocol was carried out in accordance with the Helsinki Declaration revised in 1989. All subjects were informed about the study protocol and written consents were obtained from all participants.

### Diagnosis of H pylori infection

During upper gastrointestinal endoscopy, 2 antral biopsy samples were taken for rapid urease test (CLO test) and histopathologic examination. *H pylori* was considered to be present when the rapid urease test and histological examination were positive. Biopsy samplesl were stained with hematoxylin and eosin for histopathological examination and evaluated according to the updated Sydney System[13]. The patient was considered to be *H pylori* negative if both rapid urease test and histological examination were negative. The diagnosis of *H pylori* infection was confirmed if both the urease test and histological examination were positive.

### Exclusion criteria

Exclusion criteria included recent gastrointestinal by-pass surgery, pregnancy, usage of supplemental vitamins several months prior to the study, *H pylori* eradication therapy, $H_2$ receptor antogonist or proton pump inhibitor within the last 4 wk or nonsteroidal antiinflammatory drugs (NSAIDs) within the last 2 wk prior to study, existence of diabetes mellitus, hyperlipidemia, hypertension, coronary artery disease, cerebrovascular disease, rheumatoid arthritis, renal disease, smoking, cancer, systemic or local infection.

### Samples

Blood samples were obtained following an overnight fasting. Samples were withdrawn from a cubital vein into blood tubes and immediately stored on ice at 4℃. The serum was then separated from the cells by centrifugation at 3000 r/min for 10 min.

### Laboratory methods

Serum glucose concentration was measured using commercial kits (Abbott®) in an autoanalyser (Aeroset®, Germany). Serum insulin levels were measured using an automated chemiluminescence autoanalyzer (Roche®).

### Measurement of serum total antioxidantc capacity

Total antioxidant capacity (TAC) of serum was determined using a novel automated measurement method as previously described[14]. In brief, hydroxyl radical which is the most potentbiological radical was produced. In the assay, ferrous ion solution which is present in the reagent 1 was mixed with hydrogen peroxide which is present in reagent 2. The sequential-produced radicals, such as brown-colored dianisidinyl radical cation produced by the hydroxyl radical, are also potent radicals. Using this method, antioxidative effect of the sample on the potent free radical reactions initiated by the produced hydroxyl radical, was determined. The assay achieved excellent precision values lower than 3%. The results were expressed as mmol Trolox equivalent/L.

### Measurement of total oxidant status

Total oxidant status (TOS) of serum was determined using a novel automated measurement method as previously described[15]. Oxidants present in the sample oxidized the ferrous ion-o-dianisidine complex to ferric ion. The oxidation reaction was enhanced by glycerol molecules abundantly present in the reaction medium. The ferric ion produced a colored complex with xylenol orange in an acidic medium. The color intensity, which could be measured spectrophotometrically, was related to the total amount of oxidant molecules present in the sample. The assay was calibrated with hydrogen peroxide and the results were expressed in terms of micromolar hydrogen peroxide equivalent per liter (μmol $H_2O_2$ equivalent/L).

### Determination of oxidative stress index

The ratio of TOS to TAC was accepted as the oxidative stress index (OSI). For calculation, the resulting unit of TAC was changed to mmol/L, and the OSI value was calculated according to the following formula[16]: OSI (arbitrary unit) = TOS (μmol $H_2O_2$ equivalent/L)/TAC (mmol Trolox equivalent/L).

### Insulin resistance

The insulin resistance index was calculated on the basis of fasting values for glycaemia and insulinemia, according to the homeostasis model assessment (HOMA)[17]: insulin resistance (HOMA-IR) = fasting insulinaemia (μU/mL) × fasting glycaemia (mmol/L)/22.5.

### Statistical analysis

All data were expressed as mean ± SD. Comparisons of parameters were performed with Student's *t* test and correlation analyses were performed using Pearson correlation test. $P < 0.05$ was considered statistically significant. Statistical analyses were performed by SPSS 11 statistical package.

## RESULTS

Demographic charesteristic of the subjects are shown in Table 1. There were no statistically significant differences between the two groups with regard to age, gender, and body mass indexi (BMI) and glucose level ($P > 0.05$) (Table 1).

Insulin and insulin resistance levels were significantly

**Table 1 Demographic and clinical data of *H pylori* positive and negative groups (mean ± SD)**

| Parameters | *H pylori* negative ($n$ = 48) | *H pylori* positive ($n$ = 55) | *P* |
|---|---|---|---|
| Age (yr) | 35 ± 15 | 37 ± 12 | NS |
| Sex (Female/Male) | 28/20 | 29/26 | NS |
| Body mass index (kg/m$^2$) | 22.5 ± 3.1 | 23.5 ± 1.6 | NS |
| Glucose (mg/dL) | 98.15 ± 14.97 | 97.71 ± 14.39 | NS |
| Insulin (μU/mL) | 3.61 ± 1.67 | 6.92 ± 3.86 | < 0.001 |
| HOMA-IR | 0.89 ± 0.47 | 1.67 ± 0.99 | < 0.001 |

IR: Insulin resistance; NS: Not significant.

**Table 2 Oxidative and antioxidative parameters in *H pylori* positive and negative groups (mean ± SD)**

| | *H pylori* negative ($n$ = 48) | *H pylori* positive ($n$ = 55) | *P* |
|---|---|---|---|
| TAC (mmol Trolox eq./L) | 1.70 ± 0.50 | 1.36 ± 0.33 | < 0.001 |
| TOS (μmol $H_2O_2$ equiv./L) | 5.08 ± 0.95 | 6.79 ± 3.40 | < 0.001 |
| OSI (arbitrary unit) | 3.10 ± 0.92 | 5.42 ± 3.40 | < 0.001 |

TAC: Total antioxidant capacity; TOS: Total oxidant status; OSI: Oxidative stress index.

higher in *H pylori* positive group than in *H pylori* negative group ($P < 0.001$) (Table 1).

TAC level was significantly lower in the patients *H pylori* positive group than in *H pylori* negative group ($P < 0.001$), while TOS level and OSI value were significantly higher in *H pylori* positive group than in H pylori negative group ($P < 0.001$) (Table 2).

In Pearson correlation analysis, IR was found to be significantly correlated with TAC ($r$ = -0.251, $P < 0.05$), TOS ($r$ = 0.365, $P < 0.05$), and OSI ($r$ = 0.267, $P < 0.05$).

## DISCUSSION

Information about the association of insulin resistance with *H pylori* infection is scarcely available[10,11]. Aydemir *et al*[10] reported that insulin resistance is significantly related with *H pylori* infection. However, Park *et al*[11] reported that no improvement in the metabolic parameters including insulin resistance could be observed following eradication of *H pylori*. In term of increased insulin resistance during *H pylori* infection, our results are in consistent with those of Aydemir *et al*[10]. As we did not investigate the effect of *H pylori* eradication on insulin resistance, we were not able to compare our results with those of Park *et al*[11].

To our knowledge, the association of oxidative stress with insulin resistance in *H pylori* infection has not been investigated previously. We found a significant association between increased oxidative stress and insulin resistance in *H pylori* infection. It has been reported that *H pylori* infection is associated with increased tissue and systemic oxidative stress[18]. Moreover, oxidative stress has been proposed as the root cause for the development of insulin resistance, B-cell dysfunction, impaired glucose tolerance and type 2 diabetes mellitus[19]. In addition, various antioxidants such as vitamin E, alpha-lipoic acid, and N-acetylcysteine have been shown to have improving impact on insulin resistance[20-22]. Thus, the association of insulin resistance with *H pylori* infection observed in our study seems to be due to oxidative stress induced by *H pylori*.

Many studies indicate that there are evident alterations in gastrointestinal hormone levels in *H pylori* infection[10,23-27]. *H pylori* infection has been found to decrease the expression of antral somatostation and to increase the release of acid-stimulating hormone gastrin[23]. Gastrin can inhibit glucose absorption in the small intestine[24] and amplify glucose-stimulated insulin release[25]. A link beween *H pylori* infection, serum gastrin, insulin and serum glucose concentrations has been demonstrated in dyspeptic patients[26]. During oral glucose ingestion, gastrin probably contributes very little to the insulin release. Gastrin may significantly stimulate the insulin secretion after protein-rich meals. Ordinary meal could stmulate immediate release of endogenous gastrin. The rise in serum gastrin is acute, preceding the increase in insulin concentrations[25]. Somatostatin regulates pancreatic insulin secretion and has an inhibitory effect on insulin release[10,26,27]. Decreased somatostation and increased gastrin hormone levels in patients with *H pylori* infection may play a role in the development of insulin resistance. However, in the present study, since we did not investigate gut hormones except for insulin, we could not provide any information related to this topic.

In conclusion, our findings suggest that insulin resistance seems to be associated with increased oxidative stress in *H pylori* infection. Further studies are needed to clarify the mechanisms underlying this association and elucidate the effect of adding antioxidant vitamins to *H pylori* eradication therapy on insulin resistance during *H pylori* infection.

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*RAPID COMMUNICATION*

# Analysis of serum antibody profile against *H pylori* VacA and CagA antigens in Turkish patients with duodenal ulcer

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## Abstract

**AIM:** To investigate the frequency of seropositivity against CagA, VacA proteins and to determine their independent effects on the development of duodenal ulcer (DU) in Turkish patients.

**METHODS:** The study was designed as a prospective one from a tertiary referral hospital. Dyspeptic patients who were referred to our endoscopy unit for upper gastrointestinal endoscopy between June 2003 and March 2004 and diagnosed to have DU or nonulcer dyspepsia (NUD) were included. Biopsies from the antrum and body of the stomach were taken in order to assess the current *H pylori* status by histology, rapid urease test and culture. Fasting sera were obtained from all patients and *H pylori* status of all sera was determined by IgG antibodies using an enzyme-linked immunosorbent assay (ELISA) kit. All seropositive patients were further analysed using Western blot assays detecting IgG antibodies against CagA and VacA proteins. The $\chi^2$ test was used for statistical comparison of the values and age-sex adjusted multiple regression analysis was used to determine the independent effects of CagA and VacA seropositivities on the development of DU.

**RESULTS:** Sixty-three patients with DU and 62 patients with NUD were eligible for the final analysis. Seropositivity for anti-CagA was detected in 51 of 62 (82%), and in 55 of 63 (87%) patients with NUD and DU, respectively ($P$ = no significance), and seropositivity for anti-VacA was found in 25 of 62 (40% ) and in 16 of 63 (25%) patients, with NUD and DU, respectively.

**CONCLUSION:** These findings suggest that none of these virulence factors is associated with the development of DU in the studied Turkish patients with dyspepsia.



**Key words:** *H pylori*; Western blot; CagA; VacA; Duodenal ulcer

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## INTRODUCTION

Since the discovery of *H pylori* in 1983, the diagnosis and treatment of upper gastrointestinal disease have changed greatly. A strong association has been established between colonization of the gastric mucosa by *H pylori* and various benign and malignant gastroduodenal diseases including chronic active gastritis, gastric ulceration and duodenal ulcer, gastric adenocarcinoma and gastric lymphoma of mucosa-associated lymphoid tissue type[1]. The World Health Organization and International Agency for Research on Cancer consensus group stated in 1994 that there is sufficient epidemiologic and histologic evidence *H pylori* can be clarified as a definite carcinogen[2].

However, not all infected subjects develop disease complications and such a wide spectrum of diseases associated with *H pylori* infection may depend on the heterogeneity of *H pylori* and/or the host response to the same *H pylori* strain[3]. Besides immunological factors in the host, there are specific virulence determinants in *H pylori* strains that influence the outcome of the infection. It has been suggested that both possession of the *cagA* gene and production of a vacuolating cytotoxin encoded by the *vacA* gene, are linked with the

increased pathogenecity of *H pylori* strains. The *cagA* gene encodes a 120-140 kDa protein CagA, and is a part of a large pathogenecity island[4]. Strains expressing the CagA protein have been found to induce more severe inflammation, a higher degree of gastric atrophy, a higher incidence of duodenal ulcer and gastric adenocarcinoma of intestinal type[5,6]. The *vacA* gene is present in all *H pylori* strains, however it is expressed in only 50%-65% of them, thus inducing vacuolization of cells *in vitro*[7-9]. This *vacA* gene encodes a 81-91 kDa protein VacA, which has been shown to provoke the formation of vacuoles in gastric epithelial cells[4].

Turkey is a developing country with a very high prevalance of *H pylori*. A Turkish study dealing with asymptomatic children, using the UBT as the diagnostic test, found that one child out of two under 11 years of age is infected with *H pylori*[10]. Another serology based Turkish study in asymptomatic subjects indicates that about 70% of adults have antibodies against *H pylori* in our population[11]. These data obviously indicate that infection with *H pylori* is a major health problem in our country. The aim of the current study was to evaluate the frequency of cagA and vacA seropositivity in Turkish patients with deudenal ulcer (DU) and in controls with non ulcer dyspepsia (NUD) and to determine their independent effects on the development of DU.

## MATERIALS AND METHODS

A total of 125 patients (63 with DU and 62 with NUD serving as a control group) who were referred to the Endoscopy Unit of Istanbul University, Cerrahpasa Medical Faculty between June 2003 and March 2004 were included. Inclusion criteria were the indication of endoscopy for the study of dyspeptic symptoms. Exclusion criteria were as follows: age under 18, previous gastric surgery and *H pylori* eradication treatment, consumption of antibiotics a month prior to the study, consumption of antisecretory drugs, bismuth salts or sucralfate two weeks prior to the study. A history of bleeding and coagulation disorders that are contraindications for biopsy sampling was also the reason for exclusion.The study was approved by the Ethics Committee of Istanbul University, Cerrahpasa Medical Faculty and all patients gave their written informed consent to participate in the study.

From each patient four antrum and three corpus biopsies were collected for histology (two antrum and one corpus biopsy specimens), rapid urease test (one antrum and one corpus biopsy specimens) and culture (one antrum and one corpus biopsy specimens). One antrum and one corpus biopsy specimens were placed in a CLOtest (Ballard Medical Products, Draper, Utah, USA), maintained at room temperature and read at 1 h and 24 h after sampling. Biopsy specimens were processed for histological examination according to the standard procedure. Hematoxylin and eosin staining as well as a special staining for *H pylori* (Giemsa) were performed. All biopsy samples were examined by the same pathologist (S.E), who specialises in digestive diseases.

Biopsy specimens used for bacterial culture were placed in 2 mL of phosphat-buffered saline at 4℃ and then smeared on the surface of *H pylori* agar (Biomerieux, Lyon, France) plate. Isolates were identified as *H pylori* by Gram stain morphology and the positivity of urease, catalase and oxidase.

**Table 1 Demographic characteristics of the patients included**

| Endoscopic finding | *n* (%) | Range | Male/Female (%) | % Hp (+) |
|---|---|---|---|---|
| Normal | 62 (49.6) | 36.90 ± 12.36 (18-68) | 20/42 (32/68) | 93 |
| Duodenal ulcer | 63 (50.4 ) | 43.14 ± 16.27 (18-80) | 39/24 (62/38) | 97 |

A patient was classified as *H pylori*-positive if the culture alone or both histology and rapid urease test were positive in the presence of a negative culture and as *H pylori*-negative only if all these tests remained negative.

Fasting serum samples were obtained from all patients and stored at -20℃ until assayed. The *H pylori* status of all sera was determined by enzyme-linked immunosorbent assay (ELISA; Euroimmune, Lübeck, Germany) of anti-IgG as described by the manufacturer. The cut-off value for the ELISA was 20 U/mL, antibody concentrations less than this value were considered seronegative, whereas values more than 20 U/mL were assessed as seropositive. All seropositive patients were further analysed with the Western blot technique. We used commercially available Western blot kits (Euroimmun, Lübeck, Germany) to qualitatively detect IgG antibodies against VacA (95 kDa) and CagA (120 kDa).

Continuous variables, one-way analysis of variance between the groups were calculated using. Categorical variables were analyzed with $\chi^2$ test and age-sex adjusted multiple logistic regression analysis was used to determine the independent effects of different virulence factors on the development of DU. $P < 0.05$ was considered statisticallly significant. All the statistical analyses were performed using SPSS 11 for Windows.

## RESULTS

A total of 158 patients were enrolled in the study. Eleven patients (7%) did not give their serum samples although their written informed consent was obtained, and 22 of the remaining 147 (15%) patients were seronegative by anti- IgG ELISA. These 33 patients were excluded from the final analysis.

A total of 125 patients (62 with NUD, 63 with DU) with a mean age of 40.04 ± 14.74 years (range 18-80 years) were eligible for the final analysis. Demographic characteristics of the patients are summarized in Table 1. The patients were classsified as *H pylori* positive when the culture and/or histology plus rapid urease test gave positive results. When all three tests gave concordant negative results the patient was considered negative for *H pylori*. According to these criteria, 6 of 125 (5%) patients had discordant results and 113 of the remaining 119 (95%) patients were positive for *H pylori*.

**Table 2 Percentage of seroreactivity to CagA and VacA antigens detected by Western blot in different groups of patients *n* (%)**

| | Anti-CagA IgG | Anti-VacA IgG |
|---|---|---|
| NUD (*n* = 62) | 51/62 (82) | 25/62 (40) |
| DU (*n* = 63) | 55/63 (87) | 16/63 (25) |
| *P* | 0.432 | 0.076 |
| Total (*n* = 125) | 106/125 (85) | 41/125 (33) |

NUD : Non-ulcer dyspepsia; DU : Duodenal ulcer.

The percentages of seroreactivity to CagA and VacA antigens detected by Western blot in different groups of patients are summarized in Table 2.

The percentage of seroreactivity to different antigens were as follows: anti-CagA with DU 51 of 62 (82%) patients, with NUD and in 55 of 63 (87%) patients with DU ($\chi^2 = 0.617$; $P = 0.432$), anti-VacA in 25 of 62 (40%) patients with NUD and in 16 of 63 (25%) patients with DU ($\chi^2 = 3.158$; $P = 0.076$).

Age and sex adjusted multiple regression analysis disclosed that none of these factors was an independent risk factor for the development of DU ($P = 0.736$ and $P = 0.214$ for CagA and VacA, respectively).

## DISCUSSION

*H pylori* infection is one of the most common chronic bacterial infections worldwide. Although most infected persons remain asymptomatic, 15% to 20% of *H pylori*-positive individuals develop peptic ulcer, gastric carcinoma, or mucosa-associated lymphoid tissue lymphoma[12]. However, it remains unclear why only a small number of infected patients develop such severe diseases. This phenomenon may be due to the differences in host genetics, environmental factors, and the virulence of bacterial strains.

*H pylori* strains are highly diverse[13,14]. Of the two main *H pylori* strains, type I produces a vacuolating cytotoxin, whereas type Ⅱ usually does not[15]. The genome of almost all type I strains has a gene coding for CagA, a highly immunogenic molecular weight protein, which is not present in most type Ⅱ strains. The *vacA* gene coding for vacuolating cytotoxin is polymorphic and present in all strains and the polymorphism of *vacA* accounts for the phenotypic differences between type I and type Ⅱ strains, both types synthesise a VacA protein which is active as a vacuolating cytotoxin in type I and inactive in type Ⅱ strains[15,16]. Infection with VacA-positive strains has been reported to be associated with particular gastroduodenal diseases[7,17-20] especially peptic ulcers[17, 20].

Approximately 60% of *H pylori* isolates harbor the *cagA* gene which encodes another putative virulence factor, CagA that can be variable in size (128-152 kDa)[21,22]. Serological and microbiological studies indicate that CagA-positive strains are associated with enhanced induction of local inflammatory response[23,24] and the presence of this protein has been linked to the development of peptic ulcer disease and gastric cancer[7,21,22,24-27]. However, there are geographic variations in prevalance of both VacA and CagA. Especially in East Asian countries very high prevalances of VacA and CagA-positive strains have been reported[28-30]. Based on these findings, Maeda *et al*[28] concluded that these virulence factors cannot be used as markers of particular gastroduodenal diseases. However, it was reported that the high prevalance of these factors may contribute to the characterization of *H pylori* infection in Japan.

Besides these, other reports suggest that neither VacA nor CagA positivities are associated with more serious gastroduodenal diseases. Yamaoka *et al*[31,32] examined a large number of strains from both Western and East Asian countries and constructed models to discriminate different clinical outcomes on the basis of the presence of *H pylori* putative virulence factors and concluded that none of these factors is helpful in predicting the clinical presentation.

Such conflicting results in the prevalance and clinical usefullness of these virulence factors have been observed in Turkey too. Regarding the CagA and VacA status, studies in dyspeptic patients from different regions of Turkey showed that the seropositivity is 62%-97% and 70%-76%, respectively[33-36]. However, only two of these studies[33,34] have found a significant correlation between the presence of anti-CagA antibodies and peptic ulcer. A very recent study from Turkey[37] displayed that 78% of dyspepsia patients harbour the *cagA* gene and that the *cagA* gene is significantly associated with peptic ulcer and gastric cancer. All these data indicate that the association between putative virulence factors and the development of particular gastrointestinal diseases in *H pylori*-infected individuals is still a worldwide matter of debate.

In the present study, we did not observe any significant differences in seropositivities of the studied virulence factors CagA and VacA between patients with DU and controls with NUD. The overall prevalence of CagA in seropositive patients was 85%, which is in concordance with other studies from Turkey[33-36], but the seropositivity of VacA was just 33%, a low percentage compared to previous studies[33,36], suggesting that this difference may be due to the use of commercially available different Western blot kits in these studies. We think that using VacA antigens from Turkish *H pylori* strains rather than the commercially available ones could lead to more conclusive results.

*H pylori* has a unique set of virulence factors, actively supporting its survival in the special ecological niche of the human stomach while VacA and CagA are the two major bacterial virulence factors involved in host cell modulation. Although several studies have been performed on this issue, results are still conflicting. It was reported that none of these putative virulence factors has disease specificity and that there is evidence that virulence is a host-dependent factor[38]. The primary factors responsible for the different patterns of gastritis in response to *H pylori* infection are environmental factors (e.g. diet) rather than the *H pylori* strain[38].

In conclusion, the fact that similar frequencies of CagA and VacA-positive *H pylori* strains were observed in all our dyspeptic patients, regardless of ulcer status, suggests that factors other than these may contribute to gastrointestinal pathology in patients with *H pylori* infection. Further studies are needed to eliminate the potential bias like the selection of controls with a large sample size including different diseases and ethnic groups in order to make the results robust.

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*RAPID COMMUNICATION*

# Immunohistochemical study on distribution of endocrine cells in gastrointestinal tract of flower fish (*Pseudophoxinus antalyae*)

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## Abstract

**AIM:** To detect distribution and relative frequency of endocrine cells in gastrointestinal tract of flower fish (*Pseudophoxinus antalyae*).

**METHODS:** The intestinal tract of flower fish was divided into four portions from proximal to distal; the enlarged area after oesophagus and anterior, middle and posterior intestine. Immunohistochemical method using the peroxidase anti-peroxidase complex was employed. All antisera between four portions of flower fish were compared using ANOVA.

**RESULTS:** Eleven types of gut endocrine cells were determined; they were immunoreactive for calcitonin gene related peptide, substance P, vasoactive intestinal peptide, bombesin, somatostatin-14, secretin, TrkA, TrkB, TrkC, neurotensin, neuropeptide Y, which were found in almost all portions of the gastrointestinal tract.

**CONCLUSION:** The regional distribution and relative frequency of immunoreactive cells in the flower fish, *Pseudophoxinus antalyae*, are essentially similar to those of other fish.



**Key words**: Flower fish; Gastrointestinal tract; Immunoreactive cells; *Pseudophoxinus antalyae*

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## INTRODUCTION

Gastrointestinal hormones are secreted by endocrine cells which are distributed throughout the mucosa of the gastrointestinal tract. They play important roles in the overall regulation of digestive processes such as nutrient absorption, gut motility and intestinal blood flow[1]. Fish digestive tract shows remarkable differences in function and morphology. Despite some differences in species basis, some basic histological structures are somewhat common. Differences observed at specific level should be related with food, feeding habits, body weight, shape and sex[2-4] . Furthermore, presence of a relation between feeding behaviour and basic histological structure[2] or improbability of this[3,5] have been argued.

Almost all the activities involved in the physiological control of vertebrate gut function during fasting or feeding are mediated by the neuroendocrine system. In the vertebrate gut, epithelial cells belonging to the diffuse endocrine system (DES) interact with components of the enteric nervous system (ENS) in regulating digestive functions, such as enzyme secretion, nutrient uptake and the progression of food through the alimentary canal. Concerning fish, several peptides are produced by the components of the neuroendocrine system and are involved in the communication between DES and ENS. Some of these peptides are associated with the modulation of fish alimentary behaviour[6].

Although they are one of the most common endemic species, the regional distribution and relative frequency of endocrine cells in the flower fish, *Pseudophoxinus antalyae*, have not been studied. Therefore, in the present study, the regional distribution and relative frequency of endocrine cells in the gastrointestinal tract was investigated by immunohistochemistry using 11 types of specific antisera, calcitonin gene related peptide (CGRP), substance P, vasoactive intestinal peptide (VIP), bombesin, somatostatin-14, secretin, neurotensin, neuropeptide Y (NPY), TrkA, TrkB, TrkC raised against mammalian regulatory peptides.

## MATERIALS AND METHODS

Ten adult (length 15-20 cm, weight 40-50 g) flower fish

*(Pseudophoxinus antalyae)* were used in this study without sexual distinction. After being anaesthetized with ethyl ether, the intestinal tract of flower fish was divided into four portions from proximal to distal; the enlarged area after oesophagus and anterior, middle and posterior intestine. All samples were fixed for 12 h in Bouin's solution and embedded in paraffin. Serial, transverse 6-7 μm sections of these portions were cut. Each representative section was deparaffinized, rehydrated and immunostained using the peroxidase anti-peroxidase (PAP) method[7]. Sections were treated with methanol containing 0.3% $H_2O_2$ for 30 min to block any endogenous peroxidase. Subsequently, the sections were incubated for 1 h at room temperature in normal goat serum (1:100), then stained immunohistochemically to identify specific endocrine cells using PAP. The sections were incubated with specific antisera for individual peptides for 12 h at 4℃. Details of primary antisera used in this study are listed in Table 1. After rinsing with phosphate buffered saline (PBS, 0.01 mol/L, pH 7.4), the sections were incubated in secondary antiserum (ant-rabbit IgG serum raised in goats; 1:200) for 1 h at room temperature. Sections were then washed with PBS buffer and incubated with PAP complex (1:400) for 1 h at room temperature. The peroxidase reaction was carried out in 0.02% 3, 3-diaminobenzidine tetrahydrochloride solution containing 0.01% $H_2O_2$ in Tris-HCl buffer (0.05 mol/L, pH 7.6). After immunostaining, the sections were lightly counterstained with Mayer's haematoxylin and immunoreactive (IR) cells were observed with a light microscope.

### Statistical analysis

The mean number of endocrine cells per intestinal fold that were immunoreactive to CGRP, substance P, VIP, bombesin, somatostatin-14, secretin, neurotensin, NPY, TrkA, TrkB, TrkC antisera between four portions of flower fish were compared using ANOVA. The level of significance was set at 0.001.

## RESULTS

Eleven kinds of immunoreactive cells were detected using the antisera against CGRP, substance P, VIP, bombesin, somatostatin-14, secretin, neurotensin, NPY, TrkA, TrkB, TrkC in the intestinal tract. According to the location of the gut and situation in those regions, different regional distribution and relative frequencies of these immunoreactive cells were observed (Figure 1). These differences are shown in Table 2 and Figure 2.

CGRP-IR cells were found throughout the gastrointestinal tract at various relative frequencies. Numerous CGRP-IR cells were detected in the epithelial mucosa. These cells were moderate in number in the enlarged area after oesophagus and anterior, middle intestine and then decreased slightly in the posterior intestine. Substance P-IR cells were found in all portions of gastrointestinal tract. These cells were moderate in number in the enlarged area after oesophagus and middle intestine. The number of cells was decreased in middle intestine. Cells immunoreactive for VIP were numerous in posterior intestine. VIP-IR cells, which were at highest frequency in the enlarged area after oesophagus, were found in the epithelia throughout the tract at various frequencies. These cells increased in number from the posterior intestine to the enlarged area after oesophagus.

**Table 1 List of primary antisera used in the present study**

| Antiserum | Code | Dilution | Source |
|---|---|---|---|
| CGRP | sc-28920 | 1:200 | Santa Cruz Biotech Inc. |
| Substance P | sc-9758 | 1:200 | Santa Cruz Biotech Inc. |
| VIP | NCL-VIPp | 1:200 | Nova Castra Lab, UK |
| Bombesin | NCL-BOMp | 1:200 | Nova Castra Lab, UK |
| Somatostatin | 14CL-SOMATOp | 1:200 | Nova Castra Lab, UK |
| Secretin | sc-20938 | 1:200 | Santa Cruz Biotech Inc. |
| Neurotensin | sc-20806 | 1:200 | Santa Cruz Biotech Inc. |
| NPY | sc-28943 | 1:200 | Santa Cruz Biotech Inc. |
| TrkA | sc-118 | 1:200 | Santa Cruz Biotech Inc. |
| TrkB | sc-12 | 1:200 | Santa Cruz Biotech Inc. |
| TrkC | sc-117 | 1:200 | Santa Cruz Biotech Inc. |

All antisera were raised in rabbit. CGRP: Calcitonin gene related peptide; VIP: Vasoactive intestinal peptide; NPY: Neuropeptide Y.

Bombesin-IR cells varied in number from moderate to numerous in the tract. They then decreased gradually from moderate numbers in the anterior intestine to only a few in the middle intestine. Cells that were immunoreactive for somatostatin-14 were found throughout the gastrointestinal tract except for the posterior intestine. Somatostatin-14 IR cells were demonstrated in both the enlarged area after oesophagus and middle intestine but were more numerous in the former. No somatostatin-IR cells were observed in the posterior intestine. The secretin-IR cells were restricted to the posterior intestine with a rare frequency. They were most numerous in the other portions.

In the enlarged area after oesophagus the presence of endocrine cells which were TrkA immunoreactive, was of a moderate frequency. No cells were found in other regions. TrkB and TrkC IR cells were found at a very low frequency, with the exception of the enlarged area after oesophagus, where their frequency was very low.

NPY-IR cells were demonstrated in both the enlarged area after oesophagus and middle intestine, but they were very rare. No cells were determined in the anterior and posterior intestine. Neurotensin-immunoreactive cells, which were at highest frequency in the enlarged area after oesophagus, were found in all portions at various frequencies. These cells declined in number from the anterior intestine to the middle intestine. These immunoreactive cells were found in the posterior intestine at very low frequencies.

## DISCUSSION

Endocrine cells of the gastrointestinal tract are different in the relative frequency in regional distribution and cell types, between animal species[8]. In the present study, CGRP, substance P, VIP, bombesin, somatostatin-14, secretin, TrkA, TrkB, TrkC, NPY, neurotensin-IR cells

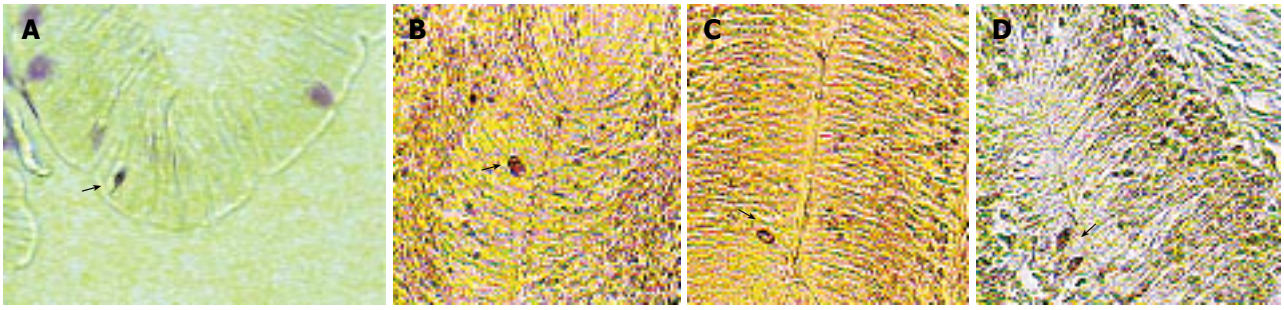


**Figure 1** Photomicrographs of immunoreactive endocrine cells in the gastrointestinal tract of the flower fish. **A**: Bombesin immunoreactive cells in the enlarged area after oesophagus; **B**: Somatostatin immunoreactive cells in the enlarged area after oesophagus; **C**: Substance-P immunoreactive cells in the enlarged area after oesophagus; **D**: Vasoactive intestinal peptide immunoreactive cells in the anterior intestine (× 450).

**Table 2 Mean number of endocrine cells per intestinal fold (mean ± SD) in flower fish (*Pseudophoxinus antalyae*) (200 intestinal folds were examined for each antisera)**

| Region | Calcitonin gene related peptide | Substance-P | Vasoactive intestinal polypeptide | Bombesin | Somato-statin-14 | Secretin | TrkA | TrkB | TrkC | Neuropep-tide Y | Neuro-tensin | Anova (F) | *P* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| The enlarged area after oesophagus | 5.00 ± 1.550 | 4.75 ± 2.217 | 20.50 ± 9.883 | 3.75 ± 2.500 | 8.50 ± 9.037 | 3.50 ± 1.291 | 4.00 ± 4.359 | 3.00 ± 100 | 5.67 ± 3.215 | 0.67 ± 0.577 | 4.33 ± 3.215 | 4.456 | 0.001 |
| Anterior intestine | 5.25 ± 2.500 | 2.75 ± 0.957 | 12.00 ± 6.481 | 2.25 ± 1.258 | 2.75 ± 1.500 | 4.25 ± 2.062 | 0.00 ± 0.000 | 2.67 ± 2.887 | 1.00 ± 1.000 | 0.00 ± 0.000 | 1.33 ± 0.577 | 6.186 | 0.000 |
| Middle intestine | 5.25 ± 2.217 | 4.25 ± 0.957 | 12.25 ± 6.702 | 1.00 ± 0.816 | 7.75 ± 1.708 | 4.75 ± 1.708 | 0.00 ± 0.000 | 1.67 ± 1.528 | 1.00 ± 1.000 | 0.67 ± 0.577 | 1.00 ± 1.000 | 8.115 | 0.000 |
| Posterior intestine | 4.50 ± 2.380 | 6.75 ± 2.217 | 7.25 ± 2.217 | 3.00 ± 0.816 | 0.00 ± 0.000 | 2.00 ± 1.44 | 0.00 ± 0.000 | 0.67 ± 1.155 | 0.67 ± 1.155 | 0.00 ± 0.000 | 0.33 ± 0.577 | 12.829 | 0.000 |

were identified in the gastrointestinal tract of the flower fish, *Pseudophoxinus antalyae*.

The appearance of CGRP-IR cells was well demonstrated in the gastrointestinal tract of the *Anguilla anguilla*[9] , the *Salmo trutta*[10]. Dezfuli *et al*[10] reported that CGRP-IR cells localized in only nerve cell bodies of the myenteric plexus, but in this study these cells were observed in surface epithelia and crypt.

It is well known substance P exerts immunostimulatory and pro-inflammatory effects[11]. Our present results on the GI tract of flower fish agree with previous reports[6,9,10,12-15].

VIP is a peptide consisting of 28 amino acids. Experiments carried out in fishes demonstrated that this peptide is found in nerves and acts as an exciting or inhibiting agent[1]. In the present study, numerous VIP immunoreactive cells were determined in all regions of the gastrointestinal tract. Similar results were reported by Dezfuli *et al*[10] who also found that VIP IR cells were sparsely distributed in the *Salmo trutta* digestive tract. VIP-immunoreactivity in the intestinal nerve fibres of myenteric plexus and ganglion cells and intestinal muscle layer has also been reported in *Anguilla anguilla*[9], *Scyliorhinus stellaris* and *Brachydanio rerio*[16], *Barbus conchonius*[12].

Bombesin is a tetradecapeptide originally isolated from the skin of the amphibian *Bombina bombin*. The presence of bombesin immunoreactivity has been reported in ECs of the gastrointestinal tract in representatives of all the major vertebrate groups except mammals, and in the enteric nerves of mammals, amphibians and fish[6]. Bombesin-like peptide may play an endocrine role in control of gastric functions such as regulation of acid secretion and gastric motility[17]. These cell types were observed in the gut of *Amia calva*[17], *Oncorhynchus mykiss*[18], *Salmo trutta*[6]. Smilar results were obtained in this study.

Somatostatin, which consists of 14 amino acids, was isolated from the hypothalamus of sheep for the first time and it could be subdivided into straight form and cyclic form[19]. As in the present study, somatostatin-14 immunoreactive cells have been determined in the intestine of many fish species, such as *Oncorhynchus mykiss*[18], species of the *Osteoglossomorpha*[20], *Pelteobagrus fuluidraca*, *Monopterus albus*, *Siniperca chuatsi*[15], *Zacco platypus*[19], *Coreoperca herzi*[9].

Secretin is a 27-amino acid polypeptide and belongs to the structurally-related peptides of the pituitary adenylate cyclase-activating polypeptide (PACAP)/glucagon superfamily[21]. In the present study, secretin-IR cells were found in the gastrointestinal tract. These findings agree well with those of previous reports[6,22] , but differ somewhat from results in the *Zacco platypus*[19] and *Coreoperca herzi*[9] where no secretin immunoreactive cells were observed.

Lucini *et al*[23] showed that Trk-like proteins (TrkA, TrkB, Trkc) IR cells were present throughout the intestine of teleost species. They also stated that this immunoreactivity was present in the myenteric plexus of whole intestinal regions containing nerve fibers in the muscle layer. In addition, Radaelli *et al*[24] reported that these cells were present in the gut of both *Pagrus major* and *Dentex dentex*. In the present study, TrkA immunoreactive cells were found only in the enlarged area after oesophagus. In the other portions of gastrointestinal tract,

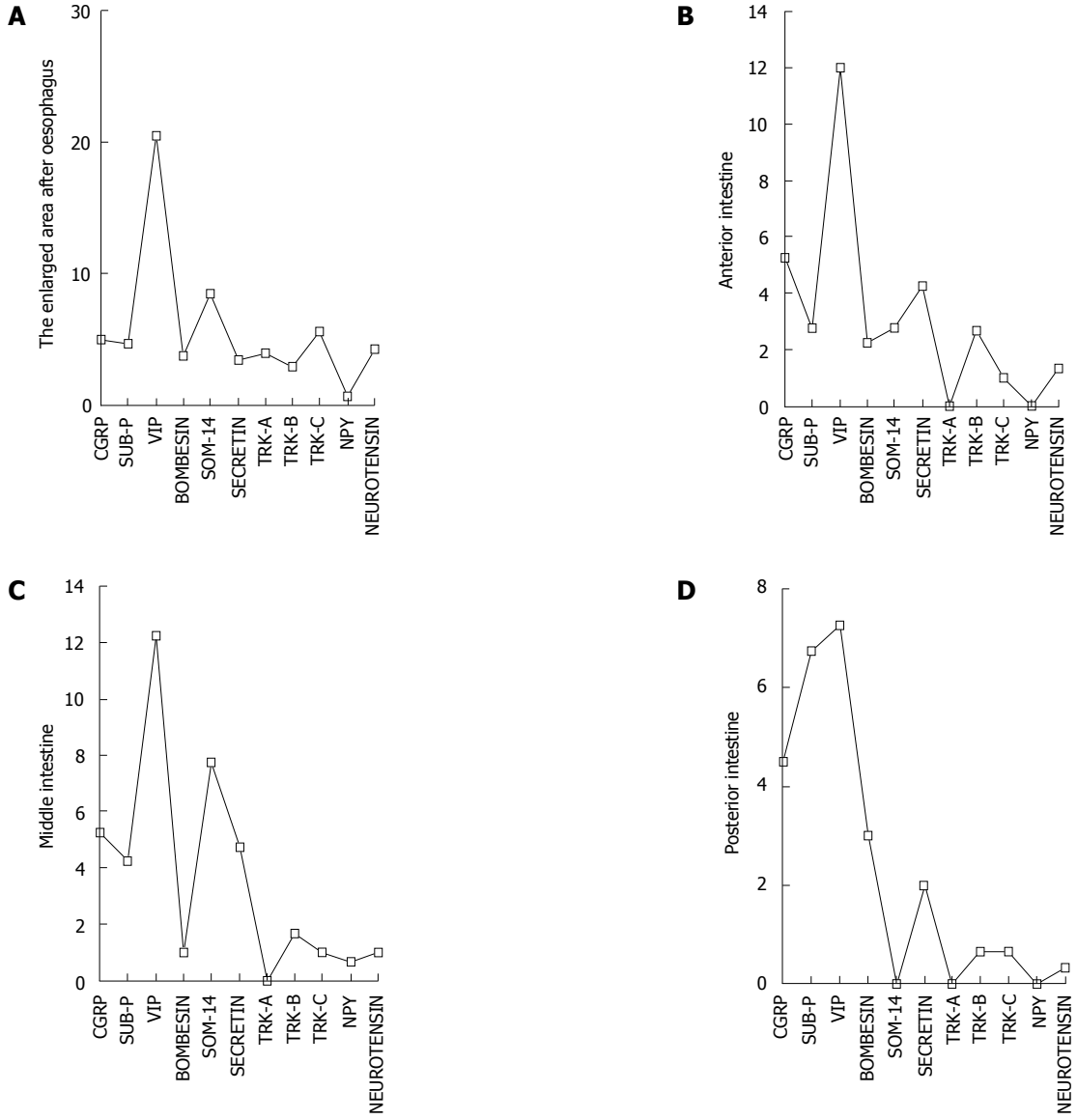


**Figure 2** Density of the immunoreactive endocrine cells in the enlarged area after oesophagus (**A**), the anterior intestine (**B**), the middle intestine (**C**) and the posterior intestine (**D**).

no cells were detected. TrkB and TrkC immunoreactive cells were scattered amongst the epithelial cells throughout the intestine, similar to that reported by Radaelli *et al*[24] and Lucini *et al*[23].

NPY is a 36-amino acid peptide that is widely and abundantly expressed in the central nervous system of all vertebrates investigated[25]. In the present study, NPY-IR cells were found in the enlarged area after oesophagus and middle intestine. These findings are consistent with those of previous reports[9,20]. Rombout and Reinecke[12] showed that neurotensin IR cells were present in the gut of *Barbus conchonius*. They also stated that this immunoreactivity was also present in the myenteric plexus of whole intestinal regions containing nerve fibres in the muscle layer. We found that, a sparse distribution of neurotensin IR cells were observed in all regions of the digestive tract.

In conclusion, the regional distribution and relative frequency of immunoreactive cells in the flower fish, *Pseudophoxinus antalyae*, are essentially similar to those of other fish[9,10,19,20,25]. However, some characteristic differences are observed in this species, which may be due to differences in the antisera tested, the methods used and/or the species investigated in the various studies.

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*RAPID COMMUNICATION*

# Hepatitis B and C virus co-infections in human immunodeficiency virus positive North Indian patients

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## Abstract

**AIM:** To determine the prevalence of hepatitis B and C virus infections in human immunodeficiency virus (HIV) -positive patients at a tertiary care hospital in New Delhi, India.

**METHODS:** Serum samples from 451 HIV positive patients were analyzed for HBsAg and HCV antibodies during three years (Jan 2003-Dec 2005). The control group comprised of apparently healthy bone-marrow and renal donors.

**RESULTS:** The study population comprised essentially of heterosexually transmitted HIV infection. The prevalence rate of HBsAg in this population was 5.3% as compared to 1.4% in apparently healthy donors ($P < 0.001$). Though prevalence of HCV co-infection (2.43%) was lower than HBV in this group of HIV positive patients, the prevalence was significantly higher ($P < 0.05$) than controls (0.7%). Triple infection of HIV, HBV and HCV was not detected in any patient.

**CONCLUSION:** Our study shows a significantly high prevalence of hepatitis virus infections in HIV infected patients. Hepatitis viruses in HIV may lead to faster progression to liver cirrhosis and a higher risk of antiretroviral therapy induced hepatotoxicity. Therefore, it would be advisable to detect hepatitis virus co-infections in these patients at the earliest.



**Key words:** Human immunodeficiency virus; Hepatitis B; Hepatitis C; Hepatitis B surface antigen; Co-infections

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## INTRODUCTION

Diseases of the hepatobiliary system are a major problem in patients with human immunodeficiency virus (HIV) infection. An estimated one-third of deaths in HIV patients are directly or indirectly related to liver disease. Liver diseases in HIV infected persons can occur due to hepatitis B virus (HBV) and hepatitis C virus (HCV) co-infections, chronic alcoholism, hepatic tuberculosis, or due to the effects of anti-retroviral therapy (ART)[1,2]. Since the principal routes for HIV transmission are similar to that followed by the hepatotropic viruses, as a consequence, infections with HBV and HCV are expected in HIV infected patients. Co-infections of HBV and HCV with HIV have been associated with reduced survival, increased risk of progression to liver disease and increased risk of hepatotoxicity associated with anti-retroviral therapy.

Worldwide, HIV is responsible for 38.6 million infections as estimated at the end of 2005[3] while HBV and HCV account for around 370 million and 130 million chronic infections respectively. Moreover, among the HIV-infected patients, 2-4 million are estimated to have chronic HBV co-infection while 4-5 million are co-infected with HCV[4]. The reported co-infection rates of HBV and HCV in HIV patients have been variable worldwide depending on the geographic regions, risk groups and the type of exposure involved[4-8]. In Europe and USA, HIV-HBV co-infection has been seen in 6%-14%[4,5] of all patients while HIV-HCV co-infection has been variably reported ranging from 25% to almost 50%[6,7] of these patients. Evidence of exposure to HBV and HCV has been found in 8.7% and 7.8%, respectively, of HIV patients from Thailand[8] in Southeast Asia.

India, with a whopping 5.2 million cases of HIV infection, has distinction of harboring the second highest number of these patients in the world. Within India also, variable co-infection rates have been reported from region to region. HIV-HBV co-infection (HBsAg positives) is reported in 6% of people from Chennai, South India[9], 7.5% in Chandigarh, Northwest India[10] and 16% in Mumbai, Western India[2]. Similarly, HIV-HCV co-infection rates also vary from 4.8%-21.4% in South India[9,11] and 30% in Western India[2] to as high as 92% in the North-East[12].

While HIV-HBV co-infection has been linked to both sexual and intravenous injection route of transmission, the HIV-HCV co-infection has predominantly been associated with non-sexual parenteral route of transmission. In India, HIV infection is predominantly acquired through heterosexual route, but no work from India has been

carried out to study the differential transmission rate of these two hepatitis viruses in HIV positive persons. With this background we set out to determine the prevalence of hepatitis B and C virus infections in HIV-positive patients coming to a tertiary care hospital located in north India.

## MATERIALS AND METHODS

### Patients and controls

The study was carried out in the Microbiology Division, Department of Laboratory Medicine at the All India Institute of Medical Sciences (AIIMS). Patients attending clinics at AIIMS were screened for HIV based on clinical suspicion after pre-test counseling and informed consent. As a routine, our laboratory follows the World Health Organization (WHO) testing strategies for HIV testing. Only the confirmed HIV positive serum samples were included in this study and were anonymously tested for hepatitis B surface antigen (HBsAg) and anti-HCV antibodies. The study period was from January 2003 to December 2005. Seroprevalence of HBsAg and anti-HCV antibodies in apparently healthy, HIV-negative organ-donors (kidney/bone-marrow) was also analyzed during the same study period and compared with the prevalence of hepatitis markers in HIV positive individuals. These organ donors are screened for viral markers as a routine work-up for their family members requiring organ transplant.

### Viral diagnosis

The serum samples which were found to be HIV positive according to WHO testing strategy Ⅲ were coded and stored at -20 degree Celsius. The coded samples were anonymously tested using enzyme linked immunosorbent assay kits at a later date for the presence of HBsAg (Cat. No.B9 280252, bio Merieux, France) and anti-HCV antibodies (DETECT-HCV™ Third generation, Cat. No. HCA 702B/700B, Adaltis, Italy). All serum samples were tested in duplicate.

### Statistical analysis

Comparison of proportions between HIV-infected and non-infected individuals was done using Chi-square tests. $P < 0.05$ was taken as significant.

## RESULTS

Sera from a total of 451 HIV-positive patients were included in this study. The retrospective demographic data of these subjects showed that out of the 451 patients, 345 (76.4%) were males and 106 (23.6%) females. The mean age of the study group was 32 years (95% CI +/- 3.2 years, range 5-70 years). The predominant mode of acquiring HIV infection was heterosexual contact (80%) followed by transfusion of blood products (6%), intravenous drug use (2.3%) and the rest unknown.

Data was available for 428 prospective organ donors who were also tested during the same period. It was presumed that these donors represent the general population and they are exposed to similar risk factors as the general population. There were 259 (60.5%) males and 169 (39.5%) females. The mean age of the donors was 38.4 years (95% CI +/- 1.1 years, range 16-67 years).

**Table 1 Seroprevalence of HBsAg and anti HCV antibodies in HIV positive patients**

| | HBsAg | HCV |
|---|---|---|
| HIV patients ($n$ = 451) | 24 (5.32)[b] | 11 (2.43)[a] |
| Males ($n$ = 345) | 23 (6.6) | 6 (1.7) |
| Females ($n$ = 106) | 1 (0.94) | 5 (4.7) |
| Controls ($n$ = 428) | 6 (1.4) | 3 (0.7) |
| Males ($n$ = 259) | 4 (1.5) | 2 (0.7) |
| Females ($n$ = 169) | 2 (1.1) | 1 (0.59) |

Values in parenthesis are percentages. Control group includes HIV negative healthy donors. [a]$P < 0.05$, [b]$P < 0.001$ *vs* controls.

### Prevalence of viral co-infections in HIV positives

Overall, the prevalence of co-infection in HIV-positive patients with hepatitis viruses was 7.76% (35 in 451). Among the co-infected patients, there were 29 males and 6 females. Triple infection with both HBsAg and HCV was not seen in any HIV patient.

The rate of HBsAg co-infection was 5.32% (24 in 451) in HIV positive patients as compared to HBsAg prevalence of 1.4% in apparently healthy donors ($P < 0.001$) (Table 1). Among males, HIV/HBV co-infection was seen in 23 of 345 (6.6%) patients while HBsAg was positive in only 4 out of 259 male donors (1.5%). Among the females, HIV/HBV co-infection was seen in only 1 of 106 (0.94%) patients while 2 out of 169 (1.1%) female donors were HBsAg positive. HBsAg co-infection rates were significantly higher in HIV positive men than in women ($P < 0.025$). HBsAg prevalence was also significantly higher in HIV males as compared to control males ($P < 0.01$), but such significance was not seen in females.

The rate of HCV co-infection was 2.43% (11 in 451) in HIV positive patients as compared to 0.70% in controls ($P < 0.05$) (Table 1). Among males, HIV/HCV co-infection was seen in 6 of 345 (1.7%) patients while only 2 out of 259 (0.7%) male donors were HCV positive. Among females, HIV/HCV co-infection was found in 5 of 106 (4.7%) patients while only 1 of 169 (0.59%) female donors was HCV positive. No statistically significant difference was seen in HCV co-infection rates between HIV positive men and women. But, the HCV seroprevalence was significantly higher in HIV positive female patients as compared to female donors ($P < 0.05$).

The majority of the HIV-infected patients comprised the 31-40 years age group (41.5%) followed by the 21-30 year age group (34.7%). Mean age of the HIV positive patients was 32 years (95% CI +/- 3.2 years) while that of the co-infected patients was 37.7 years (95% CI +/- 3.2 years). HBV-HIV co-infection was seen highest in the 31-40 year age group (45.8%) while HCV-HIV co-infection was predominant in the ≥ 51 years of age (45.4%) (Figure 1).

## DISCUSSION

The Joint United Nations Programme on HIV/AIDS (UNAIDS) estimated that 38.6 million people were living

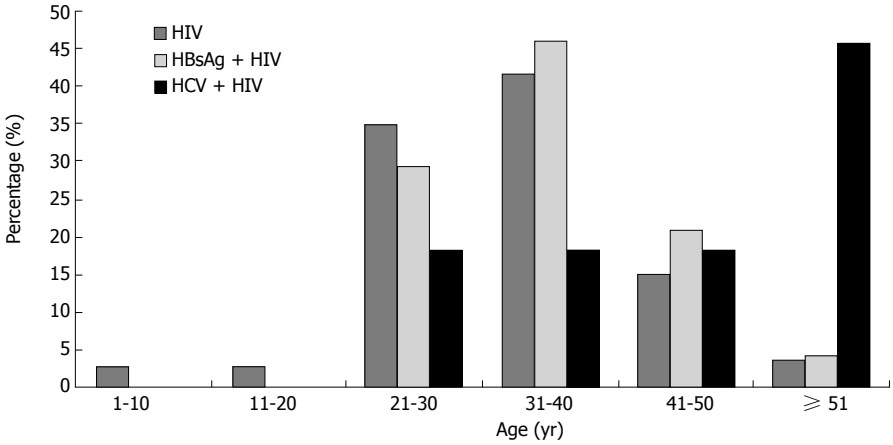


**Figure 1** Age-related distribution of HBsAg and HCV in HIV positive patients.

with HIV globally at the end of 2005[3]. India alone had the second highest number of people living with HIV (5.2 million) by the end of 2005[13]. Globally, around three million people died of the acquired immunodeficiency syndrome (AIDS) related illnesses in 2005[3]. About two-thirds of patients with AIDS develop hepatomegaly and abnormalities in serum biochemical parameters of liver function[14]. Liver damage may be directly related to HIV infection or may result from conditions such as alcoholism, prior viral hepatitis or intravenous drug abuse, which are highly prevalent in patients with HIV infection[2]. In addition, sepsis, malnutrition, or the administration of possibly hepatotoxic antiretroviral medications may also lead to liver damage[15,16].

Due to declining opportunistic infections as a result of highly active antiretroviral therapy (HAART), life expectancy of patients with HIV has increased. As a consequence, focus has shifted to the management of concurrent illnesses such as chronic HBV and HCV infections which have the potential to increase long term morbidity and mortality. As a result of the shared epidemiological factors, patients infected with HIV have a higher risk of both HBV and HCV infection as compared to those uninfected with HIV. It is very likely that the presence of HIV infection makes the transmission of hepatitis viruses more efficient, both through sexual contact as well as perinatal contact[17]. This has also been directly correlated with the degree of immunosuppression and viral load. Though HCV transmission is not widely documented through sexual contact but the presence of HIV may facilitate transmission of HCV in HIV positive individuals who otherwise have no history of blood transfusion or injection drug abuse.

Our study findings indicate that HIV-infected men and women are at a high-risk of viral co-infections, as illustrated by the high prevalence of HBsAg (5.32%) and HCV (2.43%) antibodies (Table 1). This study showed that infection with these viruses in the test group was significantly higher than the comparable group of healthy adult organ donors at the same hospital. The study group predominantly comprised of heterosexually acquired HIV infections. Also, a significant risk for HBV was seen in males. This is similar to previous reports that male gender is associated with a significant higher risk of co-infections with HBV[4]. The carriage of HBsAg in our study matches with that of Kumarasamy *et al*[9] from South India and at the National AIDS Research Institute, and of Pune (4.8%)[18] from Western India.

HCV prevalence in our study group was lower than other studies from Western countries and other parts of India. This can be related to the type of risk-groups in this study. Saha *et al*[11] reported an HCV co-infection rate of 92% in intravenous drug-users from Northeast India, while in the report by Kumarasamy *et al*[9] HCV co-infection was seen at 4.8%, but in those patients 25% had a history of blood transfusion and 50% were injection drug users (IVDU). However, in our study group IVDUs comprised only 2% of the patients while the majority of HIV infections were heterosexually transmitted. The low HCV prevalence in our data could be attributed to the mode of transmission of these viruses. While epidemiological evidence indicates sexual transmission of HCV, this occurs much less efficiently than HBV or HIV. Moreover, we need to address other possible non-sexual sources of HCV acquisition which are often overlooked[19] and several methodological shortcomings that tend to overestimate the proportion of HCV infections attributed to sexual contact. Earlier studies used first-generation antibodies for HCV assays which have a higher false positive rate than presently available third-generation assays. Moreover, only limited studies performed virological analyses to confirm that anti-HCV concordant sexual partners were infected with the same virus. In addition, other independent non-sexual factors need to be looked for such as prior history of injection drug use, tattooing, or sharing of razors and toothbrushes.

HCV-HIV co-infection in our male patients was higher than HCV mono-infection in male donors, though not statistically significant. Among HIV positives, though females showed a higher HCV co-infection rate as compared to males, the patient numbers were not comparable. Among the female group, HIV/HCV co-infection was significantly higher than HCV mono-infection in donors ($P < 0.05$). HIV co-infection appears to increase the rate of HCV transmission by sexual contact. In India, the majority of the women are in a monogamous relationship with their husbands and usually acquire HIV infection from their spouse. Therefore, while the risk for HCV acquisition in steady monogamous relationships is quite low[19], we need to look into other factors like sharing

of toothbrushes and other contaminated personal items with her husband who may be the index for the HCV infection.

At present, limited clinical information is available about the possible effects of HBV and HCV co-infection and the reciprocal interactions between these hepatotropic viruses and HIV. In the post-HAART era, with increased survival of HIV infected patients, HCV-induced liver disease has emerged as a leading cause of significant morbidity and death in this population[6,7]. Clinically, hepatitis C is a more severe disease in HIV-infected individuals rather than HCV mono-infection alone. HIV/HCV co-infected patients have a faster rate of HCV-related liver fibrosis and a more rapid progression to liver failure or hepatocellular carcinoma than HCV mono-infected persons[20-22]. In contrast, most studies have shown that HCV does not influence progression of HIV infection to AIDS or death[20].

There is also evidence that HIV may modify the natural history of HBV infection[23,24]. HIV positive subjects have higher rates of HBV chronic carriage, higher HBV replication and lower rates of seroconversion to anti-HBe and anti-HBs antibodies. The impact of HIV co-infection on the outcome of HBV infection is still controversial; while some studies have shown a rapid progression of liver fibrosis and an accelerated progression towards decompensated cirrhosis in HIV co-infected subjects[24], others have shown decreased liver necro-inflammatory processes[25]. Though some studies from the pre-HAART era described a more rapid progression to AIDS in patients having chronic HBV infection[26], post-HAART, no impact of HBV co-infection on HIV-disease progression has been detected[27].

Presently, ongoing clinical trials with pegylated interferon and ribavirin are reported to show encouraging results in HIV/HCV co-infected patients[7] while lamivudine and other combinations are also encouraging in HIV/HBV co-infections[28]. Therefore, with newer drug formulations it is now becoming evident that early initiation of therapy before marked immunosuppression sets in could be highly beneficial for the HIV infected patient, in order to decrease long term mortality and morbidity associated with these co-infections.

## CONCLUSION

Co-infection of HIV with hepatitis B and hepatitis C viruses is seen in 5.3% and 2.4% patients, respectively. This is significantly higher as compared to the prevalence of these two hepatitis virus infections in control population of the representative region of the country. Therefore, universal screening for hepatitis B and C viral infections in all HIV positive patients should urgently be started in Asia also.

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*RAPID COMMUNICATION*

# Can albumin administration relieve lung injury in trauma/hemorrhagic shock?

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## Abstract

**AIM:** To study the effect of albumin administration on lung injury in trauma/hemorrhagic shock (T/HS).

**METHODS:** Sixty experimental animals were randomly divided into three groups: rats undergoing laparotomy without shock (T/SS); rats with T/HS and resuscitation with blood plus twice the volume of shed blood as Ringer's lactate (RL), and rats with T/HS and resuscitation with blood plus additional 3 mL of 50 g/L human albumin. Expression of polymorphonuclear neutrophil (PMN) CD11b/CD18, intercellular adhesion molecule-1 (ICAM-1) of jugular vein blood and the severity of lung injuries [determined mainly by measuring activity of lung tissue myeloperoxidase (MPO) and lung injury score (LIS)] were measured after a 3-h recovery period.

**RESULTS:** All three groups showed a significant difference in the expressions of CD11b/CD18, ICAM-1, and severity of lung injury. The expressions of CD11b/CD18 in T/SS group, T/HS + RL group, T/HS + albumin group were 17.76% ± 2.11%, 31.25% ± 3.48%, 20.36% ± 3.21%, respectively ($F$ = 6.25, $P$ < 0.05). The expressions of ICAM-1 (U/mL) in T/SS group, T/HS + RL group, T/HS + albumin group were 258.76 ± 98.23, 356.23 ± 65.6, 301.01 ± 63.21, respectively ($F$ = 5.86, $P$ < 0.05). The expressions of MPO (U/g) in T/SS group, T/HS + RL group, T/HS + albumin group were 2.53 ± 0.11, 4.63 ± 1.31, 4.26 ± 1.12, respectively ($F$ = 6.26, $P$ < 0.05). Moreover, LIS in T/HS + RL group, T/HS + albumin group was 2.62 ± 0.23, 1.25 ± 0.24, respectively. The expressions of CD11b/CD18, ICAM-1 and MPO in T/HS + RL group were significantly increased compared to T/SS group ($P$ = 0.025, $P$ = 0.036, $P$ = 0.028, respectively). However, administration of 3 mL of 50 g/L albumin significantly down-regulated the expressions of CD11b/CD18, ICAM-1 and lung injury index (MPO and LIS) when compared with the T/HS + RL rats ($P$ = 0.035, $P$ = 0.046, $P$ = 0.038, $P$ = 0.012, respectively).

**CONCLUSION:** The infusion of albumin during resuscitation period can protect lung from injury and decrease the expressions of CD11b/CD18, ICAM-1 in T/HS rats.



**Key words:** Albumin; CD11b/CD18; Intercellular adhesion molecule-1; Lung injury; Trauma/hemorrhagic shock

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## INTRODUCTION

Previous studies have shown that there is obvious pulmonary microvascular injury at the early stage of trauma/hemorrhagic shock (T/HS)[1]. The polymorphonuclear neutrophils (PMNs) accumulated in lung are closely correlated with lung injury[2]. Endothelial cells can control and regulate the adhesion, recruitment and migration of white blood cells (WBC) through expressing adhesion molecules such as intercellular adhesion molecule-1 (ICAM-1), and ICAM-1 plays an important role in the process of conducting WBC firmly adherent to endothelial cells[3,4]. Colloid *versus* crystalloid resuscitation in the acute trauma remains a controversial subject[5]. Although several studies have proved the relative benefits of albumin resuscitation, no consensus has been reached by now[6]. More recently, the immunomodulatory effects of various resuscitation solutions have begun to be revaluated[7].

Trauma/hemorrhagic shock (T/HS) may lead to splanchnic ischemia-reperfusion and to gut barrier failure[8]. This sequence of events gets the gut into an inflammatory cytokine-secreting organ, which contributes to the pathogenesis of shock-induced lung injury[9]. Lung injury generally results when mediators released by systemic

inflammatory processes up-regulate PMN interactions with endothelial cells (ECs). Endothelial cells play an important role in regulating cells of blood vessel walls in response to injury. ICAM-1 is expressed on endothelial cells and is responsive to numerous inflammatory mediators[10]. It mediates both leukocyte adhesion and migration through the endothelium into tissues. Shock and trauma-induced neutrophil activation have been implicated in the pathogenesis of the adult respiratory distress syndrome (ARDS) and, as a contributory factor, in the development of the multiple organ dysfunction syndrome (MODS)[11,12]. In the study of trauma/hemorrgic shock-induced organ failure, lung often serves as an ideal model of dysfunction.

Lung injury following T/HS is a well-accepted phenomenon; the exact mechanisms of injury are not yet defined[13]. Albumin, a broadly binding protein, has been characterized as an scavenger in addition to being an anti-apoptotic agent and an antioxidant[14]. A recent study showed that albumin protected endothelial cells *in vitro* from the injury by hematin[15]. We hypothesize that albumin would protect against lung injury induced by activation of PMN in our T/HS model.

## MATERIALS AND METHODS

### Experiment design

The aim of this experiment was to determine the protective effects of albumin on lung injury in a T/HS model. Rats were randomly divided into three groups: rats undergoing laparotomy without shock (T/SS), rats with T/HS and resuscitation with blood plus twice the volume of shed blood as Ringer's lactate (RL), and rats with T/HS and resuscitation with blood plus 50 g/L human albumin (Aventis Behring GmbH, Gemany). Rats of the albumin group received 3 mL of 50 g/L human albumin (150 mg of albumin) in addition to their shed blood. In a 400-g rat, with a circulating blood volume of about 24 mL, this dose is 6 g/L, approximating the distribution of a 25-g dose of albumin in the approximately 4 L blood volume of a 60-kg man. Polymorphonuclear neutrophil (PMN) CD11b/CD18, ICAM-1 in jugular vein blood and the severity of lung injuries [determined by myeloperoxidase (MPO) and lung injury score (LIS)] were measured after a 3-h recovery period.

### Subjects

Sixty adults Sprague-Dawley (SD) rats, weighing 300-350 g, were used after a minimum acclimatization period of 7 d. These animals were randomly divided into three groups according to aforementioned experimental design. The animals were allowed free access to food and water. The animals and their diet were provided by the Laboratory Animal Center, Collage of Medicine, Zhejiang University. The animals were maintained in accordance with the guideline of the National Guide for the Care and Use of Laboratory Animals, and the experiment was approved by Local Ethical Committee of the College of Medicine, Zhejiang University.

### T/HS model

As described above[16], rats were anesthetized using intraperitoneal sodium pentobarbital (50 mg/kg). A heparinized polyethylene catheter (PE-50) was introduced into the femoral artery for measuring the arterial pressure. A right jugular vein catheter was similarly inserted for blood withdrawal and resuscitation. Laparotomy (trauma) was performed to the animals through a 5-cm midline incision with two-layer closure using 3-0 silk in a running suture. The T/HS rats were subjected to T/HS (90 min at a mean artery pressure of 30 mmHg) by withdrawing blood through the jugular vein catheter in a heparinized syringe until the pressure reached 30 mmHg. The blood pressure was maintained for 90 min by withdrawing or re-infusing the shed blood. At the end of the 90-min shock period, the HS rats were resuscitated by their shed blood (average 18 mL). The RL group received an additional crystalloid (average 18 mL) and the albumin group received an additional 3 mL of 50 g/L albumin. Sham (T/SS) animal underwent vascular cannulation and laparotomy, but had no blood withdrawn and received no resuscitation. The animals' body temperature during the experimental period was maintained at about 37℃ by using a heating pad.

### Assay of blood CD11b/CD18 and ICAM-1

After the 3-h resuscitation period, each 1 mL of blood sample from the jugular vein was taken and treated with anti-coagulant EDTANa2. The 100-μL blood sample was put into a tube with size of 12 mm × 75 mm and then 10 μL of either anti-rat CD11b or CD18 fluorescent-labeled monoclonal antibody (BD Pharmingen) was added into the tube. The samples were gently vortexed for 10 min and then placed into a dark place for 40 min. And then the red blood cells of the sample were lysed and the sample was fixed with Coahem_Q-PREP equipment (Couletr Company, USA) for 15 min on ice. After being treated with centrifugation and washed for 3 times, PMN cells obtained were analyzed for detection of adhesion molecule expression using flow cytometer (ESPLL-XL, BECKMAN, USA) according to the manufacturer's recommendation. The amounts of these neutrophils labeled with monoclonal antibody among each 10000 neutrophils were counted and the percentage was evaluated.

The expression of serum ICAM-1 in the venous blood was detected using a special Regent Box (Jianqing Company, Nanjin, China) with ELISA methods following manufacturer's instructions.

### Assay of MPO and LIS

After the 3-h resuscitation period with aforementioned different methods, rats were killed immediately, and the right lobe lung was taken. One part of lung tissue was frozen, homogenized and processed for detection of MPO with special Regent-Box (Jiancheng Bio-Technolgy Company, Nanjing, China) according to the manufacturer's recommendation. One unit of MPO activity represents the amount of enzyme that will reduce 1 μmol/L peroxide per minute. The other part of lung tissue was fixed by

**Table 1 Comparisons of the expressions of CD11b/CD18, ICAM-1, MPO, and LIS among the three groups (mean ± SD)**

| Group (*n*) | CD11b/ CD18 (%) | ICAM-1 (U/mL) | MPO (U/g) | LIS |
|---|---|---|---|---|
| T/SS (20) | 17.76 ± 2.11 | 258.76 ± 98.23 | 2.53 ± 0.11 | - |
| T/HS + RL (20) | 31.25 ± 3.48[a] | 356.23 ± 65.6[a] | 4.63 ± 1.31[a] | 2.62 ± 0.23 |
| T/HS + albumin (20) | 20.36 ± 3.21[a,c] | 301.01 ± 63.21[a,c] | 4.26 ± 1.12[a,c] | 1.25 ± 0.24[c] |
| *F* | 6.25[e] | 5.86[e] | 6.26[e] | - |

ICAM-1: Intercellular adhesion molecule-1; MPO: Myeloperoxidase; LIS: Lung injury score. [a]$P < 0.05$ *vs* T/SS group; [c]$P < 0.05$ *vs* T/HS + RL group; [e]$P < 0.05$ comparison among the three groups (ANOVA).

100 g/L formaldehyde and then was cut into olefin slice stained with standard H&E. The LIS was evaluated with OLYMPUS (Olympus, Japan) optical microscope. The LIS standard composes of three character: (1) the severity of leukocytes sequestration in the lung tissue: 0 = 0%, 1 = 0%-25%, 2 = 25%-50%, 3 = 50%-5%, 4 = 75%-100%; (2) the severity of leukocytes sequestration in lung alveolus: 0 = none, 1 = few, 2 = a lot of, 3 = almost full, 4 = absolutely full; and (3) the severity of exudation (such as fibrin, transparent membrane, edema liquor) in lung alveolus: 0 = none, 1 = few, 2 = a lot of, 3 = almost full, 4 = absolutely full.

### *Statistical analysis*

Results were expressed as mean ± SD. The data were analyzed with SPSS.11.0 statistical software. The three groups were compared using analysis of variance (ANOVA). The comparison of two groups among multiple groups was dealt with *q* test. A *P* value less than 0.05 was considered statistically significant.

## RESULTS

As shown in Table 1, we observed a significant difference in expression of CD11b/CD18, ICAM-1, and severity of lung injury ($P < 0.05$) among the three groups. The T/HS + RL and T/HS + albumin groups showed a significant increase in the expressions of CD11b/CD18, ICAM-1, and severity of lung injury compared with the T/SS group ($P < 0.05$), thereby indicating that T/HS pathological process could up-regulate the expressions of CD11b/CD18, ICAM-1, and cause lung injury. The administration of 3 mL of 50 g/L albumin significantly down-regulated the expression of CD11b/CD18 and alleviated lung injury compared with the T/HS + RL rats ($P < 0.01$). These results indicated that resuscitation with albumin may have utility in reducing CD11b/CD18, ICAM-1 expression and alleviating lung injury when compared with crystalloid in the T/HS model.

## DISCUSSION

The present study showed that PMNs infiltrated and aggregated in the lung after trauma/hemorrhagic shock (T/HS)[17]. Up-regulation of CD11b/CD18 on PMNs and ICAM-1 in endothelial cells are the molecular basis of PMNs adhering to the endothelium[18]. Trauma/hemorrhagic shock is associated with the generation of reactive oxygen species, which may contribute to delayed multiple organ system failure and death[19]. It has been shown the phenomenon of neutrophil activation is often companied with T/HS. In addition, the activated neutrophil may play an important role in the pathogenesis of lung injury or multiple organ dysfunction (MOD), multiple organ failure (MOF) in T/HS[20]. The activation of neutrophil and lung injury are often chosen as ideal quantifiable indices to assess the severity of trauma in an animal model and patients. In this study, CD11b/CD18 was chosen as the marker of PMN activation; MPO and LIS were chosen as the markers of lung injury[21].

The integrins CD11b/CD18 have been found to be involved in monocyte adhesion to endothelial cells and transendothelial migration[22,23], release of hydrogen peroxide[24,25] and oxidative activity[26]. ICAM-1 is expressed on endothelial cells and is responsive to numerous inflammatory mediators[27]. It mediates both leukocyte adhesion and migration through the endothelium into tissues[28].

The potential advantages and disadvantages of colloids during resuscitation in T/HS have been long debated[29]. Some animal experiments showed that albumin was linked with increased mortality, but which did not accord with some clinical findings that albumin could decrease mortality in the trauma population[30]. However, a more recent study has demonstrated that early albumin infusion during resuscitation period may decrease neutrophil activation in animal model[31]. Some studies indicated that resuscitation with 250 g/L albumin significantly reduced transpulmonary protein flux, bronchoalveolar lavage fluid neutrophil counts, and the degree of histopathological injury compared to resuscitation with Ringer's lactate[32].

The exact mechanism by which albumin does benefit to alleviate lung injury and down-regulate CD11b/CD18 expression is still uncertain. In addition to colloid oncotic effects during resuscitation period, a variety of other properties of albumin have been described[33]. Albumin has been shown to have antioxidant properties and proven to inhibit apoptosis in certain cell lines[10]. As a broad binding protein, albumin may bind and neutralize toxic factors and inflammatory mediators, including cytokines, eicosanods, oxidants, platelet-activating factors, complement fragments, and endotoxin[34,35]. And many of these mediators can prime or activate neutrophils and endothelial cells and cause lung injury directly or indirectly. Although the exact function and identity of the toxic factors in T/HS lymph/plasma remain unknown, it is clearly that albumin has a neutralizing effect[36].

Based on our results, the beneficial effect of albumin infusion occurred during the resuscitation period in T/HS. The results showed that up-regulated expression of PMN CD11b/CD18, ICAM-1 and the lung injury induced by T/HS could be alleviated by the infusion of albumin during resuscitation period. On the other hand, considering the ability of albumin to bind and neutralize the toxic factors,

our study further supports the hypothesis of organ injury induced by some factors released or produced by the post-ischemic intestine through mesenteric lymph pathway in T/HS[37].

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# Evaluation of the effects of combined endoscopic variceal ligation and splenectomy with pericardial devascularization on esophageal varices

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**Key words:** Esophageal varices; Endoscopic ultrasound; Color Doppler ultrasonography; Endoscopic variceal ligation; splenectomy with pericardial devascularization

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## Abstract

**AIM:** To detect the hemodynamic alterations in collateral circulation before and after combined endoscopic variceal ligation (EVL) and splenectomy with pericardial devascularization by ultrasonography, and to evaluate their effect using hemodynamic parameters.

**METHODS:** Forty-three patients with esophageal varices received combined EVL and splenectomy with pericardial devascularization for variceal eradication. The esophageal vein structures and azygos blood flow (AZBF) were detected by endoscopic ultrasonography and color Doppler ultrasound. The recurrence and rebleeding of esophageal varices were followed up.

**RESULTS:** Patients with moderate or severe varices in the esophageal wall and those with severe peri-esophageal collateral vein varices had improvements after treatment, while the percentage of patients with severe para-esophageal collateral vein varices decreased from 54.49% to 2.33%, and the percentage of patients with detectable perforating veins decreased from 79.07% to 4.65% ($P < 0.01$). Color Doppler flowmetry showed a significant decrease both in AZBF (43.00%, $P < 0.05$) and in diameter of the azygos vein (28.85%, $P < 0.05$), while the blood flow rate was unchanged. The recurrence rate of esophageal varices was 2.5% (1/40, mild), while no re-bleeding cases were recorded.

**CONCLUSION:** EVL in combination with splenectomy with pericardial devascularization can block the collateral veins both inside and outside of the esophageal wall, and is more advantagious over splenectomy in combination with pericardial devascularization or EVL in preventing recurrence and re-bleeding of varices.

## INTRODUCTION

In patients with portal hypertension, collaterals should be established to reduce the high portal pressure, and the hemodynamic indicators of such collaterals have been used for the evaluation of therapeutic effects. According to the location of veins, they can be divided into collateral veins inside and outside of the esophageal wall[1]. The collateral veins inside of the esophageal wall may form varices which can be evaluated by routine endoscopy, and are the major source of recurrent bleeding. The collateral veins outside of the esophageal wall, where hypoechic round spaces can be shown by endoscopic ultrasound (EUS), include peri-esophageal collateral veins (peri-ECVs), para-esophageal collateral veins (para-ECVs) and perforating veins. Peri-ECVs are adjacent to the esophageal wall while para-ECVs are distal to the esophageal wall, the perforating veins penetrate the esophageal wall connecting peri- or para-ECVs[2-5].

Irisawa *et al*[2-5] demonstrated that both the prevalences of perforating veins and severe peri-ECVs are positively correlated with the form of varices. The incidence of severe peri-ECVs, para-ECVs, and perforating veins in variceal recurrent cases is significantly higher than that in non- variceal recurrent cases (70%, 50% and 90% *vs* 3.6%, 32.1% and 21.4%, respectively)[6].

On the other hand, endoscopic Doppler ultrasound for measurement of azygos blood flow (AZBF) volume has been used widely in evaluation of the porto-systemic collateral circulation[7], and is a safe and useful method for monitoring portal venous flow in patients with portal hypertension.

It is reported that more than 70% of portal hypertension patients with variceal bleeding history may suffer from recurrent bleeding[8,9]. Splenectomy with pericardial devascularization, sclerotherapy, endoscopic variceal ligation (EVL) or other combined therapies have been recommended for those patients. We have previously reported that splenectomy with EVL is superior to splenectomy with pericardial devascularization in the treatment of portal hypertension[10-12]. However, whether combined EVL and splenectomy with pericardial devascularization is effective and the hemodynamic alterations in this process remain unknown.

In this study, we used EUS and color Doppler ultrasound to detect the hemodynamic alterations in collateral circulation before and after EVL in combination with splenectomy with pericardial devascularization. The hemodynamic parameters were used to evaluate its effect. An ultrasound microprobe (UMP) was adopted for the detection of collateral veins, which could provide better images of vascular structures than conventional EUS due to its higher frequency and resolution[3,13,14].

## MATERIALS AND METHODS

### Patients

From March 2001 to May 2004, consecutive patients with untreated esophageal varices caused by portal hypertension were admitted to the Third Affiliated Hospital of Sun Yat-Sen University. Patients who did not want to participate in the study, those with severe diseases in major organs other than in liver and/or older than 70 years, and those with different Child-Pugh's scores before and after all EVL sessions were excluded to avoid the influence of alterations of Child-Pugh's grade on the portal blood flow rate[5]. Finally, 43 patients who were clinically diagnosed as portal hypertension with endoscopically confirmed esophageal varices were included in this study. The clinical data of the enrolled patients are shown in Table 1.

Written informed consent was obtained from all the patients, and the study was approved by the Ethics Committee of Sun Yat-Sen University.

### Ligation of endoscopic varices

Patients underwent EVL first. After local application of lidocaine, an endoscope (GIF 240 or 260, Olympus Optical, Tokyo) was introduced, and ligation was carried out 6-12 times by placing a single rubber band (Bard Interventional Products, Tewksbury, Mass.) over a varix. The ligation was repeated every 2 wk till the complete disappearance of varices under endoscope.

### Splenectomy with pericardial devascularization

Patients underwent splenectomy with pericardial devascularization (Hassab's operation) 4-10 wk after the first EVL session as previously described by Yang and Qiu[15]. In brief, extended left subcostal incision or L incision of the left upper abdomen was used for extreme splenomegaly. After routine splenectomy, the gastric branch and 5-8 small branches of the gastric coronary veins were disconnected. The esophageal branch was disconnected and suture-ligated. The gastric posterior vein was ligated by suturing, and then the left subphrenic vein was ligated as well[16]. In addition, the arteries accompanying the veins including the left gastric, left gastroepiploic, gastric posterior and left subphrenic arteries, were disconnected.

**Table 1 Clinical data on 43 patients undergoing endoscopic variceal ligation and splenectomy with pericardial devascularization**

| | |
|---|---|
| Sex | |
| M:F | 28:15 |
| Mean age ± SD (range) (yr) | 50.8 ± 11.2 (20-66) |
| Etiology of liver cirrhosis | |
| Posthepatic | 41 |
| Schistosomiasis | 1 |
| Alcoholic | 1 |
| Liver function, Child-Pugh classification | |
| A | 16 |
| B | 18 |
| C | 9 |
| History of upper gastrointestinal bleeding | 30 |

### Color Doppler ultrasound

Color Doppler ultrasound detection was performed before the treatment and 1 wk prior to discharge. AZBF was measured with EUS duplex Doppler technique with a Pentax FG-32UA echo-endoscope (Tokyo, Japan) and a Hitachi EUB-515A ulterasound scanner (Tokyo, Japan) with a frequency of 7.5 MHz[17]. The diameter of the vessel and the blood velocity were measured, the mean value of 3 repeated measures performed at the same position was used. AZBF was calculated with the following formula: AZBF = $(\text{radius})^2 \times$ blood velocity.

### Detection of the veins inside and outside of the esophageal wall

The structure of vessels inside and outside of the esophageal wall was examined before the treatment and 1 wk prior to discharge with an Olympus UM-3R 20MHz UMP (Tokyo, Japan ) passed through the accessory channel of a GIF 250 Olympus endoscope (Tokyo, Japan). Before examination, the esophagus was filled with deaerated water through a water supply tube attached to the endoscope. The veins around the esophagus were scanned from the esophago-gastric junction (GEJ) to a point 5 cm proximal to the EGJ. Varices inside of the esophageal wall (EV) were evaluated as mild (< 5 mm in diameter), moderate (5-7 mm in diameter), and severe (> 7 mm in diameter).

According to the definition of location[3], esophageal collateral veins outside of the esophageal wall could be divided into peri-ECVs and para-ECVs. There were less than 4 mild peri-ECVs (< 2 mm in diameter) and more than 5 severe peri-ECVs (≥ 2 mm in diameter) adjacent to the muscularis externa of the esophagus, a few mild para-ECVs (< 5 mm in diameter) and severe para-ECV (≥ 5 mm in diameter) distal to the esophageal wall without contact with the muscular externa. The presentations of

**Table 2 Alteration in varices inside and outside of the esophageal wall and formation of perforating veins ($n$ = 43)**

| | Pre-treatment $n$ (%) | Post-treatment $n$ (%) |
|---|---|---|
| EV | | |
| Mild | 0 (0) | 43 (100) |
| Moderate | 26 (60.47) | 0 (0) |
| Severe | 17 (39.53) | 0 (0) |
| Peri-ECVs | | |
| Mild | 13 (30.23) | 43 (100) |
| Severe | 30 (69.77) | 0 (0) |
| Para-ECVs | | |
| Mild | 20 (46.51) | 42 (97.67) |
| Severe | 23 (53.49) | 1 (2.33) |
| Perforating veins | | |
| Detectable | 34 (79.07) | 2 (4.65) |
| Undetectable | 9 (20.93) | 41 (95.35) |

EV: Varices inside of the esophageal wall; Peri-ECVs: Periesophageal collateral veins; Para-ECVs: Paraesophageal collateral veins.

perforating veins penetrating the esophageal wall which were connected either to peri-ECV or to para-ECV, were recorded.

### Follow-up of patients

Endoscopic follow-up was performed every 12 wk after discharge of the patients. The degree of varices was evaluated at each follow-up.

### Statistical analysis

Values were expressed as mean ± SD. Statistical analysis was performed using the statistical SPSS version 10.0. Differences in numerical variables between groups were analyzed with paired $t$ test. Mann-Whitney test was used for the analysis of ranked data. Comparison of categorical data was performed by the chi square test. $P < 0.05$ (two-tailed test) was considered statistically significant.

## RESULTS

### Alterations in varices and perforating veins

Compared to pre-treatment, EV, peri- and para-ECV in varices as well as perforating veins were all improved after treatment. Patients with moderate or severe EV or severe peri-ECVs had improvement after treatment, while the percentage of patients with severe para-ECVs decreased from 54.49% to 2.33%, and the percentage of patients with detectable perforating veins decreased from 79.07% to 4.65% ($P < 0.01$, Table 2).

### Alteration in appearance of UMP images

In the UMP images before treatment, enlarged tortuous varices were found inside of the esophageal wall, while many small vessels adjacent to the muscularis externa formed a venous plexus. After treatment, the anechoic areas inside and outside of the esophageal wall disappeared or remained only tiny, while the echo of mucosa and submucosa of the esophagus enhanced. The alteration in appearance of UMP images intuitively implied the improvement in varices after treatment (Figure 1).

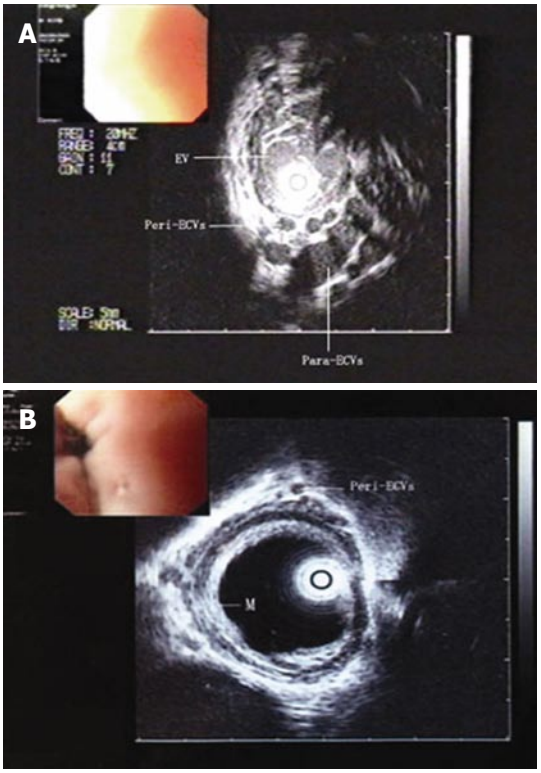


**Figure 1** Alteration in appearance of ultrasound microprobe images. **A**: Before treatment, enlarged tortuous varices were found inside of the esophageal wall, while many small vessels adjacent to the muscularis externa formed a venous plexus. **B**: After treatment, anechoic areas inside and outside of the esophageal wall disappeared or remained only tiny, while the echo of mucosa and submucosa of the esophagus enhanced. EV: Varices inside of the esophageal wall; Peri-ECVs: periesophageal collateral veins; Para-ECVs: Paraesophageal collateral veins; M: Esophageal mucosa.

**Table 3 Alteration in inside diameter, blood flow and blood flow rate of azygous vein (mean ± SD)**

| | Inside diameter (mm) | Blood flow (cm/s) | Blood flow rate (mL/s) |
|---|---|---|---|
| Pre-treatment | 10.12 ± 1.16 | 1.00 ± 0.14 | 22.73 ± 5.46 |
| Post-treatment | 7.20 ± 1.13[b] | 0.57 ± 0.05[b] | 20.15 ± 4.66 |

[b]$P < 0.01$ *vs* pre-treatment group (ANOVA).

### Alteration in azygos blood flow

Color Doppler flowmetry showed a significant decrease both in AZBF (43.00%, $P < 0.05$) and in diameter of the azygos vein (28.85%, $P < 0.05$), while the blood flow rate was uncharged (Table 3).

### Results of follow-up

A total of 40 patients were followed up for 6 mo to 1 year, with a follow-up rate of 93.0%. Three patients were not followed up due to death (1 died of liver cancer, 2 died of other diseases). The recurrence rate of esophageal varices was 2.5% (1/40, mild), while no re-bleeding cases were recorded.

## DISCUSSION

Although splenectomy with pericardial devascularization has been commonly used for portal hypertension and can control bleeding[15], re-bleeding is likely to occur because of the existing portal hyperdynamic pressure[18]. Varices cannot be eliminated by splenectomy with pericardial devascularization, and the unblocked blood flow in reverse direction in esophageal submucosa and muscular layer may increase the venous pressure at the distal part of stomach, which increases the risk of variceal bleeding.

EVL is a more effective method for portal hypertension with less side effects than surgery[19,20]. However, it causes fibrosis in mucous layer where the esophageal varices locate and has no effect on the formation of collateral circulations in the muscular layer or outside of the esophageal wall.

Nagamine *et al*[13] showed that after repeated EVL, almost all varices are undetectable in UMP imaging. However, neither collateral nor the azygous vein significantly changes its size. Similar results have been repeated by Seno *et al*[21].

In our study, not only varices were relieved, but also the sizes of collaterals and azygous vein were reduced after combined EVL and splenectomy with pericardial devascularization, suggesting that this combined therapy can almost completely relieve collateral vein varices both inside and outside of the esophageal wall.

In addition, more obvious improvement in collateral vein varices outside of the esophageal wall was found in peri-ECVs (the percentage of severe cases reduced from 69.77% to 0%) and perforating veins (the percentage of detectable cases reduced from 79.07% to 4.65%), suggesting that peri-ECVs play a more important role in the formation of esophageal varices than para-ECVs[3].

The incidence of varix and its recurrence observed in a short-term follow-up were low. However, the long-term results of the treatment in this study may be influenced by many factors, such as liver function, infection and food consumption. Therefore, the long-term effect of combined EVL and splenectomy with pericardial devascularization should be further studied.

In conclusion, combined EVL and splenectomy with pericardial devascularization can block the collateral veins both inside and outside of the esophageal wall and is more advantagious over splenectomy with pericardial devascularization or EVL in preventing recurrence and rebleeding of varices.

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*RAPID COMMUNICATION*

# Identification of differentially expressed genes in mouse hepatocarcinoma ascites cell line with low potential of lymphogenous metastasis

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## Abstract

**AIM:** To identify genes differentially expressed in mouse hepatocarcinoma ascites cell line with low potential of lymphogenous metastasis.

**METHODS:** A subtracted cDNA library of mouse hepatocarcinoma cell line with low potential of lymphogenous metastasis Hca-P and its synogenetic cell line Hca-F with high metastatic potential was constructed by suppression subtracted hybridization (SSH) method. The screened clones of the subtracted library were sequenced and GenBank homology search was performed.

**RESULTS:** Fifteen differentially expressed cDNA fragments of Hca-P were obtained which revealed 8 known genes, 4 expressed sequence tags (ESTs) and 3 cDNAs showed no homology.

**CONCLUSION:** Tumor metastasis is an incident involving multiple genes. SSH is a useful technique to detect differentially expressed genes and an effective method to clone novel genes.



**Key words:** Suppression subtracted hybridization; Liver neoplasm; Metastasis suppression genes

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## INTRODUCTION

Tumor metastasis is an incident involving multiple genes. However, the number of metastasis related genes available nowadays is very limited to elucidate the puzzling process of metastasis. Therefore, more attentions have been paid to screen candidate genes responsible for metastasis by high throughput technique. Hca-P and Hca-F are a pair of synogenetic mouse hepatocarcinoma ascites cell lines, possessing a specific potential of lymphogenous metastasis when inoculated subcutaneously into 615 mice, Hca-P showing a low metastatic potential (< 30%), while Hca-F showing a high potential (> 80%)[1]. In the current study, we employed suppressive subtracted hybridization (SSH) technique to identify differentially expressed genes specific for Hca-P in an effort to obtain candidate genes related to lymphogenous metastasis of hepatocarcinoma in mice.

## MATERIALS AND METHODS

Hca-F and Hca-P have been established and maintained by our laboratory[1]; inbred 615-mice were provided by the experimental animal center of our university.

### *Determination of lymph node metastatic rates of Hca-P and Hca-F*

Sixty inbred 615-mice were randomly divided into 2 groups. The Hca-P and Hca-F tumor cell lines were inoculated at $2 \times 10^6$ tumor cells of approximately 0.1 mL cell suspension into each mouse subcutaneously in each group. The mice were decapitated on the 28th day post-inoculation. The implanted tumor and the regional lymph nodes were removed and paraffin sections of tissues were HE stained and examined under microscope. The lymph node metastatic rates of Hca-F and Hca-P tumor cells were calculated.

### *Construction of a subtracted cDNA library by SSH*

**Preparation of total RNA and mRNA:** Isolation of total RNA was performed by TRIZOLTM(GIBCOBRL) and that of mRNA was carried out according to the

protocol of oligotex mRNA spin column purification kit (Qiagen). The quantity and integrity of mRNA were detected by ultraviolet spectrometer and by electrophoresis on a denaturing formaldehyde agarose stained by EB. mRNA of Hca-P served as tester and mRNA of Hca-F as driver. SSH was performed between tester and driver by a PCR select$^{TM}$ cDNA subtraction kit and 50 × PCR enzyme kit (Clontech, Heidelberg, Germany) following the instructions of the manufacturer.

**dscDNA synthesis and digestion with *Rsa* I :** Briefly, 2 μg aliquots of each of poly ($A^+$) mRNA from the tester and the pooled driver were subjected to dscDNA synthesis. Thereafter, they were purified by passing through Chroma spin-400 columns (Clontech, USA). Each purified dscDNA was digested with *Rsa* I .

**ligation to adaptor 1 and 2R:** The tester cDNAs were subdivided into 2 equal groups and then ligated to adaptor 1 and 2R in separate ligation reactions. Ligation efficiency analysis was performed by amplifying ligation products with G3PDH 3' primer/PCR primer 1 and G3PDH 3' primer/G3PDH 5' primer, respectively, and their intensity was compared.

**Subtractive hybridization:** Subtractive hybridization was performed by annealing an excess of driver cDNAs with each sample of adaptor-ligated tester cDNAs. The cDNAs were heat-denatured and incubated at 68℃ for 8 h. After the first hybridization, the 2 samples were mixed together and hybridized again with freshly heat-denatured driver cDNAs for 20 h at 68℃. Two rounds of hybridization would generate a normalized population of tester-specific cDNAs with different adaptors at each end. After filling in the ends, 2 rounds of PCR amplification were performed to enrich desired cDNAs containing both adaptors by exponential amplification of these products[2]. The optimized cycles for the first and second PCRs were 27 and 13 respectively to increase representation and reduce redundancy of subtracted cDNA libraries.

**Analysis of subtractive efficiency:** Secondary PCR products were used as templates for PCR amplification of human G3PDH for 18, 23, 28 and 33 cycles respectively to assure subtraction efficiency. PCR products were run on 1.8% agarose gel.

### *Ligation of the subtracted library into a TA vector*

Products of the secondary PCR reactions were cloned into a pT Adv vector (Clontech) and the resultant ligation products were then transformed into DH5α *E. coli* competent cells. The bacteria were subsequently grown in 800 μL of liquid Luria-Bertani medium and allowed to incubate for 45 min at 37℃ with shaking at 150 rpm. Thereafter, the cells were plated onto agar plates containing ampicillin (50 μg/mL), 5-bromo-4-chloro-3-indoly-b-D-galactoside (X-gal; 20 μg/cm$^2$) and iso-ploprl-b-D-thiogalactoside (IPTG; 12.1 μg/cm$^2$) and incubated overnight at 37℃. Individual recombinant white clones were picked and grown in single line pattern onto Luria-Bertani agar solid medium containing ampicillin and allowed to incubate at 37℃ for 6-7 h before single clone was picked from single-line pattern agar medium and allowed to grow in Luria-Bertani liquid medium containing ampicillin overnight at 37℃ with shaking at 150 r/min.

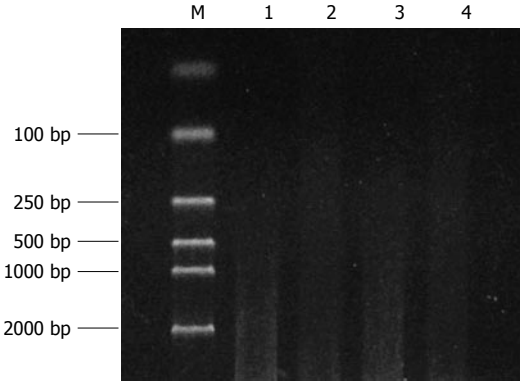


**Figure 1** The effect of *Rsa* I digestion. Lane 1, 3: cDNA of Hca-F and Hca-P cells; Lane 2, 4: cDNA of Hca-F and Hca-P cells after *Rsa* I digestion; M: DNA marker DL2000.

### *Identification of the subtracted clones*

Plasmids of candidate positive clones from subtracted cDNA library were isolated and amplified by PCR with nested primer 1 and primer 2. Meanwhile the product of PCR was detected by agarose gel electrophoresis.

### *Sequencing and BLAST homology search*

Randomly screened 14 positive clones from the subtracted cDNA library were sequenced by T7/SP6 chain termination reaction in TaKaRa (DaLian, China). Nucleic acid homology searches were subsequently performed at the National Center of Biotechnology Information (National Institutes of Health, Bethesda, Md., NCBI).

## RESULTS

### *Determination of lymph node metastatic rates of Hca-P and Hca-F*

Implanted tumors of both Hca-P tumor-bearing mice and Hca-F tumor-bearing mice were palpable on 7th day post-inoculation. On the 28th day post-inoculation, 10% Hca-P cells bearing mice developed metastatic regional lymph nodes (3/30), while 80% Hca-F cells bearing mice developed metastatic regional lymph nodes (24/30).

### *Total RNA and mRNA analysis*

The RNA samples electrophoresed on 1% agarose/EB gel exhibited 2 typical bands, corresponding to ribosomal 28s and 18s RNA, respectively, with a ratio of intensities > 2:1 and 1.9, ideal A260/A28 ratios of both samples obtained, indicating high integrity and purification of the total RNA we obtained. mRNA samples appeared as a smear with weak ribosomal RNA band: a high-quality mRNA was purified.

### *Rsa* I *digestion*

Both the digested cDNA and undigested cDNA usually presented as smears. However, their patterns were different. The digested cDNA fragments became shorter after Rsa I digestion (Figure 1).

### *Ligation efficiency analysis*

Intensity of the PCR product amplified using one gene-

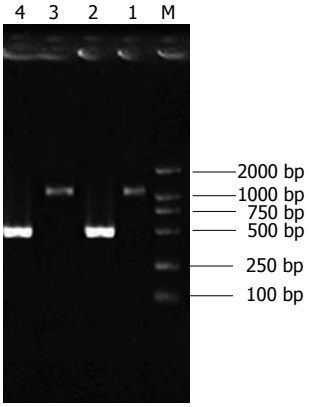


**Figure 2** Ligation efficiency analysis. Lane 1: Tester-1 as template, G3PDH 3'Primer and PCR Primer1; Lane 2: Tester-1 as template, G3PDH 3'Primer and G3PDH 5'Primer; Lane 3: Tester-2R as template, G3PDH 3'Primer and PCR Primer1; Lane 4: Tester-2R as template, G3PDH 3'Primer and G3PDH 5'Primer.

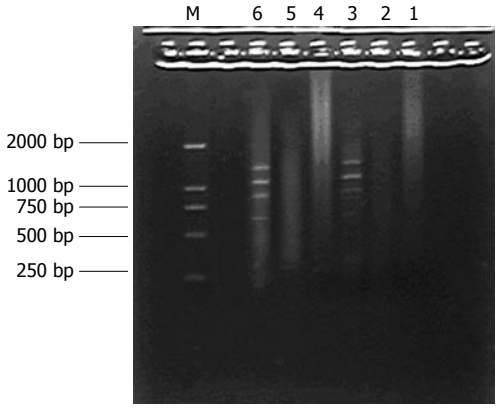


**Figure 3** The results of secondary PCR amplification. Lane 1-3: Product of primary PCR amplification, Lane 4: secondary PCR amplification product of unsubtracted cDNA, Lane 5: secondary PCR amplification product of subtracted cDNA, Lane 6: secondary PCR amplification product of PCR control cDNA, M: DNA Marker DL2000.

specific primer (G3PDH 3' primer) and PCR primer 1 was 25% more than that of PCR product amplified using two gene-specific primers (G3PDH 3' primer and 5' primer). Ligation efficiency was > 25%, ensuring enough tester cDNA in the following hybridization (Figure 2).

### *Construction of subtracted cDNA library by SSH*

PCR products of the subtracted and unsubtracted usually looked like smears with or without discrete bands. However, the patterns between them were different (Figure 3).

### *Analysis of subtractive efficiency*

Subtraction efficiency analysis showed the effectively reduced amount of non-differentially expressed genes. In unsubtracted cDNA libraries, housekeeping gene G3PDH PCR products were visible after 23 cycles of amplification and became saturated after 23-28 cycles. However, subtracted libraries required 33 cycles for G3PDH to be detected (Figure 4).

### *Differential screening of subtracted cDNA libraries*

The subtracted cDNA libraries were composed of 995 positive clones, of which 200 clones were randomly picked up and plasmids of the candidate positive clones were isolated and amplified by PCR with nested primer 1 and

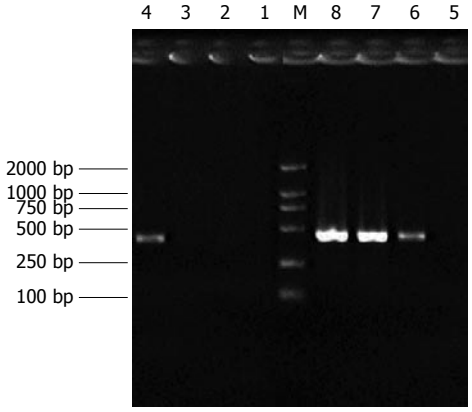


**Figure 4** Analysis of subtraction effect. PCR was performed on subtracted (Lane 1-4) or unsubtracted (Lane 5-8) secondary PCR product with G3PDH 5'Primer and 3'primer. Lanes 1, 5: 20 cycles, Lanes 2, 6: 25 cycles, Lanes 3, 7: 30 cycles, Lanes 4, 8: 35 cycles. M: DNA marker DL2000.

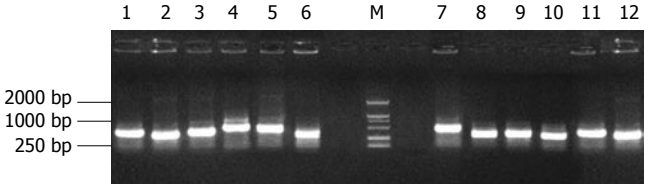


**Figure 5** The results of clone PCR amplification. There was an average insert size of 300-1000 bp. M: DNA marker DL2000.

primer 2. As a result, 189 positive clones showed PCR products of a size of 300-1000 bp (Figure 5).

### *Sequencing and homology search*

Fifteen screened clones randomly selected were sequenced and homology search (http://www.ncbi.hlm.nih.gov/BLAST/) revealed 8 known genes and 4 expressed sequence tags (ESTs). Three cDNAs showed no homology and presumably represented novel genes (Table 1).

## DISCUSSION

Tumor metastasis, as the leading cause of tumor related death, is a process involving multiple genes and their products. Elucidation of the gene expression profiles specific for tumor cells with different potential of metastasis might help in the understanding of the molecular mechanisms of metastasis. As one of the high throughput screening techniques, SSH technique has two distinct advantages: (1) it boasts a high subtraction efficiency; (2) it harbors an equalized representation of differentially expressed sequences which can separate effectively both high and low copy expressed genes mainly because of normalization[2]. von Stein *et al*[3] found about 94% positive rate in their research. Thus they considered confirmation of differentially expressed genes by Northern blot analysis for each clone obtained was probably unnecessary.

For a long time, studies have focused on the angiogenesis of tumors, but the roles of lymphatic vessels

**Table 1 Homologue searching of the sequenced cDNA fragments from SSH library**

| Clone seriel number | Size (bp) | Sequence identity |
|---|---|---|
| 1-3 | 508 | Mus musculus Telomere repeat binding factors TRF1 |
| 3-5 | 543 | Mus musculus Telomere repeat binding factors TRF2, |
| 9-6 | 489 | Mus musculus maspin |
| 15-4 | 335 | Mouse chromosome 3 clone RP 6-126M1 |
| 11-7 | 386 | Mouse 7 d embryo whole body cDNA RIKEN full-length enrich library, clone 2210102k3 |
| 16-3 | 503 | Mouse 5 d liver cells cDNA RIKENfull -length library enriched library, clone E330462F4 |
| 10-1 | 549 | Mouse chromosome 17 clone RP 26-122M3 |
| 13-7 | 502 | Mouse chromosome 4 clone RP13-110N10 |
| 9-8 | 340 | EST-mouse |
| 6-6 | 411 | EST-mouse |
| 11-2 | 390 | EST-mouse |
| 20-9 | 399 | EST-mouse |
| 23-3 | 470 | Unknown |
| 17-6 | 486 | Unknown |
| 12-3 | 344 | Unknown |

in tumor growth and metastasis were neglected. However, it is well known that lymphatic metastasis is mainly responsible for the spread of epithelial malignant tumors, and is closely related to the prognosis of patients.

Hca-P and Hca-F are a pair of synogenetic mouse hepatocarcinoma ascites cell lines presenting a specific potential of lymphogenous metastasis with a significant difference in their potential of metastasis[1]. Candidate genes involved in lymphogenous metastasis are supposed to be among the differentially expressed genes.

Using Hca-P as a tester, Hca-F as a driver, and we employed SSH technique to identify differentially expressed genes specific for Hca-P(low metastatic potential) so as to obtain candidate suppressor genes of lymphogenous metastasis. Fifteen screened clones randomly selected were sequenced and homology search revealed 8 known genes as $TRF_1$, $TRF_2$(telomere repeat binding factor 1, 2) ; maspin; mouse 7 days embryo whole body cDNA, RIKEN full-length enriched library clone 2210102k3; mouse 5 days liver cells cDNA, RIKEN full-length enriched library, clone E330462F4; mouse chromosome 3 clone RP 6-126M1;mouse chromosome 17 clone RP 26-122M3 and mouse chromosome 4 clone RP13-110N10. Studies showed $TRF_1$, and $TRF_2$ play important roles in genome stabilization [4-11] and are down-regulated in some malignant cell lines and tumor tissues[12-18]. In hematopoietic carcinogenesis, gene expression of telomerase suppressors such as TRF and TIN2, is decreased. mRNA encoding $TRF_1$ and $TRF_2$ when gastric cancer becomes more deeply invaded**,** is significantly decreased, indicating a negative association with tumor progression. Of the 8 known fragments, one showed high homology to mouse Maspin gene. Serving as one of the few p53-targeted genes involved in tumor invasion and metastasis, Maspin, a member of the serpin family, has been reported to suppress metastasis and angiogenesis in breast and prostate cancers, and is closely correlated with their clinical manifestations[19-31]. It indicates that SSH in our study is capable of enriching metastasis related genes.

Another 5 known fragments were attributed to embryo genes. Embryo genes AFP and CEA are overexpressed in hepatocarcinoma and other malignant tumors, indicating a possible association between embryo development and tumor. Embryo genes were also found in our previously established SSH library which contains candidate tumor metastasis genes[32]. These data showed that up-regulated expression of embryo genes during metastasis is not a casual event. Their roles in tumor metastasis need to be clarified. Moreover, 4 cDNA fragments demonstrated homology with 4 ESTs-mouse and 3 cDNA fragments showed no homology and presumably represented novel genes[33].

In summary, the findings of our study suggest that the lymphatic invasiveness of tumor cells is determined by multiple genes and co-factors with complex cellular signal pathways. Further functional study of the candidate novel genes might provide clues to molecular mechanism of tumor metastasis.

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*CASE REPORT*

# Cytomegalovirus-associated colitis causing diarrhea in an immunocompetent patient

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## Abstract

Cytomegalovirus (CMV) colitis rarely occurs in immunocompetent patients. We report a case of disabling and life threatening diarrhea in an immunocompetent elderly woman due to CMV colitis. The diagnosis of CMV was based on histological examination of tissues biopsied at colonoscopy, positive CMV antigen and high CMV-IgM titer in peripheral blood samples and a good response to systemic gancyclovir treatment. We conclude that CMV should be considered in the differential diagnosis of colitis in elderly immunocompetent patients.



**Key words:** Cytomegalovirus; Colitis; Immunocompetent; Diarrhea; Gancyclovir

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## INTRODUCTION

The seroprevalence of cytomegalovirus (CMV) in the general population is high (40%-100%)[1]. Most CMV infections are acquired in the perinatal period, infancy or early adulthood[2]. The majority of primary CMV infections in immunocompetent adults are asymptomatic or associated with mild mononucleosis-like syndrome. To our knowledge, there have been only anecdotal reports of clinically significant CMV gastrointestinal infection in healthy adults. In these cases, CMV disease developed with community acquired, acute primary infection, through blood transfusions and sexual transmission[3,4].

## CASE REPORT

A 76-year-old woman was admitted with a week history of diffuse abdominal pain and one passing of watery diarrhea a few hours before admission. She had well controlled type 2 diabetes mellitus and hypertension. Twenty years ago, she had undergone a right mastectomy and chemotherapy treatment for breast carcinoma. She had not received chemotherapy for at least 15 years. On admission, her physical examination revealed minimal tenderness in the right abdomen without reboundness. Laboratory investigations showed a leucocytosis of $15 \times 10^9$/L (normal range, 3.5-9.5 $\times$ $10^9$/L) and mildly elevated liver enzymes of SGOT 75 (normal range, 15-40) IU/L and SGPT 129 (normal range, 15-40) IU/L. Bilirubin levels were normal. HIV serology was negative.

During the initial days of her hospitalization, the diarrhea worsened and was accompanied by bloody and pussy rectal discharge. She had a mild fever of 38℃. Electrolyte disturbances (hypokalemia and hypomagnesemia) were attributed to the diarrhea and were easily corrected. Albumin levels decreased to 2.4 (normal 3.6-5.5) g/L. Stool cultures were negative, and Clostridium Difficile toxin was not detected in three stool samples. Computed tomography of the abdomen demonstrated atherosclerosis of the abdominal aorta and thickening of the sigmoid colon. Colonoscopy revealed several fibrin-coated deep ulcers in the rectum and sigmoid colon. Mucosal biopsy showed acute inflammation, ulceration and signs of regeneration with marked atypia. CMV immunostain was positive in a few cells. Active CMV infection was confirmed by high IgM CMV titer and CMV antigen in peripheral blood samples.

The patient was treated with intravenous gancyclovir for one week and then with oral valgancyclovir for additional five weeks. Her abdominal pain and diarrhea settled within a few days of initial treatment. Her blood studies normalized. The patient remained asymptomatic during 12 mo of follow-up.

## DISCUSSION

CMV colitis is associated with constitutional symptoms as well as gastrointestinal symptoms such as diarrhea, hematochezia, tenesmus, urgency and abdominal pain. The disease is usually limited to the left colon, with endoscopic findings indistinguishable from ulcerative colitis and Crohn's disease. Diagnosis requires both serologic and histologic criteria. CMV produces a characteristic cytopathic effect, consisting of a large 25 to 35 μm cell containing a basophilic intranuclear inclusion, which is sometimes surrounded by a clear halo and is frequently

associated with clusters of intracytoplasmatic inclusions[5]. Immunohistochemistry of the biopsied tissue using monoclonal antibodies and in-situ DNA hybridization enhances the sensitivity of the histopathologic analysis[6]. Positive IgM titer for CMV, CMV antigen in the blood and positive polymerase chain reaction in blood or urine confirm the diagnosis.

In a meta-analysis of outcome of CMV colitis in immunocompetent hosts (44 cases), the rate of spontaneous remission was 31.8%, but > 50% for patients less than 55 years of age. Death occurred in 31.8% of patients greater than 55 years of age[7]. The decline in humoral and cellular immunity, and the higher prevalence of co-morbidity in older patients may explain these findings. The highest mortality rates were associated with immune modulating conditions, such as diabetes mellitus, renal failure and malignancies. Young (< 55 years) and otherwise healthy patients usually are able to recover from CMV infection without the need for antiviral therapy. Antiviral therapy with gancyclovir or foscarnet is mandatory for older patients and for patients with immune modulating conditions.

In conclusion, CMV colitis, although rare in immunocompetent patients, should be considered when more common etiologies for severe diarrhea have been excluded. Timely diagnosis and treatment are essential in order to improve the outcome of elderly patients or patients with serious co-morbidities.

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***World Journal of Gastroenterology*** ISSN 1007-9327


# Meetings

## MAJOR MEETINGS COMING UP

First Biennial Congress of the Asian-Pacific Hepato-Pancreato-Biliary Association
March 2007
Fukuoka, Japan
<http://www.congre.co.jp/1st-aphpba>/

American College of Gastroenterology Annual Scientific
20-25 October 2006
Las Vegas, NV

14th United European Gastroenterology Week, UEGW
21-25 October 2006
Berlin, Germany

APDW 2006: Asian Pacific Digestive Week 2006
26-29 November 2006
Lahug Cebu City, Philippines

## EVENTS AND MEETINGS IN THE UPCOMING 6 MONTHS

Falk Symposium 151: Emerging Issues in Inflammatory Bowel Diseases
24-25 March 2006
Sydney - NSW
Falk Foundation e.V.
symposia@falkfoundation.de

10th International Congress of Obesity
3-8 September 2006
Sydney
Event Planners Australia
enquiries@ico2006.com
www.ico2006.com

Easl 2006 - the 41st annual
26-30 April 2006
Vienna, Austria
Kenes International

Prague hepatology 2006
14-16 September 2006
Prague
Foundation of the Czech Society of Hepatology
veronika.revicka@congressprague.cz
www.czech-hepatology.cz/phm2006

12th International Symposium on Viral Hepatitis and Liver Disease
1-5 July 2006
Paris
MCI France
isvhld2006@mci-group.com
www.isvhld2006.com

Falk Symposium 152: Intestinal Disease Part I, Endoscopy 2006 - Update and Live Demonstration
4-5 May 2006
Berlin
Falk Foundation e.V.
symposia@falkfoundation.de

Falk Symposium 153: Intestinal Disease Part II, Immunoregulation in Inflammatory Bowel Disease - Current Understanding and Innovation
6-7 May 2006
Berlin
Falk Foundation e.V.
symposia@falkfoundation.de

ILTS 12th Annual International Congress
3-6 May 2006
Milan
ILTS
www.ilts.org

Internal Medicine: Gastroenterology
22 July 2006-1 August 2006
Amsterdam
Continuing Education Inc
jbarnhart@continuingeducation.net

6th Annual Gastroenterology And Hepatology
15-18 March 2006
Rio Grande
Office of Continuing Medical Education
cmenet@jhmi.edu
www.hopkinscme.net

World Congress on Gastrointestinal Cancer
28 June 2006-1 July 2006
Barcelona, Spain
c.chase@imedex.com

International Conference on Surgical Infections, ICSI2006
6-8 September 2006
Stockholm
European Society of Clinical Microbiology and Infectious Diseases
icsi2006@stocon.se
www.icsi2006.se/9/23312.asp

7th World Congress of the International Hepato-Pancreato-Biliary Association
3-7 September 2006
Edinburgh
Edinburgh Convention Bureau
convention@edinburgh.org
www.edinburgh.org/conference

Society of American Gastrointestinal Endoscopic Surgeons
26-29 April 2006
Dallas - TX
www.sages.org

Digestive Disease Week 2006
20-25 May 2006
Los Angeles
www.ddw.org

Annual Postgraduate Course
25-26 May 2006
Los Angeles, CA
American Society of Gastrointestinal Endoscopy
www.asge.org/education

American Society of Colon and Rectal Surgeons
3-7 June 2006
Seatlle - Washington
www.fascrs.org

## EVENTS AND MEETINGS IN 2006

10th World Congress of the International Society for Diseases of the Esophagus
22-25 February 2006
Adelaide
isde@sapmea.asn.au
www.isde.net

Falk Symposium 151: Emerging Issues in Inflammatory Bowel Diseases
24-25 March 2006
Sydney - NSW
Falk Foundation e.V.
symposia@falkfoundation.de

10th International Congress of Obesity
3-8 September 2006
Sydney
Event Planners Australia
enquiries@ico2006.com
www.ico2006.com

Easl 2006 - the 41st annual
26-30 April 2006
Vienna, Austria
Kenes International

VII Brazilian Digestive Disease Week
19-23 November 2006
www.gastro2006.com.br

International Gastrointestinal Fellows Initiative
22-24 February 2006
Banff, Alberta
Canadian Association of Gastroenterology
cagoffice@cag-acg.org
www.cag-acg.org

Canadian Digestive Disease Week
24-27 February 2006
Banff, Alberta
Digestive Disease Week Administration
cagoffice@cag-acg.org
www.cag-acg.org

Prague Hepatology 2006
14-16 September 2006
Prague
Foundation of the Czech Society of Hepatology
veronika.revicka@congressprague.cz
www.czech-hepatology.cz/phm2006

12th International Symposium on Viral Hepatitis and Liver Disease
1-5 July 2006
Paris
MCI France
isvhld2006@mci-group.com
www.isvhld2006.com/

Falk Seminar: XI Gastroenterology Seminar Week
4-8 February 2006
Titisee
Falk Foundation e.V.
symposia@falkfoundation.de

European Multidisciplinary Colorectal Cancer Congress 2006
12-14 February 2006
Berlin
Congresscare
info@congresscare.com
www.colorectal2006.org

Falk Symposium 152: Intestinal Disease Part I, Endoscopy 2006 - Update and Live Demonstration
4-5 May 2006
Berlin
Falk Foundation e.V.
symposia@falkfoundation.de

Falk Symposium 153: Intestinal Disease Part II, Immunoregulation in Inflammatory Bowel Disease - Current Understanding and Innovation
6-7 May 2006
Berlin
Falk Foundation e.V.
symposia@falkfoundation.de

14th United European Gastroenterology Week
21-25 October 2006
Berlin
United European Gastroenterology Federation
www.uegw2006.de

World Congress on Controversies in Obesity, Diabetes and Hypertension
25-28 October 2006
Berlin
comtec international
codhy@codhy.com
www.codhy.com

Asia Pacific Obesity Conclave
1-5 March 2006
New Delhi
info@apoc06.com
www.apoc06.com/

ILTS 12th Annual International Congress
3-6 May 2006
Milan
ILTS
www.ilts.org

XXX Panamerican Congress of Gastroenterology
11-16 November 2006
Cancun
www.panamericano2006.org.mx

Internal Medicine: Gastroenterology
22 July 2006-1 August 2006
Amsterdam
Continuing Education Inc
jbarnhart@continuingeducation.net

6th Annual Gastroenterology And Hepatology
15-18 March 2006
Rio Grande
Office of Continuing Medical Education
cmenet@jhmi.edu
www.hopkinscme.net

Hepatitis 2006
25 February 2006-5 March 2006
Dakar
hepatitis2006@mangosee.com
mangosee.com/mangosteen/hepatitis2006/hepatitis2006.htm

World Congress on Gastrointestinal Cancer
28 June 2006-1 July 2006
Barcelona, Spain
c.chase@imedex.com

International Conference on Surgical Infections, ICSI2006
6-8 September 2006
Stockholm
European Society of Clinical Microbiology and Infectious Diseases
icsi2006@stocon.se
www.icsi2006.se/9/23312.asp

5th International Congress of The African Middle East Association of Gastroenterology
24-26 February 2006
Sharjah
InfoMed Events
infoevent@infomedweb.com
www.infomedweb.com

7th World Congress of the International Hepato-Pancreato-Biliary Association
3-7 September 2006
Edinburgh
Edinburgh Convention Bureau
convention@edinburgh.org
www.edinburgh.org/conference

13th International Symposium on Pancreatic & Biliary Endoscopy
20-23 January 2006
Los Angeles - CA
laner@cshs.org

2006 Gastrointestinal Cancers Symposium
26-28 January 2006
San Francisco - CA
Gastrointestinal Cancers Symposium
Registration Center
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Society of American Gastrointestinal Endoscopic Surgeons
26-29 April 2006
Dallas - TX
www.sages.org

Digestive Disease Week 2006
20-25 May 2006
Los Angeles
www.ddw.org

Annual Postgraduate Course
25-26 May 2006
Los Angeles, CA
American Society of Gastrointestinal Endoscopy
www.asge.org/education

American Society of Colon and Rectal Surgeons
3-7 June 2006
Seatlle - Washington
www.fascrs.org

71st ACG Annual Scientific and Postgraduate Course
20-25 October 2006
Venetian Hotel, Las Vegas, Neveda
The American College of Gastroenterology

AASLD 57th Annual - The Liver Meeting™
27-31 October 2006
Boston, MA
AASLD

New York Society for Gastrointestinal Endoscopy
13-16 December 2006
New York
www.nysge.org

## EVENTS AND MEETINGS IN 2007

9th World Congress on Gastrointestinal Cancer
20-23 June 2007
Barcelona
Imedex
meetings@imedex.com

***Gastro 2009, World Congress of Gastroenterology and Endoscopy London, United Kingdom 2009***

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All contributions should be written in English. All articles must be submitted using a word-processing software. All submissions must be typed in 1.5 line spacing and in word size 12 with ample margins. The letter font is Tahoma. For authors from China, one copy of the Chinese translation of the manuscript is also required (excluding references). Style should conform to our house format. Required information for each of the manuscript sections is as follows:

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Data that are not statistically significant should not be noted. $^{a}P<0.05$, $^{b}P<0.01$ should be noted ($P>0.05$ should not be noted). If there are other series of *P* values, $^{c}P<0.05$ and $^{d}P<0.01$ are used. Third series of *P* values can be expressed as $^{e}P<0.05$ and $^{f}P<0.01$. Other notes in tables or under

illustrations should be expressed as $^1F$, $^2F$, $^3F$; or some other symbols with a superscript (Arabic numerals) in the upper left corner. In a multi-curve illustration, each curve should be labeled with ●, ○, ■, □, ▲, △, *etc.* in a certain sequence.

### *Acknowledgments*

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Authors: the first author should be typed in bold-faced letter. The surname of all authors should be typed with the initial letter capitalized and followed by their name in abbreviation (For example, Lian-Sheng Ma is abbreviated as Ma LS, Bo-Rong Pan as Pan BR). Title of the cited article and italicized journal title (Journal title should be in its abbreviation form as shown in PubMed), publication date, volume number (in black), start page, and end page [PMID: 11819634]

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### *Format*

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

1 **Grover VP**, Dresner MA, Forton DM, Counsell S, Larkman DJ, Patel N, Thomas HC, Taylor-Robinson SD. Current and future applications of magnetic resonance imaging and spectroscopy of the brain in hepatic encephalopathy. *World J Gastroenterol* 2006; **12**: 2969-2978 [PMID: 16718775]

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

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

*In press*

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

*Organization as author*

4 **Diabetes Prevention Program Research Group**. Hypertension, insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension* 2002; **40**: 679-686 [ PMID: 12411462 ]

*Both personal authors and an organization as author*

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

*No author given*

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

*Volume with supplement*

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

*Issue with no volume*

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

*No volume or issue*

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

**Books**

*Personal author(s)*

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

*Chapter in a book (list all authors)*

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

*Author(s) and editor(s)*

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

*Conference proceedings*

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

*Conference paper*

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

**Electronic journal** (list all authors)

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

**Patent** (list all authors)

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

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Authors should always cite references that are relevant to their article, and avoid any inappropriate references. Inappropriate references include those that are linked with a hyphen and the difference between the two numbers at two sides of the hyphen is more than 5. For example, [1-6], [2-14] and [1, 3, 4-10, 22] are all considered as inappropriate references. Authors should not cite their own unrelated published articles.

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Present 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 $\chi^2$ (in Greek), related coefficient as $r$ (in italics), degree of freedom as $\gamma$ (in Greek), sample number as $n$ (in italics), and probability as $P$ (in italics).

### *Units*

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

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***Italics***

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*LEADING ARTICLE*

# How to establish a first-class international scientific journal in China?

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## Abstract

Hundreds of scientific journals are published in China. However, only scores of them are included in Science Citation Index by the Institute for Scientific Information, with impact factors of only 1 or less. Thus, how to establish a first-class international scientific journal in China is an important but difficult topic that deserves extensive exploration. *World Journal of Gastroenterology* (*WJG*) sets a good example although it has experienced setbacks on the road towards success. Concepts and pursuits that affirm the overall development direction, innovation and dreams that provide impetus and aspiration for higher objectives, team work and unique pattern that assure excellent quality and service, and culture and environment that also determine the speed and direction of the development, are believed to be the major factors contributing to the success of *WJG*. It is recommended that the effective resolution to the above issue is to learn from Chinese examples such as *WJG* rather than from "how foreign journals do".



**Key words:** First-class scientific journal; Concepts and pursuits; Innovation and dreams; Team work and unique pattern; Culture and environment; *World Journal of Gastroenterology*

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## INTRODUCTION

In 2005, China Ministry of Science and Technology organized and implemented a research project on "How to establish a first-class international scientific journal in China". This issue is indeed an important but difficult topic that deserves extensive exploration. Both the editorial workers and the administrative authorities of scientific journals should seriously respond to it. In the meantime, I believe that the effective resolution to this issue is to learn from "Chinese cases"rather than from "how foreign journals do".

*World Journal of Gastroenterology* (*WJG*) was formally established by Dr. Lian-Sheng Ma on October 1, 1995. As an administrative leader of the Science Committee of Shanxi Province which is in charge of scientific journals, I have witnessed the growth and development of *WJG* over the past 12 years, but never made any public comments on the journal. Today, I wish to make some points by incorporating the *WJG* case into the above mentioned topic.

## CONCEPTS AND PURSUITS

As a macroeconomist, I know nothing about scientific journals. However, I am astonished to find that many journals have the same weaknesses as enterprises do. Some experts have reported that many Chinese enterprises suffer from "short-life syndrome" with an average lifespan of 2.9 years. It has also been said that some large Chinese corporations suffer from "small enterprise disease" characterized by severe opportunism in the strategic plan, lack of supporting sections in organization structure, collaboration inside the teams, and the uniform core values of the corporation. Similarly, it has been said that Chinese scientific journals are in short of creative and innovative papers, and abundant in third-class and "cloned" papers, which not only is a great waste of academic resources but also results in an extremely bad impact on scientific development in China. In my opinion, Chinese scientific journals share one common weakness, i.e. lack of concept and pursuit. This is just like the situation in Shanxi Province, where the negative impact driven by so-called resource economy has become increasingly obvious. Unauthorized mining, frequent mine explosion, vicious competition and corruption all can be attributed to the lack of concepts. Shanxi economy is an example that is cursed by "its resources". The misfortune resulting from the lack of concepts is shown by the horrible economic development without any conceptual restriction. Similarly, the development of scientific journals without any conceptual restriction is disastrous since it brings about enormous scientific "rubbish" and academic corruption,

which is known as "concept-lacking syndrome"in China.

Concepts mean belief, pursuits, and direction. Its core is the concept of values. The world well-known United States Military Academy at West Point has a history of over 200 years. The Academy's special educational concepts are "nation, honor and responsibility", which represent the spirit of nation, honor for society, team and personal responsibility. With such concepts, the Academy has educated two presidents (Ulysses S. Grant and Dwight D. Eisenhower), many famous generals, and a lot of business leaders. Whether an entrepreneur pursues a career or profits is the criterion to judge his/her personality. What is the objective to establish an academic journal? I said to Dr. Ma 10 years ago that "You should not be involved in any academic journal if you just want to make money". He got what I meant very clearly. Frankly, I did not believe he was able to establish an academic journal at that time because he lacked resources. Moreover, I did not understand his intention or ambition. On the one hand, he was already a famous doctor, and made a lot of money from his herbal inventions at that time, and thus could live a very comfortable life. On the other hand, it requires quantities of human and financial resources to take many risks to establish an academic journal. To choose establishing an academic journal as a career means hard work, suffering and even tears.

With high quality and excellent service as his concepts, Dr. Ma has devoted himself to and sacrificed a lot for *WJG*. As a man in his forties, his hair has turned grey. Fortunately, he finally succeeded. In the article entitled "The reasons for *WJG*'s success", which describes that the ultimate criterion to test a scientific journal is whether or not researchers in the field put it in their reading list. *WJG* has now successfully passed the criterion. *WJG* is an open and free international journal, and also the only weekly journal in gastroenterology in the world. The latest published papers in *WJG* are always sent to doctors and researchers free of charge by email. In addition, an average of about 30 000 online visits to the *WJG* website is recorded every day.

*WJG* has proposed a higher objective. At present 47 journals in Gastroenterology worldwide are included in the Science Citation Index Expanded (SCI-E). These first-class journals are also classified into three grades. Dr. Ma plans to invest one hundred million Yuan to make *WJG* an upper-middle level journal among these journals within 5 to 10 years. He is exploring a unique way to establish an international first-class scientific journal in China with persistent pursuits for quality and services.

## INNOVATION AND DREAM

Almost all the industries find it hard to adapt to the new environment in which there is a changing process from an industrialized society to a knowledgeable society, and do not know what to do next. I attended a recent forum on education reform during which a Japanese expert introduced education reform in Japan. "The most lacking nowadays are concepts and ideas" he said emotionally. It is really difficult to depict the pattern or model of scientific journals in the future. It was reported in a survey that only less than 5% Chinese enterprises have made a professionally-standard, complete and feasible strategicaly developed plan by scientific means. More than 90% of companies have no long term plan on how to survive. There is a famous byword at Harvard University, that is, a successful man is distinguished not due to his knowledge and experience, but due to his way of thinking. It has been said that the future cannot be forecasted or adapted, but can only be created. What is a first-class international scientific journal in China? This question can only be thought and answered by ourselves. Creativity is always limited by knowledge, experience and way of thinking. As a president of a publisher or an editor-in-chief of a journal, one must have the specific ability to shape innovative thinking, and to improve his thinking to a higher level.

Over the past decade, the most that Dr. Ma and I have discussed is the creativity of *WJG*. Dr. Ma telephoned me almost every week after my retirement, telling me his new ideas, from the administrative system to the flow remodeling, from scientific editing to electronic editing, from human resources to the internal structure management, and from exiting journals to resource integration in the same field. He is pursuing innovation in every aspect of editing a journal, which is the major reason that I have been greatly interested in *WJG*. On January 9, 2006, President Jin-Tao Hu pointed out at the National Congress of Science and Technology that China shall become a country with innovation in the next 15 years. This is a new starting point to a new road. Now officials in every industry are thinking about the issue on how to exploit a path with independence, innovation and Chinese characteristics. Then, how to establish an international first-class scientific journal in China? Taking the laggard reality in China into consideration, I would suggest that innovation must be comprehensive and systematic, rather than individualized, revolutionary, and evolving, in order to catch up or even overtake the international levels. Moreover, innovation in the administrative system is much more important than innovation in technology. I believe that one can find all answers to the question if he/she sticks to the two principles of innovation.

Now *WJG* Press, based on the current achievements and experience, is building an innovative system that includes journals as the main body, and the laboratory and pharmaceutical companies as supporting points, and is exploring a new model that combines journal publication with manufacturing, education and research. Dr. Ma always tries hard to make the best *WJG* once he identifies the right way, with the strategic design and practical implementation being carried on almost at the same time. *WJG* was publicly issued on June 29, 1995 as a seasonal journal. It became bimonthly from 1998 to 2002, monthly in 2003, biweekly in 2004, and then weekly in 2005, with one big jump each year over the last three years. Despite such significant achievements, I described each jump as the "thrilling jump" (Karl Max). I have warned Dr. Ma that if the jumping failed, the injured would be Dr. Ma himself but not *WJG*. Fortunately, Dr. Ma succeeded. Now he is thinking how to integrate resources of the famous journals in the field, and is planning to publish *World Journal of Oncology* and *World Journal of Chinese Medicine*, and to create a

broader platform for medical communications. In this way, an innovative chain will be created to optimize resource allocation, just like the supply chain management in the enterprises. I believe that he will succeed again this time since the innovation will not be limited in one journal or one step, and thus will improve the whole academic level.

## TEAM AND PATTERN

Only a first-class team can publish first-class journals. The *WJG* editorial board is composed of 760 experts in gastroenterology from 52 countries. Many famous scientists have been invited to write reviews and editorials for *WJG*. Its permanent editorial team is composed of those outstanding staff with doctors, masters of bachelor degree, and has become more competitive and efficient.

There were some bad comments at the beginning of *WJG* when there were not many resources such as financial support for journals from the government and experienced editors. There was a doubt on the success of *WJG* that was run by an "individual". Sometimes, I wonder how many emergent novelties have been killed by such an attitude and way of thinking. Everything has its own developing and improving process, which is from small to large and from weak to strong. The Editor-in-Chief is the key to a journal, just like an entrepreneur to an enterprise. A recent book "The World Is Flat" by an American writer, Thomas L. Friedman, describes that the world has become smaller and smaller. In the opinion of the author, the world has experienced three "shrinking" processes. The first one took place in the fourteenth century, and is characterized by the expansion of countries. At that time, Columbus discovered the New Continent, which opened the sea route and thus reduced the distances between countries. The second one took place in the industrial revolution, and is characterized by the expansion of enterprises (multinational corporations). At that time, the development and production of the railway, electricity and telecommunication instruments further reduced the size of the world. The third one is taking place now following the significant development of information network, and is characterized by the expansion of personal power. I heard recently that there is a news agency in the United States run by a single person. Therefore, we should not underestimate or ignore the ability of any individuals. To some extent, even an individual is able to influence the whole world.

Composition of a team is always influenced by the strategic orientation and the tactical policy, and restricted by the administration system and operation mechanisms. *WJG* has two principles. One principle is to operate according to the international standards. Dr. Ma has described to us the general situation of international standards and market operation patterns in 15 aspects, such as administrative system, editing criteria, paper quality, online submission, invitations for editorials and reviews on highlighted topics, peer review, external communications, printing and distribution, *etc*. This year, 75.8% of manuscripts submitted to *WJG* were from authors outside of China. The other principle is to improve with informatization. In some senses, internationalization means internet communications. *WJG* always improves continuously its business flow in order to match its administration pattern with informatization. I read a worksheet about the editing process of *WJG*, which is composed of 29 steps for scientific editing, 36 steps for electronic editing, and more than 10 other basic steps. Scientific and strict regulations and operational patterns are the basis of a successful *WJG*. The quality of a journal must be improved with time, and its development must represent the changing world. Unfortunately, some journals are sill run with the management pattern designed for the outdated planned economy. These journals lack in vitality and compatibility despite abundant human resources and financial support. Now is the time when live fish eat dead fish, and fast fish eat slow fish, but not necessarily big fish eat small fish. This is due to the choice of the market rather than the instruction of government. I used to recommend the pattern of *WJG* to some of my friends in the field. They all made good comments on the pattern but could not learn from it very well. Structure and administrative pattern are determined by the system. Therefore, it is the issue of the system that we must resolve before we find an international first-class scientific journal in China.

## CULTURE AND ENVIRONMENT

A journal, or an enterprise or a university, or an institute, or even a development zone can only grow in a certain culture and environment. They are all the products of the culture and environment. Why is the United States of America so strong? Is it just because of its military power and technology? No, it is because of its culture based on its democratic construction and citizen quality. Michael Bott, a famous American scholar has pointed out that the advantage that is based on culture is the most difficult to copy or replace, the most fundamental, the most everlasting and thus the most pivotal advantage in competition. China is investigating how to develop the "middle part" these days. Some experts believe that the key to developing the "middle part" is reshaping of the local culture. What I learned from the growing process of *WJG* is that some aspects of our culture really need improvement. Some government officials created difficulties for *WJG*, and some authorities turned their noses at *WJG*. In addition, some "readers" abused *WJG* or even insulted the publisher with whatever words they could create on the internet, such as"*WJG* is a bad journal", "I feel shamed that my paper is published in *WJG*". What kind of culture is it where you cannot do anything? Who are the victims of such a culture? We must have courage to reflect and animadvert on the outdated culture, and investigate these details in depth in order to found an international first-class scientific journal. One may get some insight from the book "Details Determine the Success".

The culture in the Silicon Valley or the Tech-Garden of Zhongguan Village is non-traditional. Individual behavior is determined by the profits and dreams. There is no authority or god. There are only heroes from commonality. So Silicon Valley is the place for youngsters to begin their career, to carve out, and to realize their dreams. Persons in Silicon Valley always respect losers, because they know

that the losers have taken the risks and paved the path for the victors towards the hi-tech peak. Mistakes and failures are unavoidable in its growing process. *WJG* was refused to be included in SCI in 2004 and 2005, which was a big blow to Dr. Ma. He made intensive investigations, and found out the reasons. The *WJG* self citation rate of 94%, which was rather high and made its impact factor up to 2.532 in 2002, was the major reason for the refusal. High impact factor and compositor were usually induced by high self citation rate, which resulted in a misleading of the influence of *WJG* in the field. Dr. Ma reduced its self citation rate to 15.87% in 2005, and fortunately *WJG* recovered by SCI this year. This is probably a good lesson. In addition to Dr. Ma's hard work, the success of *WJG* is also attributed to the support from some related authorities, such as China Ministry of Science and Technology, National Administration of News and Publication, Natural Science Foundation Committee of Shanxi Province, and many experts and readers. Professor Fa-Zu Qiu, member of the Chinese Academy of Sciences, aged 93, and Mr. Xu-E Li, former vice-director of China National Science and Technology Committee, both gave *WJG* tremendous support. Thus, *WJG* is very fortunate to have such support. We must learn from the growing process of the international first-class scientific journals if we want to found such a journal. The processes are always more splendid than results. Every time when *WJG* steps forward, I would remind Dr. Ma that happiness of success is temporary while the suffering and challenging are everlasting. Indeed, the most valuable personality of modern entrepreneurs is willingness to pursue "suffering" and challenge.

I have studied a book "Laws Cannot Change the Society: How to Reform France", written by a famous French sociologist, Michel Crozier. In his opinion, with the development of society, communication between individuals becomes more and more complicated, the society becomes more and more fragile, and thus the outdated social administrative systems become weaker and weaker and the laws can no longer resolve the social problems. The impetus of the society is the human resources, especially those intellectuals with great potentials. Only by motivating their enthusiasm, can the social reform be performed. Generally, those who are engaged in scientific journals are the nation's backbone.

In April 2006, *WJG* held the first working meeting of the year. I was initially invited to give a lecture on the relation between morality and quality, but I was unable to attend the meeting. Later, Dr. Ma told me an interesting story. One day he attended lectures on two topics delivered by Professor Wu-Zong Zhou from University of St. Andrews, St. Andrews, the United Kingdom. The first topic was "Why to Publish Scientific Papers", and the second one was "Strict Academician Attitude Ensures the Quality of Scientific Papers". During the lecture, someone raised a comment, saying "we are coming here to learn how to write papers, not to accept moral education". Surprisingly, about two thirds of the audience applauded for the comment. Professor Zhou shook his head and could not say anything. So did I after I heard the story. There is an extensive debate on the comparison of the development between China and India in recent two years. Some people believe that intellectual community which represents the advanced productivity is better qualified in India than in China. The former American President Bill Clinton also holds this viewpoint. Last year, I read an article by Dr. Nan-Ping Yuan, the consul general of the Chinese Consulate in Bombay, India. The title was "What Is stronger in India than in China?". It describes that Indian intellectuals are determined to retain their independent personality with consistency and conscience. They do not compromise to obtain any benefits. They sympathize and help the weak. They possess excellent academic quality, without flippancy and the urge for a quick success and an instant benefit. They rarely have the concepts such as "job-hopping" and "becoming official and making money" in mind. I believe that all these characteristics that Indian intellectuals possess are the core compatibility that a journal or a region requires.

*WJG* has represented China to compete on the international academic stage without any national financial support. Many experts and scientists in Western countries praise this kind of intellectual such as Dr. Ma as "scientific fighters" and "academic faeries". At least, we should show our sympathy and respect to those who dedicate themselves to their national academy.

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*EDITORIAL*

# Current concepts and controversies in the treatment of alcoholic hepatitis

Catherine Rongey, Neil Kaplowitz

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## Abstract

The treatment of alcoholic hepatitis remains one of the most debated topics in medicine and a field of continued research. In this review, we discuss the evolution of scoring systems, including the recent development of the Glasgow alcoholic hepatitis score, role of liver biopsy and current treatment interventions. Studies of treatment interventions with glucocorticoids, pentoxifylline, infliximab, s-adenosyl-methionine, and colchicine are reviewed with discussion on quality. Glucocorticoids currently remain the mainstay of treatment for severe alcoholic hepatitis.



**Key words:** Alcoholic hepatitis; Treatment; Glucocorticoid; Biopsy; Scoring system

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## INTRODUCTION

The treatment of alcoholic hepatitis is one of the most debated topics in medicine. The prevalence of the disease, its high fatality rate, and the elusiveness of cure keeps this disease in the forefront of topic reviews and scientific investigations.

Alcoholic liver disease accounts for over 12 000 deaths per year and 300 000 years of potential life lost in the United States[1]. Age adjusted death rate from alcohol induced liver disease accounts for 40% of deaths from cirrhosis or 28% of all deaths from liver disease[2]. Alcoholic liver disease is one of the top ten leading causes of death in developed countries, responsible for 3% (1.8 million) of all deaths[3]. While alcoholic hepatitis is common, its pathogenesis, predictors for survival, and treatment remain debated.

There have been several excellent reviews on the treatment of alcoholic hepatitis in the past year[4,5]. The focus of our review is to expand on the treatment of alcoholic hepatitis while addressing the role of scoring systems and liver biopsy.

## PATHOGENESIS

While the histology of alcoholic hepatitis is well characterized, the pathogenesis remains uncertain. A number of hepatocellular and inflammatory processes including the potential involvement of innate and adaptive immunologic responses are under investigation.

The variety of treatment options in alcoholic hepatitis share a common treatment goal of blocking the myriad of innate immunologic responses which include macrophage release of chemokines and cytokines, TNF-$\alpha$, IL-1$\beta$, IL-6 and IL-8, in addition to adaptive immune responses to acetaldehyde and hydroxyethyl radical formation[6,7].

While the immunologic responses are varied, they share similar end results of apoptosis, necrosis, inflammation and fibrosis. The history and basis of treatment interventions in alcoholic hepatitis are centered on blocking one or more of the harmful mechanisms found in the animal model with the hope, in human trials, of providing survival benefit (Table 1).

## SCORING SYSTEMS

There are several scoring systems applied toward predicting survival in alcoholic hepatitis. Many clinically employed scoring systems, however, are derived from related liver diseases and later validated for alcoholic hepatitis. As patient risk stratification and allocation of treatment are entirely dependent on a system to gauge short-term mortality, it is important to develop a scoring system that accurately predicts survival versus mortality.

First developed as risk stratification method for patients undergoing shunt surgery and later refined[8,9], the Child Turcotte Pugh (CTP), score has evolved as the

**Table 1 Immunologic responses and directed therapies in alcoholic hepatitis**

| Cytokines | Mechanism | Mediators | Treatment |
|---|---|---|---|
| Cytokines | | | |
| TNFα | Via TNFR1 signaling in hepatocytes and Kupffer cells (KC) | Apoptosis, necrosis, KC production of cytokines, potential cytoprotective effect | Glucocorticoids; Pentoxifylline; Infliximab |
| IL-6, 8 | Lymphocyte and neutrophil activation, release of acute phase reactants | Inflammation, fibrosis | Glucocorticoids |
| Antigenic adduct | Oxidation of ethanol, binding to proteins forming antigenic adducts | Adaptive immunity | Glucocorticoids |
| Chemokines | Attract leukocytes and increase adhesion molecules | Inflammation | Glucocorticoids |
| Oxidative Injury | | | |
| S-adenosyl-methionine (SAMe) | Precursor for glutathione, defense mechanism against oxidative stress, increase methylation | Protective role of SAMe | SAMe |
| Hypoxia/ischemic injury | Hypermetabolic state | Insufficient oxygen | Propylthiouracil |

most clinically used prognostic tool in cirrhotic patients. Patients are stratified into three categories based on point assignment of objective and subjective measures of liver function (Appendix A). The clinical application of the CTP score applied to cirrhotic patients has been validated[10,11]. Cited over 1700 times, the CTP score remains one of the most important clinical predicting tools in patients with cirrhosis[12]. However, in the setting of alcoholic hepatitis, there is evidence that other scoring systems may better predict survival.

The Maddrey Discriminant Function (DF) score, unlike the CTP, is derived from a clinical trial studying the efficacy of corticosteroid therapy in patients with alcoholic hepatitis[13]. Admission prothrombin time and serum bilirubin are independently and significantly associated with mortality and serve as the key variables in calculating the DF score. Further modified, in combination with the presence of encephalopathy, a DF score greater than 32 predicts greater than 50% mortality[14]. The DF score has subsequently been used to stratify patients in trials studying the efficacy of corticosteroid treatment in patients with alcoholic hepatitis[14,15]. While the DF score provides risk stratification, concerns of center to center variability of prothrombin time measurement[16], significant mortality in patients with DF score less than 32 and low test specificity have led some investigators to suggest alternative scoring systems[17].

The Mayo Endstage Liver Disease (MELD) Score, like the CTP score, was developed to predict survival in patients undergoing decompressive therapy for portal hypertension[18,19] (Appendix A). Unlike the CTP score, the MELD score is derived from prospective data and lacks subjective measurements of liver function. Using a derived formula, the MELD score is calculated using prothrombin time, creatinine and bilirubin. All of which are factors Maddrey *et al* found to be significant in his original study. However, unlike the Maddrey Discriminant Function, the MELD score is employed as a continuous assessment of liver function and includes creatinine, a marker of development of hepatorenal syndrome. It would be interesting to evaluate the Maddrey DF as a continuous marker of liver function.

The MELD score has been validated in predicting survival in patients with end-stage liver disease and chronic liver disease[19,20]. Three retrospective studies suggest that MELD is equivalent to DF in predicting survival in patients with alcoholic hepatitis[22,23]. One study found MELD to be more predictive of survival if calculated after the first week of hospitalization[23]. This result may reflect the fact that patients with a higher likelihood of dying decline in the first few weeks of hospitalization rather than improved test sensitivity. In summary, admission MELD, DF and CPT did not differ significantly from each other (Table 2).

Citing concerns of low specificity of the Maddrey DF score and the difficulty of identifying an optimal cut-off point of the MELD score[24,25], Forrest *et al* introduced the Glasgow alcoholic hepatitis score (GAHS)[16] (Appendix A). Using retrospective data from patients presenting with alcoholic liver disease, the authors use stepwise logistic regression to identify variables associated with mortality. In Table 2, we include the results of Forrest *et al*.'s study with separate comparisons between GAHS *versus* DF and GAHS *versus* MELD from the validation portion of the trial. We compared the accuracies of each test *via* Chi-square analysis. GAHS is more accurate than DF in 28 and 84 d mortality prediction but equivalent to MELD in 28 d mortality prediction. The reported specificities of both MELD and DF in this study are quite low, especially in comparison with other studies. However, the reported low specificity of DF significantly affected its comparative predictive capacity, *via* chi-square analysis, against GAHS.

Alcoholic hepatitis scoring systems are in evolution. MELD and DF appear to be equivalent in predicting mortality. The higher specificity and overall accuracy of the GAHS, if confirmed, may make it a better screening tool in the clinical trial. It would be interesting to review previous studies assessing the efficacy of glucocorticoid treatment using GAHS as the scoring system. For clinicians, however, a test with a higher sensitivity would be desirable as their primary goal is to maximize the number of patients receiving a treatment benefit. Therefore, the DF score may be preferred in the clinical setting as

**Table 2 Sensitivity and specificity of scoring systems for alcoholic hepatitis: sensitivities (Sen)/specificities (Spec)**

| Study | Patients | Study design | Predictive Mortality | MELD Sen/Spec (%) | DF Sen/Spec (%) | CTP Sen/Spec (%) | Glasgow Sen/Spec (%) | Conclusions |
|---|---|---|---|---|---|---|---|---|
| Sheth *et al*[21] 2002 | 34 | Retrospective | 30 d | ⩾ 11<br>86/82 | ⩾ 32<br>86/48 | N/A | N/A | MELD equivalent to DF |
| Kulkarni *et al*[17] 2004 | 41 | Retrospective | 28 d | N/A | ⩾ 33<br>66.7/61.5 | N/A | N/A | DF ⩾ 32 is appropriate.<br>High mortality in DF < 32 |
| Dunn *et al*[22] 2005 | 73 | Retrospective | 90 d | ⩾ 21<br>75/75 | ⩾ 37<br>88/65 | N/A | N/A | MELD equivalent to DF |
| Srikureja *et al*[23] 2005 | 202 | Retrospective | Not given | Admission: ⩾ 18<br>85/84<br>Wk 1: ⩾ 20<br>91/85 | ⩾ 32<br>83/84 | ⩾ 12<br>76/80 | N/A | Admission MELD equivalent to DF |
| Forrest *et al*[16] 2005 | 134 | Retrospective | 28 d<br>84 d | N/A | ⩾ 32<br>28 d<br>96/27<br>84 d<br>95/31 | N/A | ⩾ 9<br>28 d<br>81/61<br>84 d<br>78/66 | GAHS more accurate in predicting mortality compared to DF |
| Forrest *et al*[16] 2005 | 46 | Retrospective | 28 d<br>84 d | ⩾ 11<br>28 d<br>92/29<br>84 d<br>92/29 | N/A | N/A | ⩾ 9<br>28 d<br>75/68<br>84 d<br>69/67 | GAHS more accurate in predicting 84 d mortality<br>GAHS equivalent to MELD in predicting 28 d mortality |

it captures more patients at risk of dying than does the GAHS score.

## LIVER BIOPSY

Since it was reported first by Mallory *et al*[26] the morphology of alcoholic liver disease has been well described[27]. The details and significance of morphologic progression from steatosis to cirrhosis, as it relates to survival and treatment, continue to be refined.

Employed as part of an entry and stratification criteria in a few clinical trials[13,15,28-31], liver biopsy for staging and predicting survival has been replaced by scoring systems. Although survival did not differ between the glucocorticoid trials that used pre-randomization biopsies versus scoring system, we think that clinical trials should enroll biopsy-proven cases. Biopsy confirmation of alcoholic hepatitis accurately defines the patients eligible for clinical trials and, in our view, is preferred if fever, leukocytosis and hepatic bruit are absent.

While histologic changes from steatosis and steatohepatitis to cirrhosis are known, correlating degree of steatosis with liver function and survival is currently under investigation. A study by Duvoux *et al*[32] finds a correlation between low grade steatosis and advanced liver failure as well as lowered sensitivity to steroid treatment. However, patients with low grade steatosis had higher Maddrey discriminant function scores, which can also predict poor survivals.

The role of liver biopsy in defining prognosis and treatment of alcoholic hepatitis in the clinical setting remains unclear. A thorough patient history and physical examination has a reported sensitivity and specificity of 91% and 96% in diagnosing alcoholic hepatitis[33]. However, from the above study, four cases out of 103 were misdiagnosed as alcoholic hepatitis upon review of biopsy specimen. It is generally accepted to perform a liver biopsy if the diagnosis of alcoholic hepatitis is either in question or a concomitant pathology, such as hepatitis C, is suspected[34]. Approximately 35%-40% of alcoholics are infected with HCV[35,36] and experience higher mortality rates than patients with alcoholic liver disease alone[37,38].

A patient history and physical exam cannot, however, consistently distinguish between and determine the extent of alcoholic hepatitis alone versus alcoholic hepatitis with concomitant cirrhosis. A biopsy can provide useful prognostic and diagnostic information. Patients with alcoholic hepatitis and cirrhosis have significantly higher 1- and 5-year mortality compared to patients with cirrhosis alone[39,40]. Presence of perivenular fibrosis, steatosis and giant mitochondria in a known alcoholic may herald the transition from alcoholic hepatitis to cirrhosis; a transition which could be prevented with abstinence[41-43]. In clinical practice, we recommend transjugular(given the presence of coagulopathy and/or ascites) liver biopsy in cases in which it is difficult to distinguish the contribution of alcoholic hepatitis and end-stage cirrhosis, especially when the hallmarks of alcoholic hepatitis, leukocytosis, fever and hepatic bruit are absent. Since treatments are associated with complications, we believe it is prudent to be confident of the diagnosis before using steroids.

## TREATMENT

### *Glucocorticoids*

First studied in the treatment of alcoholic cirrhosis in

**Table 3 Randomized controlled glucocorticoid trials in treatment of alcoholic hepatitis (% Death)**

| Study | Glucocorticoid | Patient | Severity assessment | HE | Placebo All (%) / HE (%) | Steroid All (%) / HE (%) | RRR (95% CI) All % / HE % | NNT (95% CI) All / HE | Quality score |
|---|---|---|---|---|---|---|---|---|---|
| Porter *et al*[125] 1971 | Methylprednisolone | 20 | Self derived | 16 | 7/9 (77)[b]<br>7/8 (88)[c] | 6/11 (55)[b]<br>6/8 (75)[c] | N/A | N/A | 5 |
| Helman *et al*[28][a] 1971 | Prednisolone | 37 | Self derived | 15 | 6/17 (35)<br>6/6 (100) | 1/20 (5)<br>1/9 (11) | 86 (-0.06-0.98)<br>84 (0.28-0.96) | 3 (2-18)<br>1 (1-2) | 2 |
| Campra *et al*[31] 1973 | Prednisone | 54 | Self derived | 18 | 9/25 (36)<br>8/10 (80) | 7/20 (35)<br>4/8 (50) | N/A | N/A | 2 |
| Blitzer *et al*[52] 1977 | Prednisolone | 28 | Self derived | 5 | 5/16 (31)<br>1/2 (50) | 6/12 (50)<br>2/3 (67) | N/A | N/A | 5 |
| Lesesne *et al*[49][a] 1978 | Prednisolone | 14 | Self derived | 14 | 7/7 (100)<br>7/7 (100) | 2/7 (29)<br>2/7 (29) | 67 (0.05-0.88)<br>67 (0.05-0.88) | 2 (1-4)<br>2 (1-4) | 3 |
| Shumaker *et al*[30] 1978 | Methylprednisolone | 27 | Self derived | 12 | 7/15 (47)<br>4/6 (67) | 6/12 (50)<br>2/6 (33) | N/A | N/A | 5 |
| Maddrey *et al*[13][a] 1978 | Prednisolone | 55 | DF | 15 | 6/31 (19)<br>6/10 (60) | 1/24 (4)<br>1/5 (20) | 79 (-0.67-0.97)<br>67 (-1.1-0.95) | 6 (-3-111)<br>3 (-1-16) | 4 |
| Depew *et al*[126] 1980 | Prednisone | 28 | Self derived | 28 | 7/13 (54)<br>7/13 (54) | 8/15 (53)<br>8/15 (53) | N/A | N/A | 4 |
| Theodossi *et al*[53] 1982 | Methylprednisolone | 55 | Self derived | 34 | 16/28 (57)<br>10/14 (71) | 17/27 (63)<br>17/20 (85) | N/A | N/A | 3 |
| Mendenhall *et al*[48] 1984 | Prednisolone | 178 | Self derived | 61 | 50/88 (57)<br>10/30 (33) | 55/90 (61)<br>11/31 (36) | N/A | N/A | 3 |
| Carithers *et al*[14][a] 1989 | Methylprednisolone | 66 | DF | 33 | 11/31 (36)<br>9/19 (47) | 2/35 (6)<br>1/14 (7) | 84 (0.3-0.96)<br>85 (-0.06-0.98) | 3 (2-9)<br>2 (2-7) | 5 |
| Ramond *et al*[15][a] 1992 | Prednisolone | 61 | DF | 19 | 16/29 (55)<br>7/10 (70) | 4/32 (13)<br>2/9 (22) | 77 (0.4-0.9)<br>68 (-0.15-0.9) | 2 (2-5)<br>2 (1-12) | 5 |

[a]$P < 0.05$ for survival benefit as reported by study authors; Note percent death at 28 d in placebo *versus* steroids is shown in each box for all cases[b] and for those with hepatic encephalopathy. [c]RRR, NNT is calculated from published data of those studies that reported a significant survival benefit. HE: Hepatic Encephalopathy; DF: Maddrey Discriminant Factor; CP: Child Turcotte Pugh; N/A: non significant difference in mortality as reported by study authors; Self derived: Criteria derived by study team not including CP, DF or MELD.

1960[44], the use of glucocorticoids remains perhaps the most studied and debated intervention. Reported successes of glucocorticoids are variable and appear largely dependent on the nature of the trial.

The rationale for the use of glucocorticoids is centered upon blocking the cytotoxic and inflammatory pathways in alcoholic hepatitis. Glucocorticoids decrease circulating inflammatory cytokines such as TNF-α, ICAM-1 expression, and have demonstrated short term histologic improvement in the treatment of alcoholic hepatitis[45].

It is difficult to provide a simple summary of results for glucocorticoid trials in alcoholic hepatitis. While all trials appeared to have been controlled, few have high quality scores given the variable definition of randomization and blinding in each trial[46,47].

The trials vary by inclusion/exclusion criteria, glucocorticoid type, scoring system, length of treatment and co-interventions. The study by Mendenhall *et al* is particularly difficult to interpret as essentially three different intervention arms, oxandrolone, prednisolone and prednisone, are employed[48]. Lesesne *et al* compares prednisolone to a 1600 caloric intake diet which is below the estimated caloric needs of most hospitalized patients; his trial is not placebo controlled[49]. Furthermore, the variation in type of glucorticoid, dose, and treatment length makes it difficult to provide treatment guidelines for physicians.

We did a computerized search using the MEDLINE database from 1971 to August 2005 using the search headings of "steroids", "corticosteroids", "alcoholic hepatitis", "hepatitis, alcohol", "randomized" and "English". We obtained additional trials by manually searching through retrieved trials and review articles. Randomized trials including corticosteroids in the treatment of alcoholic hepatitis with the outcome measure of mortality are summarized in Table 3. The trial results are summarized by percentage death in each group including number of patients with hepatic encephalopathy and their percentage mortality. Relative risk reduction (RRR), number needed to treat (NNT) with their associated 95% confidence intervals are calculated for trials that reported a significant benefit in survival. Given that hepatic encephalopathy is a known predictor of mortality, we have also calculated RRR and NNT for those patients; again only in trials that reported a significant survival benefit. We employed the Jadad score as an assessment of trial quality. The Jadad score is one of the few validated measures of randomized trial quality[46,50,51]. Out of a maximum score of five, points are assigned based on the method of randomization, double blinding and description of withdrawals/drop-outs.

The results are variable. Five trials reported a significant

survival benefit with glucocorticoids[13-15,28,49]. RRR ranged from 67% to 86%, with NNT varying between 2 and 6. As reported in the above trials and noted in our table, glucocorticoid treatment significantly reduces mortality in patients with hepatic encephalopathy. The latter trials by Carithers *et al* and Ramond *et al*, selectively treated patients with discriminant function scores greater than 32, supporting a more discriminate use of glucocorticoids[14,15].

Of the trials which reported a non-significant benefit, there are four trials which report higher mortality in the glucocorticoid group[30,48,52,53]. While glucocorticoids are relatively benign in the short term for most patients, the remaining three trials remind clinicians that there are significant complications with their use. Blitzer *et al* reported a higher number of fungal infections in the steroid group contributing to the greater percentage of deaths in the steroid group when compared to placebo. However, his steroid treatment group contains a higher proportion of patients with elevated total bilirubin, when compared to placebo, which may contribute to his study result[52]. Included in our discussion of nutrition in the treatment of alcoholic hepatitis, Cabre *et al*'s study found 31% (11/35) mortality in the steroid group with 91% of deaths attributable to infection[54]. It is important to recognize the potentially serious infectious complications secondary to steroid treatment.

Subsequent meta-analyses, while still yielding conflicting results, began to delineate which patients would most benefit from glucorticoid treatment. The first meta-analysis on this topic, conducted by Imperiale and McCullough in 1990 (which antedates more recent trials), finds a protective efficacy of glucocorticoids in higher quality trials, particularly those that exclude patients with gastrointestinal bleeding but include patients with hepatic encephalopathy[55]. They found a protective efficacy of 34% overall (95% CI, 15%-48%) for patients with hepatic encephalopathy. Imperiale and McCullough's study applies quality scores which are important in the setting of such trial heterogeneity. In their paper, quality scores are assigned by independent assessors. Quality scores we use in this paper are derived from Jadad *et al*, and have been used in assessing the quality of randomized clinical trials[46]. We do not find a significant association between high Jadad score and trial survival benefit. However, Jadad *et al* score does not consider baseline equivalence of compared groups, use of co-therapies and adequate potency of principal therapy. The variance of survival amongst the trials may have more to do with patient inclusion/exclusion criteria and the self-derived scoring systems than trial quality and adherence to randomization and double blinding.

Meta-analysis conducted by Christensen *et al*, did not find an overall treatment benefit, after attempting to control for confounding variables[56]. Controlling for confounding variables without direct access to individual study data can be difficult given the heterogeneity of prior trials. A subsequent study, by Mathurin, Mendenhall, Carithers *et al*[57] pooled raw data from their respective trials based on DF score (greater than 200 patients with DF $\geqslant$ 32 in placebo versus steroids) and found a survival benefit. If the DF < 32, there was a > 90% survival without steroids. The conclusions from the above study provide a more definitive treatment guideline for clinicians. In patients with DF $\geqslant$ 32, treatment with glucocorticoids improves short term, 28 d, survival with mortality decreasing from 35% in controls to 15% with steroids.

The longer term benefit of glucocorticoids are difficult to assess given the variable long- term clinical trial definitions (1.5 mo, 6 mo, 1 year)[54,48,58] and each of the existing three trials reported different outcomes: harm, no benefit and benefit[54,48,58]. It is also difficult to assess long-term benefit as alcoholic hepatitis is likely to recur unless the patient abstains.

### Recommendations

A review of the literature supports a more discriminate use of glucocorticoids in patients with a Maddrey discriminant function score $\geqslant$ 32. If there is no evidence of gastrointestinal bleeding or infection, the frequent concomitant presence of hepatic encephalopathy provides an even stronger support for the use of glucocorticoids. A study by Mathurin *et al* suggests a simple method to identify patients who are most likely to respond to glucocorticoids. Patients with an 'early change in bilirubin levels' (ECBL), i.e. a bilirubin level at 7 d lower than the bilirubin level on the first day of treatment, were significantly more likely to survive and respond to steroid treatment[59]. Discontinuation of glucocorticoid treatment in the non-responder group, i.e. patients that did not have an ECBL at 7 d, did not appear to result in adverse events[5]. Devising methods to target the patient groups most likely to benefit are important in maximizing treatment benefit, avoiding unnecessary complications of treatment and streamlining the treatment decision process.

Glucocorticoids, while providing a benefit in a select group of patients, are not without risks and should be used with caution in patients with infectious complications and gastrointestinal bleeding. Further trials that are larger in sample size, involving multiple centers and with an active comparator, i.e. pentoxifylline, are needed to better delineate the true effect of glucocorticoids. Finally, as scoring systems are changing, repeat studies may be needed to reassess the treatment effect of glucocorticoids employing MELD and GAHS.

### Anabolic steroids

There is a measurable and clinically apparent decline in gonadal function in patients with alcoholic liver disease[60-62]. In 1938, administration of androgens appeared to enlarge the liver of cirrhotic rats, thereby suggesting that perhaps androgens could reverse the process of fibrosis[63]. This lead to the first clinical trial where 12 patients with alcoholic cirrhosis were injected with large doses of testosterone with 'some improvement'[64].

A 2003 Cochrane systematic review could not demonstrate a significant effect of anabolic-androgenic steroids on the mortality of patients with alcoholic liver disease[65]. Three trials[48,66,67] included in the analysis and two trials[68,69] excluded from the analysis are trials in which all participants have alcoholic hepatitis.

**Table 4 Infliximab trials in the treatment of alcoholic hepatitis**

| Study | Design | Patients | Treatment | Results |
|---|---|---|---|---|
| Spahr *et al*[76] 2002 | Randomized | 20 | All patients: prednisone for 28 d<br>Randomized d 0<br>R1: Infliximab 5 mg/kg<br>R2: Placebo | Improved Maddrey score<br>No significant difference in survival, histology or adverse outcomes |
| Tilg *et al*[77] 2003 | Case Series | 12 | Infliximab 5 mg/kg | 83% (10/12) survived at median 15 mo<br>No mention of infection |
| Mookerjee *et al*[78] 2003 | Case Series | 10 | Infliximab 5 mg/kg times one | 72 h assessment<br>Significant reduction in laboratory parameters<br>Increased hepatic and renal blood flow |
| Naveau *et al*[79] 2004 | Randomized | 36 | All patients: Prednisone for 28 d<br>R1: Infliximab 10 mg/kg<br>R2: Placebo | Significantly higher rate of infections in treated group<br>Non-significantly higher rate of death in treated group<br>Study stopped secondary to adverse events in treatment group |

Reflective of the conclusions derived from the systematic review, Bonkovsky *et al*'s study[67] and Mendenhall *et al*'s 1984 study[48] did not find a significant survival advantage in the anabolic steroid group in the placebo. In Mendenhall *et al*'s study, patients in the oxandrolone group were treated for 30 d. They report, however, in subgroup analysis that patients with moderate hepatitis treated with oxandrolone seemed to have survival advantage 6 mo post treatment. As the subgroup analysis did not include patients that had died within the first two months of treatment, the results should be taken with some caution.

Currently, anabolic steroids are not recommended for the treatment of alcoholic hepatitis.

### Pentoxifylline

Pentoxifylline is a suppressor of tumor necrosis factor alpha (TNF-α), prevents leukocyte adherence to vascular endothelium and down regulates the expression of intercellular adhesion molecule-1 in monocytes[70]. The main signaling pathway is through type 1 tumor necrosis factor receptor, TNFR1. Elevated levels of TNF-α are predictive of poor survival in alcoholic hepatitis[71,72]. Other effects of this drug may contribute to its action such as its effects on membrane fluidity which determine its use in peripheral vascular disease.

First studied by McHutchison *et al* in 1991, in patients with severe alcoholic hepatitis (defined as DF score $\geqslant$ 32), pentoxifylline reduced the development of hepatorenal syndrome, and as a consequence mortality, in comparison to patients who received placebo[73]. A subsequent double blind placebo controlled trial, Akriviadis *et al*, from the same center, supports McHutchison's findings[74]. There did not appear to be any complications as a consequence of pentoxifylline treatment. As noted by Dr. Mathurin[5], the latter study showed no improvement in liver function tests. The reported improved survival was accounted for by a reduction in the development of hepatorenal syndrome in the treatment group. This finding is in sharp contrast to the glucocorticoid trials which demonstrate an improvement in liver function and in survival compared to placebo.

A small sample size, retrospective, observational study by McAvoy *et al*, published as an abstract, finds a treatment benefit with pentoxifylline only in patients stratified to GAHS $\geqslant$ 9, but not in patients with DF $\geqslant$ 32[75]. As raw numbers are not available at press time, it is difficult to draw a meaningful conclusion from his study.

Recommendations: Pentoxifylline may reduce mortality from hepatorenal syndrome in the setting of severe alcoholic hepatitis but further studies are needed to confirm these findings. Aside from the need for head to head comparative trials with steroids, one wonders if the combination of the two treatments might exhibit an additive benefit.

### Infliximab

Infliximab, used in the treatment of Crohn's disease, rheumatoid arthritis and psoriasis, is a chimeric mouse/human antibody which binds to tumor necrosis alpha, blocking its effects[76]. Preliminary trial data was encouraging. Three trials reported either better survival than predicted, improved Maddrey score or laboratory parameters[76-78]. The largest and most comprehensive trial studying the efficacy of prednisone and infliximab in the treatment of alcoholic hepatitis was terminated early when a significantly higher number of deaths occurred in the treatment group[79]. The study received some criticism for its use of high dose of infliximab and infusion protocol which varied from previous studies. In this study, investigators cited prior studies in Crohn's[80] and rheumatoid arthritis[81] in which there did not appear to be a relationship between dose of infliximab and rate of infection[78]. Furthermore, Dr. Naveau contends, perhaps infliximab is not the TNF-α blocking agent for alcoholic hepatitis (Table 4).

An open label uncontrolled pilot study on etanercept in the treatment of moderate to severe alcoholic hepatitis was completed[82]. Of the 13 patients treated, 7 had Maddrey DF greater than 32 and two of the seven died within 32 d. Etanercept was discontinued in 3 patients secondary to infection, hepatorenal decompensation and gastrointestinal bleeding. Therefore, there is no particular evidence one way or the other to suggest a beneficial or detrimental effect of treatment. As etanercept targets soluble TNF, whereas infliximab targets both soluble and

membrane bound TNF, it is uncertain what the advantages/disadvantages are of this distinction in the setting of this specific disease target.

Infliximab is not currently recommended for the treatment of alcoholic hepatitis, outside of clinical trials. Although concerns have been raised about increased risk of infection, the more disturbing aspect has been recent warnings of acute liver failure in patients with Crohn's disease and rheumatoid arthritis treated with infliximab[83]. This risk may preclude its use in patients with underlying severe liver injury who are less capable of withstanding an additional insult to the liver.

### Nutrition

There are multiple etiologies for weight loss and malnutrition in patients with years of alcohol abuse. Weight loss can be reflective of years of substitution of alcohol for more than 50% of other calories[84], malabsorption of dietary fat and nutrients[85] and the induction of a catabolic state resulting in skeletal muscle depletion[86].

Recently reviewed[4] parenteral and enteral nutrition, while improving liver function in a few studies in alcoholic hepatitis[87-89] has yet to demonstrate a change in clinical outcome.

Mendenhall *et al* have done the most extensive assessment on the effect of protein calorie malnutrition (PCM) and protein energy malnutrition (PEM) on survival and liver function. The results of their interventional studies are shown in Table 5. The observed associations between degree of malnutrition, as calculated by PCM or PEM score, and severity of liver disease[90-92] as well as improvement of survival with improved PCM score[93] serve as the basis for determining the effect of nutritional intervention on survival and liver function in alcoholic hepatitis[69,94].

Nutritional interventions such as caloric amount, type, mode and duration of supplementation vary among the trials. For example, the 1600 caloric nutritional intervention in Lesesne *et al*'s study is below that of most hospitalized patients. A positive correlation between nutritional intake and survival, if present, would not be expected. Much as in the case of the glucocorticoid literature, it would be difficult to provide clinical recommendations when the treatment interventions and outcomes vary. Furthermore, it is difficult to draw meaningful clinical conclusions. While nitrogen balance improves in the nutrition intervention arm, survival remains unchanged.

The majority of trials did not find a survival advantage in nutritional support. There are two trials which showed a survival advantage. Nasrallah *et al*'s study is smaller and both groups receive a 3000 kCal diet with protein, which is an intervention treatment in some studies. Mendenhall *et al,* found a later survival advantage, 6 mo post treatment, in the moderately malnourished group. This is in contrast to Mezey *et al*'s study which did not find a survival advantage up to two years after treatment.

Recommendations: It is important to assess nutritional status of patients in order to recognize and treat the distinct nutritional deficiencies inherent in alcoholic cirrhosis and hepatitis. At this time, however, nutritional supplementation during acute presentation of alcoholic hepatitis does not appear to affect survival.

### Colchicine

The final histologic stage in alcoholic liver disease is cirrhosis. Found to inhibit liver fibrosis in rats[95], colchicine's anti-fibrotic activity presented a theoretical possibility of preventing liver fibrosis in humans.

Three clinical trials in the setting of alcoholic hepatitis[96,97] and a Cochrane database review in the setting of alcoholic and non-alcoholic liver fibrosis[98] fail to find a benefit in the treatment of alcoholic hepatitis with colchicine. Recently published and not included in the cochrane review, the largest trial studying long-term colchicine in the setting of alcoholic cirrhosis did not find a therapeutic benefit when compared to placebo, in concordance with prior literature[99]. Colchicine is not currently recommended for the treatment of alcoholic hepatitis.

### S-adenosyl-methionine

SAMe, produced from methionine by adenosylmethionine synthetase, is important in the metabolism of nucleic acids, structure and function of cell membranes and as a precursor of glutathione. Glutathione may be protective in alcohol induced liver injury[100]. However, in liver disease there is an impairment of enzyme activation of methionine which cannot be corrected by methionine supplementation[101]. In the setting of alcoholic hepatitis, there is a measurable decrease in hepatic methionine, SAMe and glutathione levels[102]. In animal studies, administration of SAMe increased glutathione levels, attenuated ethanol induced liver injury as well as liver injury caused by other hepatotoxins[103-106].

In a 2001 Cochrane systematic review[107], SAMe has yet to consistently demonstrate a significant beneficial effect on the mortality in the setting of alcoholic liver disease. None of the analyzed trials in the systematic review targeted patients with alcoholic hepatitis. The largest multi-center and highest Jadad quality scoring trial, by Mato *et al*, treated patients with alcoholic cirrhosis with SAMe for up to two years[108]. There was an overall decline in mortality in the treatment group compared to placebo, but did not reach significance. Excluding patients with Child's C cirrhosis, however, did yield a significant mortality benefit.

There are currently two NIH funded trials studying the effect of SAMe on the mortality in the setting of alcoholic cirrhosis. There has yet to be a trial studying the effect of SAMe administration on survival in the setting of acute alcoholic hepatitis.

SAMe is currently not recommended in the treatment of acute alcoholic hepatitis.

### Propylthiouracil (PTU)

Found to reduce hypoxic hepatocellular injury in ethanol fed rats[109], subsequent animal studies confirm PTU's protective role against oxidative and ischemic liver injury[110]; similar hepatic injuries are found in patients with alcoholic hepatitis[111].

In a 2001 Cochrane systematic review[112], PTU did

**Table 5 Interventional studies on nutrition and alcoholic hepatitis**

| Study | Design | Patients | Intervention | Findings |
|---|---|---|---|---|
| Lesesne *et al*[49] 1978 | Randomized | 14 patients, alcoholic hepatitis and encephalopathy | 7 controls, 1600 Kcal diet<br>7 study, prednisolone | Reduction in mortality in the prednisolone arm |
| Galambos *et al*[127] 1979 | Case series | 11 patients, alcoholic hepatitis | 4, enteral hyperalimentation<br>7, parenteral hyperalimentation | No difference in mortality<br>Increased nitrogen balance in study group |
| Nasrallah *et al*[128] 1980 | Randomized | 35 patients, alcoholic hepatitis | All received 3000 kcal 100g protein diet<br>18 control<br>17 study, 70-85 gram of intravenous amino acid | Lower mortality in the study group |
| Diehl *et al*[129] 1985 | Randomized | 15 patients, alcoholic hepatitis | All allowed to consume hospital diet ad libitum<br>10 controls, glucose solution<br>5 study, glucose solution + amino acids | Increased nitrogen balance in study group<br>No difference in clinical and biochemical markers of liver disease |
| Mendenhall *et al*[94] 1985 | Randomized | 57 patients, moderate-severe alcoholic hepatitis | 34 controls, 2500 cal diet<br>23 study, Hospital diet + Hepatic Aid | No difference in mortality<br>Improvement in nutritional parameters in intervention group |
| Calvey *et al*[130] 1985 | Randomized | 64 patients, alcoholic hepatitis | 32 controls, standard diet<br>32 study, standard diet + 2000 kCal + 10 g nitrogen | No difference in biochemical or clinical parameters |
| Soberon *et al*[131] 1987 | Case series | 14 patients, alcoholic hepatitis | 6 with adequate nutritional status, hospital diet<br>8 with poor baseline nutritional status, nasoduodenal diet, 35 kCal/kg per day | No difference in mortality<br>Increased nitrogen balance in study group |
| Simon *et al*[87] 1988 | Randomized | 12 patients, moderate alcoholic hepatitis<br>22 patients, severe alcoholic hepatitis | Moderate Group<br>6 control, standard diet<br>6 study, PPN<br>Severe Group<br>12 control, standard<br>10 study, PPN | No difference in mortality<br>Improved in biochemical tests in severe group |
| Bonkovsky *et al*[67] 1991 | Randomized | 39 patients, moderate to severe alcoholic hepatitis | 9, standard therapy<br>8, oxandrolone + standard therapy<br>10, PPN<br>12, oxandrolone + standard therapy + PPN | Improved biochemical parameters |
| Mezey *et al*[88] 1991 | Randomized | 52 patients, alcoholic hepatitis | 28 control, dextrose solution<br>26 study, dextrose + amino acid | No difference in mortality during hospitalization and 2 yr after treatment |
| Mendenhall *et al*[69] 1993 | Randomized | 273 patients, severe alcoholic hepatitis | 136 control<br>137 study, oxadrolone + enteral nutrition | No difference in mortality overall<br>Improvement in mortality in moderately malnourished group(19%) versus control (51%) at 6 mo post treatment |
| Cabre *et al*[54] 2000 | Randomized | 71 patients, severe alcoholic hepatitis | 36, prednisolone<br>35, enteral tube 2000 kCal/d | No difference in overall mortality<br>Higher early mortality in nutrition *versus* higher follow up mortality on steroids |
| Alvarez *et al*[132] 2004 | Case series | 13 patients, severe alcoholic hepatitis | 13, prednisolone + TEN 2000 kCal/d | 15% death during treatment<br>67% of patients developed infections during treatment -no deaths due to infections |

not provide a significant survival benefit in the setting of alcoholic liver disease. All of the analyzed six studies (3 of which were published only in abstract) included patients with alcoholic hepatitis[110,113-117].

Contrary to animal studies, hepatic histologic improvement with PTU administration is not replicated in clinical trial literature. PTU also does not appear to have a measurable effect on splanchnic hemodynamics in the setting of alcoholic cirrhosis[118].

While the systematic review did not find a significant association between PTU and adverse events, one trial was discontinued when higher mortality rates were observed in the PTU group[110]. Furthermore, there are case reports and several reviews on fulminant hepatic failure and hepatitis[119-123] secondary to PTU in addition to leukopenia[124] Propylthiouracil is not recommended for the treatment of alcoholic hepatitis.

## CONCLUSIONS

The treatment of alcoholic hepatitis continues to evolve as our understanding of the disease process expands. As it does so, however, it is important that our clinical trials attempt to achieve the highest quality possible. Trials

designed to replicate treatment effect should be done with treatment dosages and duration that can be employed in the clinical setting.

Further modification of scoring systems and streamlining methods to identify patients most likely to respond to treatment continue to improve as we seek to minimize risk of treatment while maximizing survival gain.

At the present, we recommend corticosteroids for patients with alcoholic hepatitis and DF $\geqslant$ 32, providing there is not evidence of gastrointestinal bleeding. In patients with active infection, we delay treatment until antibiotic control of infection is achieved. Given the various glucocorticoids and dosages employed in clinical trials, it is difficult to provide clinicians evidence based guidelines on type of glucocorticoid, dosage and length of treatment. We currently recommend using the lowest effective dose of prednisone or prednisolone studied in the literature. As prednisone is less costly, we prescribe prednisone 40 mg daily for up to 28 d. If no improvement in bilirubin is seen after 7 d, we recommend stopping glucocorticoids as suggested by Mathurin[59]; switching to pentoxifylline is a reasonable alternative in that situation. Although primary treatment with pentoxifylline holds some promise, the evidence of its efficacy is not as robust as that with steroids.

Although the focus of this article is treatment, preventing the occurrence of disease is important. From physician screening for alcohol abuse to community wide education in a culturally sensitive manner on the risks of alcohol abuse are important health service fields.

## APPENDIX A

**Child-Turcotte with pugh modification**

| Score | 1 | 2 | 3 |
|---|---|---|---|
| Prothrombin time (INR) | < 4 s (< 1.7) | 4-6 s (1.7-2.3) | > 6 s (> 2.3) |
| Bilirubin (mg/dL) | < 2 | 2-3 | > 3 |
| Albumin (g/dL) | > 3.5 | 3.5-2.8 | < 2.8 |
| Ascites | None | Slight | Moderate |
| Encephalopathy | 0 | 1-2 | 3-4 |

**Maddrey criteria**

| | Score indicating |
|---|---|
| Initial | Poor prognosis |
| 4.63 × prothrombin time (seconds) + serum bilirubin (mg/dL) | > 93 |
| Modified | |
| 4.6 (patients prothrombin time-control time) + serum bilirubin (mg/dL) | > 32 |

**Glasgow alcoholic hepatitis score**

| Score | 1 | 2 | 3 |
|---|---|---|---|
| Age | < 50 | $\geqslant$ 50 | - |
| WCC ($10^9$ /L) | < 15 | $\geqslant$ 15 | - |
| Urea (mmol/L) | < 5 | $\geqslant$ 5 | - |
| PT ratio | < 1.5 | 1.5-2.0 | > 2.0 |
| Bilirubin (mmol/L) | < 125 | 125-250 | > 250 |

GAHS $\geqslant$ 9 predictive of poor prognosis. MELD = 3.8 × $\log_e$ (bilirubin (mg/dL)) + 1.2 × $\log_e$ (INR) + 9.6 × $\log_e$ (creatinine (mg/dL)); One can also calculate the MELD score at the following internet address: www.mayoclinic.org/gi-rst/mayomodel7.html.

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*EDITORIAL*

# Occult hepatitis C virus infection: A new form of hepatitis C

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## Abstract

Occult hepatitis C virus (HCV) infection is a new recently characterized entity. This occult infection can be present in two different clinical situations: in anti-HCV negative, serum HCV-RNA negative patients with abnormal liver function tests and in anti-HCV positive subjects with normal values of liver enzymes and without serum HCV-RNA. This review describes recent studies of occult HCV infection in both kinds of patients.



**Key words:** Hepatitis C virus-RNA; Liver; Peripheral blood mononuclear cells

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## INTRODUCTION

The etiology of liver disease is unknown in approximately 10% of patients with abnormal results on liver function tests. Some authors have reported that occult hepatitis B virus could be the cause of a proportion of these cryptogenic chronic hepatitis[1,2] cases, but no conclusive results have been yielded.

In January 2004, the role of occult hepatitis C virus (HCV) infection in chronic liver disease of unknown etiology was first described by Castillo *et al*[3]. This study included 100 patients with persistently long-standing abnormal liver function test results:alanine aminotransferase (ALT) and/or gamma-glutamyl transpeptidase (gamma-GTP). All known causes of liver diseases were excluded, and they were repeatedly anti-HCV and serum HCV-RNA negative. All these patients underwent a liver biopsy. A reverse-transcription polymerase chain reaction found that 57% of them had HCV-RNA in their liver. These results were also confirmed by *in situ* hybridization. In addition, 48/57 (84%) of these patients with occult HCV infection also had the antigenomic HCV-RNA strand in the liver tissue, indicating an ongoing viral replication. The HCV genotype found in the liver of the patients was 1b, which was demonstrated by a commercial genotyping assay and by amplification and sequencing of the HCV-core region. Moreover, 70% of the patients with intrahepatic HCV-RNA in liver also had viral RNA in their peripheral blood mononuclear cells (PBMC). Finally, liver necroinflammatory activity and fibrosis were observed in a significantly higher proportion of patients with occult HCV infection than in those without intrahepatic HCV-RNA. In summary, this paper identified a new form of hepatitis C virus infection called "occult HCV infection". It is characterized by the presence of HCV-RNA in the liver in the absence of serological markers of infection (anti-HCV and serum HCV-RNA negative).

The existence of this kind of occult HCV infection has also been found by other authors. Thus, Stapleton and colleagues have reported several studies on "seronegative" HCV infection in patients with cryptogenic liver disease and persistently abnormal results of liver tests[4,5]. Editorials or editors' comments have also been devoted to the role and significance of occult HCV infection, recognizing this infection as a new entity that should be taken into account for the diagnosis of patients with liver diseases of unknown cause[6-10].

Once occult HCV infection was identified, different research fields were developed: (1) To find an alternative to the liver biopsy for the diagnosis of occult HCV infection. (2) To study if HCV replicates or not in the PBMC of patients with occult HCV infection. (3) To compare the clinical, biochemical and histological characteristics of occult with chronic HCV infection. (4) To compare virus-specific T-cell responses in patients with occult and with chronic HCV infection. (5) To assess the possible role of occult HCV infection in the development of hepatocellular carcinoma. (6) To study the prevalence of occult HCV infection in other risk populations such as hemodialysis patients. (7) To assess the efficacy of antiviral therapy for occult HCV infection. (8) To study other possible clinical situations of occult HCV infection.

## ALTERNATIVES IN DIAGNOSIS OF OCCULT HCV INFECTION

Although HCV-RNA is also detected in the PBMC of a high percentage of patients with an occult HCV infection, the gold standard for diagnosis of this occult viral infection

is detection of HCV-RNA in liver cells. However, because of the invasive nature of the liver biopsy, other alternatives were studied in an attempt to increase the sensitivity of the diagnostic tests in serum. Taking into account previous data recorded in patients with chronic hepatitis C[11], we performed a study with 21 patients diagnosed as having occult HCV infection (HCV-RNA positive in liver but negative in serum) and compared detection of viral RNA in plasma, PBMC and whole-blood[12]. All cases had negative results for HCV-RNA in plasma. In 3 (14%) patients, viral RNA was detected in whole-blood while HCV-RNA could be detected in PBMC of 57% of the included cases. Thus, using whole-blood as the source for HCV-RNA detection does not improve the sensitivity of the diagnosis of occult HCV infection. Testing for HCV-RNA in PBMC is much more reliable in identifing patients with an occult HCV infection when a liver biopsy is not available.

## OCCULT HCV REPLICATION IN PBMC

One important question regarding the transmission of occult HCV infection was whether the virus could replicate in PBMC. To study this issue, 18 patients who had been diagnosed with occult hepatitis C by testing for HCV-RNA in their liver biopsy and who also had HCV in their PBMC were selected for this study[13]. By a strand-specific RT-PCR it was found that 61% of the patients had the antigenomic HCV-RNA strand in their PBMC, indicating that HCV was replicating in these cells. So, although the patients with occult HCV infection do not have detectable circulating virions, they could be potentially infectious.

## CHRONIC VERSUS OCCULT HEPATITIS C

Once occult HCV infection is identified, one important question is if the clinical characteristics of this infection differ from those usually found in chronic hepatitis C. Trying to answer this question, the biochemical, virological and histological features of a group of 68 patients with occult HCV infection were compared with those of a group of 69 patients with histologically proven chronic hepatitis C[14]. Groups were matched with respect to gender, age and known time duration of the disease. Triglycerides and cholesterol values were significantly higher in occult HCV infection, while alanine aminotransferase, gammaglobulin, alpha-fetoprotein and iron levels were significantly higher in patients with chronic hepatitis C. The number of patients who had necroinflammatory activity and fibrosis in the liver biopsy was significantly higher in the group with chronic hepatitis C than in the group with occult HCV infection, but no difference was found in the percentage of patients with liver steatosis between both groups. Finally, as could be expected, the percentage of HCV-infected hepatocytes (determined by *in situ* hybridization) was significantly lower in patients with occult HCV. Thus, it was concluded that occult HCV infection is a milder disease, with less liver damage than chronic hepatitis C. Nevertheless, as patients with occult hepatitis C may present dyslipidemic disorders, studies on the natural history of occult HCV infection should be performed to prove the role of occult HCV as the cause for liver injury in these patients.

## CELLULAR IMMUNE RESPONSES IN OCCULT HCV INFECTION

Why does occult HCV infection induce a less aggressive disease than chronic HCV infection? The immunological system of the patients could be involved in this situation.

Quiroga *et al*[15] have performed a study in an attempt to determine if the cellular immune response of patients with occult HCV infection is different from that of patients with chronic hepatitis C. This work compared 50 patients with occult HCV, 141 with chronic hepatitis C and 21 patients with cryptogenic liver disease (all known causes of liver disease were discarded, including an occult HCV infection). Overall, 26/50 (52%) of patients with occult HCV infection had $CD4^+$ T-cell proliferative responses. These responses were significantly more frequent in patients with occult HCV than in the group of patients with chronic hepatitis C (37/141: 26%; $P = 0.0016$) or in individuals with cryptogenic liver disease (1/21: 5%; $P < 0.001$). HCV-specific T-cells of patients with occult HCV infection proliferated more commonly in response to NS3 and NS4 proteins, and the peripheral blood mononuclear cells derived T-cell lines from these patients produced gamma interferon. Finally, patients with occult HCV infection had significantly higher amounts of HCV-specific $CD8^+$ T-cells than patients with chronic hepatitis C. In summary, HCV-specific cellular immune responses are more frequent in occult HCV infection than in chronic hepatitis C. Thus, patients with an occult HCV infection had a better immune response and this could be the cause of the milder disease that these patients have in comparison to those with chronic hepatitis.

These results seem to suggest that the clinical differences observed between occult and chronic hepatitis C are a consequence of the host's immunological system.

## OCCULT HCV INFECTION AND HEPATOCELLULAR CARCINOMA

Hepatocellular carcinoma (HCC) is one of the most common malignancies throughout the world. In a small proportion of cases, the etiology agent associated with the development of liver cancer is unknown. Two different groups, one from Japan[16] and the other from Italy[17], have reported the presence of occult HCV infection in non-tumoral and tumoral liver tissues of patients with HCC who were negative to serological HCV markers. Therefore, it seems that occult HCV infection may play a role in the development of HCC however, as discussed above, more studies on this issue should be performed.

## OCCULT HCV INFECTION IN RISK POPULATIONS

In the presence of elevated levels of liver enzymes, hemodialysis patients are screened for hepatotropic viral infections: hepatitis B surface antigen, anti-HCV and/

or serum HCV-RNA. Nevertheless, in some patients the etiology of their elevated liver enzymes cannot be established as they are negative for all serological viral markers (including serum HCV-RNA). To investigate whether these hemodialysis patients are affected by an occult HCV infection, 6 Spanish Hemodialysis Units enrolled 42 patients with abnormal liver function tests of unknown etiology[18]. By strand-specific RT-PCR and by *in situ* hybridization it was found that 26/42 (62%) of the patients had HCV-RNA in their PBMC. These patients with occult HCV infection had significantly higher ALT values than the negative ones. In addition, HCV was replicating in the PBMC of 15/26 (58%) of the cases of occult infection. In summary, although these patients are serum HCV-RNA negative they could be potentially infectious as HCV is replicating in their PBMC, thus preventive measures to avoid HCV spread in hemodialysis units must be considered.

## ANTIVIRAL TREATMENT IN OCCULT HCV INFECTION

Regarding the possible efficacy of antiviral therapy in occult hepatitis C, Pardo *et al*[19] treated 10 patients with occult HCV infection with pegylated-interferon plus ribavirin for 6 mo, followed by a post-treatment follow-up period of 6 mo. Although all the patients were infected with HCV genotype 1b, they were treated for 6 mo instead of 12 mo (the currently accepted duration of antiviral therapy for patients with chronic hepatitis C and genotype 1b), because they were serum HCV-RNA negative and the percentage of infected hepatocytes is lower than in chronic hepatitis C[14]. At the end of treatment, 80% of the patients had normalized ALT levels and cleared HCV-RNA from PBMC. However, at the end of the post-treatment follow-up only 30% of the patients maintained a complete response (HCV-RNA negative in PBMC and normal ALT levels). Five patients (2 of them were complete responders) underwent a second liver biopsy after treatment and, although none of them lost HCV-RNA in liver, a significant decrease was observed in the amount of intrahepatic viral RNA in comparison to the basal levels. Moreover, in 3 patients liver necroinflammatory activity and fibrosis score had decreased with respect to the pre-treatment histological diagnosis.

It can be concluded that, as reported in chronic hepatitis C (see below), antiviral therapy in occult HCV infection does not lead to a complete eradication of HCV infection, yet it may be useful as liver damage improves. Thus, treatment with pegylated-interferon and ribavirin of patients with occult HCV infection and a stage of liver fibrosis of 2 or more seems advisable.

## OTHER FORMS OF OCCULT HCV INFECTION

As commented before, occult HCV infection is characterized by the absence of anti-HCV and of serum HCV-RNA, but viral RNA is detectable in liver and PBMC. However, occult HCV infection may exist in other clinical situations such as in anti-HCV positive patients who are serum HCV-RNA negative and who present normal liver function tests.

One of these populations is the "healthy" HCV-carriers. These patients, who are anti-HCV positive with undetectable serum viral RNA and normal ALT levels, are considered to be subjects who have cleared HCV infection after exposure to HCV. To verify if these patients could have HCV-RNA in their liver, we performed a study with 12 "healthy" anti-HCV carriers[20]. These anti-HCV positive patients were serum HCV-RNA negative and had persistently normal ALT values for a 29 ± 19 mo follow-up period. These patients underwent a programmed interventional laparoscopy and gave their consent for obtaining a liver biopsy specimen during the laparoscopy. The genomic HCV-RNA strand was detected in the liver of 10/12 (85%) of these subjects and it was also found that HCV was replicating in the hepatocytes of the 10 patients in question. All of them were infected by HCV genotype 1b, as demonstrated by sequencing of the HCV genome amplified from the liver. Viral RNA was also found in the PBMC of 6/12 patients and 5 (83%) out of these 6 also had HCV replication in PBMC. Another research group[21] has also demonstrated the presence of HCV infection and replication either in the liver or PBMC of nearly 90% of anti-HCV positive patients with normal ALT values.

Occult HCV infection has also been identified in a similar cohort of patients: those with chronic hepatitis C who have responded to an antiviral therapy with loss of circulating HCV-RNA and normalization of ALT levels. Several papers have reported the presence of an occult HCV infection (persistence of HCV-RNA) in the liver and in PBMC of sustained responders[22-24]. However, other authors have not found viral RNA in these patients[25,26]. These discrepancies could be due to different preservation methods of the liver biopsies, to differences in the sensitivity of the methods employed for HCV-RNA detection, different geographical incidence, *etc*. Thus, further studies are needed to know the real prevalence of occult HCV in complete responder patients. One of the possible consequences of occult HCV infection is the persistence of liver necroinflammation in an important number of sustained responder patients[23,24].

The case of a patient with chronic hepatitis C who cleared serum HCV-RNA with normalization of ALT levels in whom HCV infection reactivated following prednisone therapy after 8.5 years of HCV-RNA negativity has been published. Thus, occult HCV infection should be taken into account when these anti-HCV positive patients with normal ALT levels undergo immunosuppressive therapies[25].

## CONCLUSIONS

Occult hepatitis C infection is a new entity that should be taken into account for the diagnosis of patients with a liver disease of unknown origin. Future works should deal with its possible incidence and pathologic relevance in immunosuppressed or HIV coinfected patients, drug abusers or subjects who had received multiple blood transfusions. On the other hand, it would be convenient to perform epidemiological studies on occult HCV infection

among health care staff, patients on hemodialysis, etc to know the prevalence and spread of this infection in these populations. It is also very important to determine the possible incidence and consequences of occult HCV infection in blood donated and transplanted patients.

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*REVIEW*

# Role of ethanol in the regulation of hepatic stellate cell function

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## Abstract

Evidence has accumulated to suggest an important role of ethanol and/or its metabolites in the pathogenesis of alcohol-related liver disease. In this review, the fibrogenic effects of ethanol and its metabolites on hepatic stellate cells (HSCs) are discussed. In brief, ethanol interferes with retinoid metabolism and its signaling, induces the release of fibrogenic cytokines such as transforming growth factor β-1 (TGFβ-1) from HSCs, up-regulates the gene expression of collagen I and enhances type I collagen protein production by HSCs. Ethanol further perpetuates an activated HSC phenotype through extracellular matrix remodeling. The underlying pathophysiologic mechanisms by which ethanol exerts these pro-fibrogenic effects on HSCs are reviewed.



**Key words:** Ethanol; Acetaldehyde; Hepatic stellate cells; liver fibrosis; Type I collagen gene; Transcription factors; Transforming growth factor β-1

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## INTRODUCTION

Ethanol abuse is a leading cause for morbidity and mortality throughout the world. It affects many organ systems, most notably the liver causing both acute and chronic liver disease, and the central nervous system[1-3]. Hepatic cirrhosis resulting from alcohol abuse is one of the principal causes of liver-related morbidity and mortality. In the liver, excess ethanol leads to three pathologically distinct disorders, namely fatty liver (alcohol-associated hepatic steatosis), alcoholic hepatitis and cirrhosis. Alcohol-associated hepatic steatosis is the most common form of liver injury and is reversible with abstinence[3-5]. More serious forms of alcoholic liver disease (ALD) include alcoholic hepatitis characterized by persistent inflammation of the liver, and cirrhosis, characterized by progressive hepatic fibrosis. The pathogenesis of ALD is poorly understood, in part because no simple animal model exists that reproduces the full spectrum of the human disease, including the development of cirrhosis[1,4]. In addition, there is considerable variation among individuals in their susceptibility to ALD, so that among people drinking similar amounts, only a proportion develops cirrhosis[1,3-5].

Almost all ingested ethanol is metabolized in the liver. Two major enzyme systems, namely the oxidative and non-oxidative pathways, mediate the initial phase of ethanol metabolism[1,5] (Figure 1). The oxidative pathway comprises the alcohol dehydrogenases (ADH) and members of the cytochrome P450 system (predominantly CYP2E1)[5-7]. This pathway generates acetaldehyde. Acetaldehyde is subsequently metabolized to acetate via the mitochondrial enzyme acetaldehyde dehydrogenase (ALDH). Although acetaldehyde is oxidized to acetate by ALDH, the kinetics of this reaction is sufficiently slow to allow for the accumulation of acetaldehyde in humans or animals consuming alcohol[1,2,5]. The non-oxidative pathway of ethanol metabolism involves the esterification of ethanol with fatty acids to form fatty acid ethyl esters (FAEE), a reaction catalyzed by FAEE synthases[1,5].

Ethanol and its metabolites including acetaldehyde cause liver damage through several interrelated pathways[1,2,8,9]. The oxidation of ethanol is associated with a change in hepatocyte redox homeostasis which can lead to a number of metabolic disorders including lactic acidosis, hyperlipidaemia and hyperuricaemia. Chronic ethanol consumption does not influence ADH activity, but has a profound stimulatory effect on microsomal enzymes, particularly CYP2E1[1,2]. This is in part responsible for the development in alcoholic liver diseases, a rise in oxygen consumption, the excessive production of free radicals and an increase in the metabolism of ethanol, vitamin A and testosterone. Ethanol and acetaldehyde have deleterious effects both direct and indirect, for example by generating reactive oxygen species (ROS) and causing damage to

the intestinal mucosal barrier[1,10]. Cellular oxidative stress that is caused by the relative imbalance between free radical generation and insufficient anti-oxidant defense mechanisms, including reductions in glutathione, vitamin E and phosphatidylcholine, may be a principal mediator for the progression of alcoholic liver disease[1,2,10].

Steatosis, hepatitis and fibrosis seen in persons with ALD are a consequence of complex pathophysiological events involving various cell types within the liver including neutrophils, sinusoidal endothelial cells (SECs), Kupffer cells (KCs), hepatic stellate cells (HSCs) and hepatocytes. Recently, many studies have demonstrated that ethanol and its metabolites including acetaldehyde directly activate HSCs, the principal fibroblastic cell type within the liver[8,9,11]. Ethanol and acetaldehyde directly promote the production of transforming growth factor beta-1 (TGFβ-1) and several extracellular matrix (ECM) constituents including type I collagen by HSCs[8,9,11].

This article reviews recent advances in our knowledge on the effects of ethanol and its metabolites on HSCs.

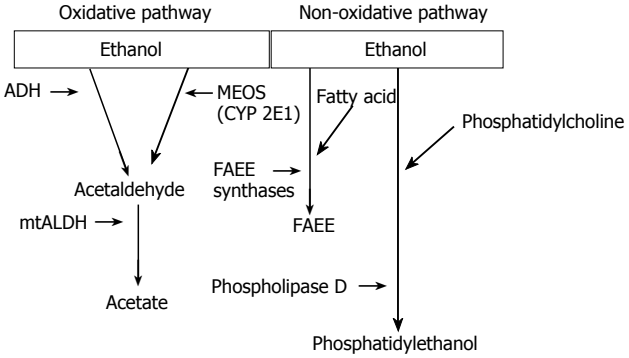


**Figure 1** Metabolism of ethanol in the liver via oxidative and non-oxidative pathways. Oxidative pathway: In the first step of oxidation, ethanol is converted to acetaldehyde. Alcohol dehydrogenase (ADH) is the major enzyme. The microsomal ethanol-oxidizing system (MEOS) involves several cytochrome P450 proteins, of which cytochrome P450 2E1 (CYP2E1) is the major constituent. In the second oxidative step, acetaldehyde is rapidly metabolized to acetate by mitochondrial acetaldehyde dehydrogenase (mtALDH). Non-oxidative pathway: The non-oxidative pathway of ethanol metabolism involves the esterification of ethanol with fatty acids to form fatty acid ethyl esters (FAEE), a reaction catalyzed by FAEE synthases. The non-oxidative pathway also generates phosphatidylethanol *via* phospholipase D.

## DIRECT EFFECTS OF ETHANOL ON HSCS

A central event in liver fibrosis is the activation of HSCs, which represents a transition from a quiescent vitamin A-rich cell type to a vitamin A-deficient, proliferative, fibrogenic and contractile myofibroblast. Activated HSCs demonstrate altered cell behaviors including proliferation, chemotaxis, fibrogenesis, contractility, matrix degradation, retinoid loss, leukocyte chemotaxis and cytokine release. In total, these changes result in excess ECM deposition which is reabsorbed, culminating in the development of liver fibrosis.

HSCs derived from the intragastric ethanol infusion model of ALD demonstrate an activated phenotype including an increase in collagen I and DNA synthesis[12], expression of α-smooth muscle actin (α-SMA) and depletion of retinyl palmitate[13].

### *Effect of ethanol on vitamin A metabolism within HSCs*

HSCs are the major site of vitamin A storage in healthy adults. Vitamin A in HSCs is in the form of retinyl esters located in cytoplasmic lipid droplets[14]. The three active forms of vitamin A, namely retinol, retinal and retinoic acid (RA) are important regulators of cell proliferation and differentiation, binding to 2 distinct families of ligand-activated transcription factors: the retinoic acid receptor (RARs: RARα, RARβ and RARγ) and the retinoid X receptor (RXR)[15]. The natural ligand for the RARs is all trans-retinoic acid (ATRA). Published data indicate that HSCs from healthy rats express mRNAs in the RARs and RXRs[16].

Nutritionally reduced levels of serum and hepatic vitamin A have been reported in persons with ALD and in animal models of the disease[17,18]. In HSCs, ethanol significantly inhibits RA production[19] and reduces the retinol level[20]. Acetaldehyde exposure results in a reduction in RARβ message and protein in HSCs[21]. There are several other possible mechanisms by which ethanol can interfere with retinoid metabolism in the liver[19], including decreased vitamin A uptake, enhanced degradation of vitamin A in the liver, enhanced vitamin A mobilization from the liver to other organs, and degradation by ethanol of RA into polar inactive metabolites *via* induction of cytochrome P4502E1.

The activation and differentiation of HSCs are characterized by proliferation and an increase in the production of ECM proteins together with a loss of cellular retinoids. Therefore, it is plausible that ethanol-induced RA metabolism in HSCs could play a role in the development of alcohol-related liver fibrosis and cirrhosis.

### *Ethanol, HSC proliferation and α-SMA expression*

Linolenic acid ethyl esters (LAEE), one of the FAEE products of non-oxidative ethanol metabolism, may promote HSC proliferation[22]. This effect is thought to be modulated through increased cyclin E and cyclin-dependant kinase 2 (CDK2) activities[22]. Ethanol, acetaldehyde and lactate, in contrast, have no direct effect on HSC proliferation[23,24].

Ethanol induces early protein expression of α-SMA in cultured HSCs compared to controls[25,26]. Chen and colleagues[27] likewise reported that α-SMA mRNA expression in HSCs is significantly enhanced by exposure to acetaldehyde. However, Poniachik *et al*[24] were unable to replicate this finding. Hence, the effects of ethanol on HSC proliferation and α-SMA expression remain controversial.

### *Effects of ethanol on ECM production by HSCs*

HSC activation is characterized by an increase in the production of ECM, mainly collagen types I and Ⅲ. In addition, HSC activation is associated with alterations in both types of collagen, matrix-degrading metalloproteinases (MMPs) and their inhibitors, tissue inhibitors of metalloproteinases (TIMPs)[28]. Failure of matrix degradation leads to ECM accumulation and progressive hepatic fibrosis[28].

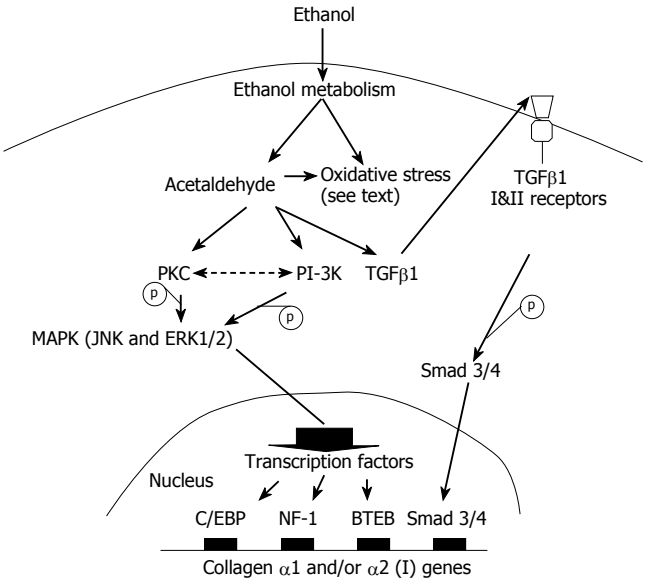


**Figure 2** Possible mechanisms for the direct profibrotic effects of ethanol on hepatic stellate cells. Acetaldehyde, together with ethanol or acetaldehyde-derived oxidative stress, induces latent TGFβ1 and TGFβ receptor activation that subsequently leads to Smad3/4 activation and binding to the promoter of collagen I genes. PKC and/or PI-3K kinases are also activated by acetaldehyde. Both kinase systems activate their downstream components, including ERK1/2 and JNK. As a result, C/EBP, NF-1 and/or BTEB transcription factors are activated and therefore up-regulate collagen I gene transcription.

Numerous studies have shown that ethanol and/or its metabolites regulate the expression of multiple components of the ECM in HSCs. Both ethanol and acetaldehyde induce α1 (I) collagen mRNA expression in HSCs[29-33] but not hepatocytes[30]. This effect is protein synthesis-independent[29]. Acetaldehyde increases the steady-stage levels of α2 (I) gene expression[21,34] and the production of type I collagen protein[29-31] in HSCs. Acetaldehyde likewise up-regulates the mRNA expression of MMP-2 and fibronectin in human HSCs[35,36].

### *Signaling pathways that mediate type I collagen gene transcription in HSCs exposed to ethanol*

The mechanisms by which ethanol and its metabolites regulate ECM gene and/or protein expression in HSCs have not been completely elucidated (Figure 2). Several centers have reported that the MAPK and PI-3K pathways are involved[35,37,38]. Anania and colleagues[37] noted that in rat HSCs, phospho-JNK is elevated following exposure to acetaldehyde. Inhibition of JNK by curcumin at low doses reduces acetaldehyde-induced steady-state levels of endogenous α1 (I) collagen mRNA expression[37]. Phosphorylated ERK and p38 are detectable but not significantly elevated. It seems likely therefore that JNK is the principal mediator of acetaldehyde-induced α1 (I) collagen gene up-regulation in rat HSCs. These finding are consistent with those previously reported by Chen *et al*[38]. In contrast, in human HSCs, ERK1/2 and the PI-3K pathway appear to be triggered by acetaldehyde, leading to α2 (I) collagen and fibronectin gene up-regulation[35].

The protein kinase C (PKC) pathway may also play a role in the up-regulation of collagen gene transcription following exposure to ethanol, since PKC is upstream of ERK1/2 and JNK[35,39,40]. Acetaldehyde-elicited α2 (I) collagen and fibronectin gene expression in human HSCs is inhibited by calphostin C (a PKC inhibitor). This PKC inhibitor also reduces the enhancing effect of acetaldehyde on α1 (I) collagen mRNA expression in cultured mouse and human HSCs[35,39]. Other experiments noted that acetaldehyde increases the translocation of PKC activity to membrane fractions[39] and both α1 (I) and α2 (I) collagen gene transcription in a calcium-independent manner[39].

### *Transcription factors that mediate type I collagen gene expression in response to ethanol*

The modulation of gene expression in response to an exogenous or endogenous stimulus occurs through alterations in any one of the steps of gene transcription, mRNA stability, protein translation or protein degradation. Transcription factors are generally classified according to the conserved motifs within either their activation- or DNA- binding domains[41,42]. The binding of transcription factors at DNA-binding sites brings them into proximity with RNA polymerase Ⅱ and components of the transcription complex that assemble in the 5'untranscribed region of genes[42,42]. Transcription factors are then able to exert either a positive or a negative influence on the rate at which the transcription complex transcribes the gene of interest. Transcriptional control of acetaldehyde-induced type I collagen gene expression might be regulated through CCAAT/enhancer-binding proteins (C/EBP), nuclear factor-I (NF-I), basic transcription element binding (BTEB) protein as well as activating protein-1 (AP-1)[34,38,43,44]. The precise mechanisms however, remain to be clarified.

A C/EBP binding site is present in the α1 (I) collagen promoter between -365 and -335 of the transcription start site[44]. Transfection of the α1 (I) collagen promoter mutated at the C/EBP binding site results in unresponsiveness to acetaldehyde, indicating that this site is essential for the collagen gene transcription effect of acetaldehyde[34,44]. C/EBP consists of 6 members. The principal form present in activated HSCs is C/EBPβ[44]. In turn, four C/EBPβ isoforms with approximate molecular weights of 45, 43, 35 and 20 kDa have been identified in activated rat HSCs, with the 35-kDa isoform being predominant[44,45]. Attard *et al*[44] noted that activation of the α1 (I) collagen promoter by acetaldehyde in HSCs is most likely consequent upon an increase in this isoform and increased protein/DNA binding to the C/EBP binding site.

Another report suggests that acetaldehyde-induced α1 (I) gene expression in rat HSCs requires the binding of the acetaldehyde-inducible transcription factor BTEB to a GC box (-1484 to -1476) on the promoter of this gene[43]. In keeping with this proposal, blocking BTEB protein production, results in a reduction in acetaldehyde-induced α1 (I) collagen gene expression[43]. In an extension of the previous report, additional data suggest that acetaldehyde can firstly induce AP-1 activation in HSCs[38,43] and then the activated AP-1 can bind to AP-1 responsive elements in the BTEB promoter to stimulate BTEB expression. The BTEB protein, in turn, stimulates the expression of the α1 (I) gene in HSCs[38,43].

Nuclear factor I (NF-I), a CCAAT binding trans-

cription factor, is also known to bind to and activate the α1 (I) and α2 (I) collagen promoters[46]. Acetaldehyde-induced enhancement of the α2 (I) collagen promoter in activated HSCs is associated with increased binding of NF-I to a consensus consequence located at -352 to -104 bp from the transcriptional start site[34,46,47].

These data suggest that the transcription factors C/EBP, BTEB and NF-1 bind to and activate type I collagen gene transcription through each of them and/or *via* synergic effects, though further characterization of these effects is required. Whether these collagen gene transcription signaling pathways (after exposure to ethanol), are regulated by acetaldehyde itself, or in concert with other profibrogenic mediators such as oxidative stress or TGFβ1 is presently uncertain. The available data are discussed below.

### *Increased oxidative stress responses and TGFβ1 play an important role in the regulation of type I collagen gene transcription in HSCs*

**Oxidative stress:** Increased oxidative stress is present in the liver after both acute and chronic ethanol administration[48]. Ethanol-induced oxidative stress within hepatocytes can occur acutely through ethanol metabolism or chronically following the induction of CYP2E1[2,10]. The oxidative metabolism of ethanol in hepatocytes elicits a range of mediators including ROS. CYP2E1 in particular has been shown to generate ROS including the superoxide anion, hydrogen peroxide ($H_2O_2$) and hydroxyethyl free radicals[2,49]. Other sources of free radical generation by ethanol include NADH oxidation by aldehyde oxidase[50].

HSCs contain the enzymes of oxidative ethanol metabolism including ADH and P450 proteins[51-53]. Yamada and Oinonen[52] observed that CYP2E1 is present in rat HSCs as high as 21% of that found in hepatocytes. CYP2E1 is also detectable in the rat hepatic stellate cell line, HSC-T6[51]. In HSC-T6 cells overexpressing ethanol-inducible CYP2E1, time- and dose-dependent induction in collagen α2 (I) mRNA together with increased $H_2O_2$ production by ethanol has been observed. Antioxidants, including catalase (an $H_2O_2$ scavenger) prevent this increase in collagen α2 (I) mRNA expression[51]. Because ethanol can be oxidized to acetaldehyde by the peroxidative activity of catalase[54], this decrease in collagen α2 (I) expression by catalase suggests that ethanol-derived acetaldehyde is not responsible for this effect. Svegliati-Baroni *et al*[11] and Greenwel *et al*[35] have also provided evidence to support the concept that increases in mouse α1 (I) and human α2 (I) collagen gene expression in HSCs by acetaldehyde are linked to elevated $H_2O_2$ production. For example, acetaldehyde-elicited type I collagen gene expression can be blocked by the addition of catalase[11,35], and is in part, TGFβ1-independent[11]. It is known that $H_2O_2$ activates MAPK pathways[49] and this activity might enhance the binding of the down stream transcription factors to acetaldehyde-responsive elements within the type I collagen promoter. Likewise, leptin induces $H_2O_2$ production and contributes to TIMP-1 expression in HSCs[55]. Collectively, these data suggest that increased $H_2O_2$ generation during the metabolism of ethanol by HSCs might play a critical role in their activation.

**TGFβ-1:** Ethanol and acetaldehyde increase autocrine TGFβ1 expression in HSCs. In turn, TGFβ1 is able to up-regulate type I collagen gene expression[32,34,38]. Anania and colleagues[34] noted that the effects of acetaldehyde-induced TGFβ1 in the regulation of α2 (I) collagen gene expression are mediated by a factor or factors that bind to nuclear factor I (NF-I) consensus sequence located at the -352 to -104 region of the α2 (I) gene promoter. Acetaldehyde further increases the secretion of both latent and active forms of TGFβ1 in cultured rat HSCs[38], and induces the expression of the type Ⅱ TGFβ receptor which is required for all TGFβ-mediated signaling events[38]. In transient transfection experiments, the combination of TGFβ1 and acetaldehyde could result in greater activation of the mouse α2 (I) collagen promoter than either TGFβ1 or acetaldehyde alone[34]. Taken together, these observations suggest that TGFβ1 could play a key role in acetaldehyde-induced collagen I gene activation.

Chen *et al*[38,43] have noted that acetaldehyde stimulates latent TGFβ1 secretion and TGFβ type Ⅱ receptor gene expression. BTEB might be the principal transcription factor binding to the GC box of the type Ⅱ TGFβ receptor gene promoter[38]. The authors proposed a model wherein acetaldehyde activates signal transduction pathways including PKC, JNK and ERK, leading to activation of AP-1. AP-1 is proposed to activate the gene expression of BTEB. BTEB then up-regulates TGFβ type Ⅱ receptor gene expression in HSCs. By stimulating latent TGFβ1 activation and secretion, as well as up-regulating the expression of TGFβ type Ⅱ receptor, acetaldehyde activates TGFβ1 signaling, which eventually enhances expression of the α1 (I) collagen gene in HSCs[38].

The precise molecular mechanisms by which acetaldehyde elicits TGFβ1 production in HSCs are largely unknown. Acetaldehyde might directly bind to the TGFβ1 gene promoter leading to its activation. Alternatively, acetaldehyde might bind to other gene promoters of transcription factors that in turn activate the TGFβ1 gene.

## OTHER DIRECT FIBROGENIC EFFECTS OF ETHANOL ON HSCS

Rodriguez-Fragoso and his colleagues[56] investigated the effects of the activity of urokinase type plasminogen activator (uPA) in the CFSC-2G stellate cell line and demonstrated that acetaldehyde (175, 250 and 350 μmol/L) enhances uPA gene expression. This is accompanied with a concomitant increase in production of type I collagen. uPA plays an important role in matrix remodeling under a wide range of physiological and pathological conditions, activates TGFβ1 and induces proliferation of HSCs[57,58]. Furthermore, profibrogenic mediators including IL-6, TNF-α, malondialdehyde (MDA) and intracellular GSSG have been reported to increase in CFSC-2G cells treated with ethanol or acetaldehyde[59-61].

Malondialdehyde-acetaldehyde (MAA) -protein adducts induce a dose- and time-dependent increase in the secretion of chemokines including monocyte chemoattractant protein (MCP)-1 and macrophage inflammatory protein (MIP)-2 as well as an increase in

the production and expression of intercellular adhesion molecule-1 (ICAM-1) in activated rat HSCs[62,63]. These effects may contribute further to the activation of HSCs and the subsequent development of alcohol-associated liver fibrosis.

## ACTIVITY OF ETHANOL/ACETALDEHYDE ON PANCREATIC STELLATE CELLS

Rat pancreatic stellate cells (PSCs) exhibit features similar to those of HSCs[64-66]. These cells are abundant in alcoholic chronic pancreatitis in humans, suggesting a central role of this cell type in pancreatic fibrosis[65]. An effect of ethanol on the modulation of PSCs has been documented. Ethanol and acetaldehyde increase α-SMA protein and type I collagen synthesis in PSCs[67], likewise enhance PSC MMP-2 and TIMP-2 gene expression as well as TIMP-2 protein secretion[67-69]. Both ethanol and acetaldehyde increase the activation of all 3 subfamilies (ERK1/2, JNK/SAPK and p38 kinase) of the MAPK pathway in PSCs. Only p38 MAPK is responsible however, for the induction of α-SMA and α1 (I) collagen gene expression[70]. Moreover, ethanol and acetaldehyde-induced MAPK activation can be blocked by the antioxidant N-acetyl-cysteine, suggesting a role of oxidative stress in signal transduction[68,71].

## CONCLUSION

Ethanol can be metabolized in hepatocytes and stellate cells to generate acetaldehyde and other metabolites. Ethanol and/or its metabolites including acetaldehyde have direct effects on HSC activation. These effects might be mediated by ethanol/acetaldehyde and/or ethanol/acetaldehyde-induced oxidative stress and TGFβ1 expression which activate relevant signaling pathways leading to the binding of transcription factors to the type I collagen gene promoter (Figure 2). As a result, ethanol augments the production of extracellular matrix proteins. Ethanol also stimulates the production of other profibrotic mediators, including IL-6, TNF-α and uPA. Taken together, these effects of ethanol/acetaldehyde on HSCs play an important role in the development of alcohol-associated liver fibrosis. Characterization of the key genes initiating and perpetuating the process of HSC activation by ethanol helps to further elucidate the molecular mechanisms of alcohol-associated liver fibrosis. In the future, it is hoped that specific, directed pharmacological agents can be selected and/or developed that target these mechanisms and thereby prevent or retard the fibrogenesis induced by alcohol.

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*REVIEW*

# Methylation in esophageal carcinogenesis

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## Abstract

Genetic abnormalities of proto-oncogenes and tumor suppressor genes have been demonstrated to be changes that are frequently involved in esophageal cancer pathogenesis. However, hypermethylation of CpG islands, an epigenetic event, is coming more and more into focus in carcinogenesis of the esophagus. Recent studies have proved that promoter hypermethylation of tumor suppressor genes is frequently observed in esophageal carcinomas and seems to play an important role in the pathogenesis of this tumor type. In this review, we will discuss current research on genes that are hypermethylated in human esophageal cancer and precancerous lesions of the esophagus. We will also discuss the potential use of hypermethylated genes as targets for detection, prognosis and treatment of esophageal cancer.



**Key words:** Methylation; Esophageal cancer; Tumor suppressor gene; Carcinogenesis

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## INTRODUCTION

Esophageal cancer is one of the least studied and deadliest cancers, with a remarkable geographical distribution and a low likelihood of cure[1]. Therefore, the current challenges in the management of esophageal cancer are to obtain a better understanding of the underlying molecular biological alterations to provide new treatment options. Cancer of the esophagus exists in two main forms with different etiological and pathological characteristics-esophageal squamous cell carcinoma (ESCC) and esophageal adenocarcinoma (EAC)[1]. It is well known that esophageal carcinogenesis is a multistage and progressive process which includes basal cell hyperplasia (BCH), dysplasia (DYS), carcinoma in site (CIS) and advanced esophageal carcinoma[1,2]. A variety of genetic lesions are involved in esophageal carcinogenesis, including gene amplifications, loss of heterozygosity(LOH) or homozygous deletions, mutations, and chromosomal rearrangements[1,2]. From the above mentioned genetic lesions , mutations are greatly focused on. The vast majority of esophageal cancers have mutations of the TP53 and p16 genes at an early stage followed by mutations in genes such as APC, Rb and cyclin $D_1$ at later stages of progression[3]. Furthermore, the alteration of the gene mutations may show different types in ESCCs and EACs. For example, ESCCs and EACs show distinct patterns of TP53 mutations, namely a high prevalence of G > A transitions at CpG sites in EACs whereas in ESCCs a higher prevalence of G to T transversions and mutations at A:T base pairs is present[4].

Genetic mutation of genes that inhibit the formation of tumors has long been known to be one of the main driving forces in the development of cancer[1]. However, recent data have focused our attention to the contribution of epigenetics to tumorigenesis. In tumorigenesis of the esophagus, the epigenetic inactivation of genes is as an important a driving force as the inactivation of genes by mutation[5]. 'Epigenetic' events, i.e. heritable changes in gene function which cannot be explained by changes in DNA sequence, are composed of histone acetylation, the chromatin structure and DNA methylation[5]. DNA methylation seems to be the most important mechanism for "epigenetic change" at present[5,6]. Through a process of post-replicative covalent modification catalysed by DNA methyltransferases(DNMTs), the DNA of mammalian cells contains a 'fifth base', namely 5-methylcytosine. The most frequent target for this modification is cytosine in the context of the dinucleotide CpG[5]. Throughout the genome CpG dinucleotides are found at one-fifth of their predicted frequency[6]. In marked contrast to the genome-wide underrepresentation of CpGs, there are regions of the genome termed CpG islands which have maintained their expected frequency of the dinucleotides. And the CpG islands are often found within the promoter of genes[6,7]. It has been known for many years that, in general terms, there is an inverse relationship between the density of promoter methylation and the transcriptional activity of a gene[8,9]. The mechanism of gene silencing by promoter hypermethylation has recently been shown to be related to the recruitment of repressor protein complex, resulting in

de-acetylation of the chromatin and histone, thus barring access to the active transcription complex. However, the actual mechanisms by which DNA methylation modulates gene expression have remained elusive[7,10]. The assays for detection of cytosine methylation can be divided into two groups: restriction enzyme-based and bisulfite treatment-based[11,12]. The former employs the inhibition of certain restriction enzymes by methylation of their recognition sites as an indicator for the presence of methylation. The latter translates the epigenetic information of cytosine methylation in primary sequence differences by converting unmethylated cytosine to uracil whereas methylated cytosine remains unaltered. The bisulfite-converted genomic DNA can be analyzed by a wide variety of PCR-based methods[11,12]. Methylation is needed for the normal development of cells. And genome stability and normal gene expression are largely maintained by a fixed and predetermined pattern of DNA methylation[13]. Aberrant methylation confers a selective growth advantage that results in cancerous growth[13]. From various lines of evidence, it is known that the methylation pattern of the cancerous cell is associated with a broad genomic hypomethylated state that is often accompanied by a more regional and locus-specific hypermethylated pattern[7]. The presence of alterations in the profile of DNA methylation in cancer was initially thought to be exclusively a global hypomethylation of the genome that would possibly lead to massive overexpression of oncogenes whose CpG islands were normally hypermethylated[14]. Nowadays, however, this is considered to be an unlikely or, at least, incomplete scenario. The popularity of the concept of demethylation of oncogenes leading to their activation is in clear decadency[14,15]. Hypermethylation of CpG islands located in the promoter regions of tumor suppressor genes(TSGs) is now firmly established as an important mechanism for gene inactivation[16,17,18]. The particular genes that are hypermethylated in tumor cells are strongly specific to the tissue of origin of the tumor. A profile of CpG island hypermethylation exists according to the tumor type[15]. The mechanism responsible for this type of pattern remains largely unclear. Moreover, accumulating evidence indicates that CpG island hypermethylation is an early event in cancer development and, in some cases, may precede the neoplastic process[19]. Therefore, such profiles would provide invaluable insight into mechanisms underlying the evolution of each tumor type and will provide new molecular markers. This review will focus on the current understanding of DNA methylation abnormalities in esophageal cancer and discuss how this knowledge contributes to our understanding of the pathogenesis of esophageal cancer.

## STUDIES OF GENE PROMOTER HYPERMETHYLATION IN ESOPHAGEAL CANCER

Putative tumor suppressor genes, involving apoptosis, cell adherence, DNA repair, and the cell cycle, have been investigated for hypermethylation by various techniques in esophageal cancer. Below, we have chosen to describe those genes that have been most extensively studied in the past and that have been shown to undergo epigenetic changes in esophageal carcinoma.

### *p14$^{ARF}$, p15 and p16*

The 9p21 chromosomal band is one of the most frequently altered genomic regions in human cancers[20]. Within a short distance of 50 kb, a gene cluster consisting of three genes, p14$^{ARF}$, p15 and p16, is harbored. All of which have putative tumor suppressor roles[20,21,22]. Inactivation of p14$^{ARF}$, p15 and p16 genes has been observed in many types of human cancers including ESCC[21,22,23]. For example, the results from immunohistochemical analysis indicated that p16 expression was present in only 3 out of 22 ESCC cases[24]. Some studies showed that germline mutations in the p16 gene might be related to familial melanoma[25], but another study found the mutation of the p16 gene in esophageal cancer was rare[26]. Hemizygous and homozygous deletion at 9p21 are widely considered to be one of the primary mechanisms of p16/p15 inactivation[26]. Recently, however, aberrant methylation of the CpG islands at the promoter regions of p16 and p15 genes was reported in many cancers and was associated with loss of transcription[8,27]. Abbaszadegan *et al*[28] assessed a large family with clustering of ESCC in northeastern Iran and found aberrant p16 promoter methylation in 64% of ESCC family members and none in normal volunteers. By analyzing the p14$^{ARF}$, p15, and p16 genes individually in 40 ESCCs, Xing *et al*[29] detected aberrant promoter methylation of the p16 gene in 40%, of p14$^{ARF}$ in 15%, and of p15 in 13% tumor samples. They further detected homozygous deletion of p16 in 18%, of p14$^{ARF}$ in 33%, and of p15 in 40% tumor samples, and detected no mutation in the p14$^{ARF}$ and p16 genes[29]. Hardie *et al*[30] reported that hypermethylation of the p16 promoter is detected in 85% (18/21) of EACs while p16 mutations are uncommon (1.9%; 1/54). Another report found that in 50 Barrett's esophagus-associated EACs, hypermethylation of p16 was present frequently (54%), but hypermethylation of p14$^{ARF}$ was absent[27]. The above results suggest that p14$^{ARF}$, together with p15, is a primary target of homozygous deletion, whereas p16 is the hypermethylation hotspot in human esophageal cancer.

### *The FHIT gene*

The FHIT gene is located at chromosome 3p14.2 and encodes a polypeptide of 147 amino acids[31]. FHIT allelic deletions and reduced or absent FHIT protein expression have been observed in a variety of tumors suggesting a putative tumor suppressor function[31,32]. In ESCCs, the CpG island in the FHIT promoter region was hypermethylated in 25 of the 36 (69%) analyzed cases, significantly correlated with the deletion of FHIT protein expression[33]. Methylated ESCC cell lines exhibit re-expression of the FHIT gene and demethylation in the CpG islands after treatment with demethylating agent 5-aza-2'-deoxycytidine[34]. Another report showed that aberrant methylation of FHIT was found in 85 of 257 (33%) ESCCs[35]. These findings suggest that methylation of the 5' CpG islands of the FHIT gene is closely

associated with transcriptional inactivation and might be involved in tumor development of the esophagus.

### *The RARβ2 gene*

The retinoic acid receptor-beta2 (RARβ2) gene located at 3p24 has been intensively studied in many cancers and found to have defective function, thus making it a candidate TSG[36]. RARβ2 expression was detected in 88% (14/16) of normal esophageal tissues and only 54% (84/162) of esophageal carcinomas[36]. And 14 of 20 (70%) ESCC samples had hypermethylation of the RARβ2 promoter[37]. Another group reported that 34 of 47 (73%) primary resected ESCC samples showed RARβ2 methylation[38]. After 5-aza-2'-deoxycytidine treatment the expression of RARβ2 was reversed in two RARβ2-downregulated ESCC cell lines[39]. These results identified methylation as the underlying mechanism for this frequent loss of RARβ2 in esophageal cancer.

### *The APC gene*

The adenomatous polyposis coli (APC) gene, located on chromosome 5q21, is a TSG in the wnt signaling pathway[40]. APC shows frequent LOH in esophageal carcinomas, and the prevalence of mutations in the APC gene in esophageal carcinomas is low[41]. Hypermethylation of the promoter region of the APC gene occurred in abnormal esophageal tissue in 48 of 52 (92%) patients with EAC, in 16 of 32 (50%) patients with ESCC, but not in matching normal esophageal tissues[41]. So methylation of the promoter region of this gene constitutes an alternative mechanism of gene inactivation in esophageal carcinoma.

### *The ER gene*

The estrogen receptor (ER) gene at chromosome 6q, which has growth and metastasis suppressor activity in many different cell types, is widely expressed in tissues other than breast, and is methylated in 51% of EAC patients[42].

### *The MGMT gene*

The human enzyme $O^6$-methylguanine-DNA methyltransferase (MGMT) is located on chromosome band 10q26, and protects the cell from guanine methylation by irreversibly transferring the alkyl group of the $O^6$-methylguanine to a specific cysteine residue within the molecule[43]. Approximately 20% of tumor cell lines lack MGMT activity and are highly sensitive to alkylating agents[44]. In established cancer cell lines, MGMT expression appears to be correlated with methylation in the promoter of the gene[44]. The gene has been shown to be methylated in 46/119 (39%) ESCC, but all 21 normal esophageal tissues had unmethylated MGMT[45]. Another report studied the role of DNA hypermethylation in the loss of expression of MGMT during the development of ESCC, and found that 5 of 17 (29%) normal esophagus, 10 of 20 (50%) BCH, 8 of 12 (67%) DYS, and 13 of 18 (72%) ESCC samples had DNA hypermethylation in the MGMT promoter region, showing a gradual increase with the progression of carcinogenesis, while the frequency of the loss of MGMT mRNA and protein expression progressively decreased from normal to BCH, DYS, and ESCC, and it was highly correlated with MGMT promoter hypermethylation[46].

### *The E-cadherin gene*

E-cadherin gene on chromosome 16q22.1 encodes a Mr 120 000 transmembrane glycoprotein expressed on the surface of epithelial cells. In epithelial tissues, E-cadherin mediates homophilic, $Ca^{2+}$-dependent intercellular adhesion that is essential for the maintenance of normal tissue architecture[47]. Loss of E-cadherin expression occurs in a variety of human tumors and is correlated with invasion and metastasis, and activation of E-cadherin results in the growth inhibition of tumor cell lines[48]. E-cadherin can be targeted by both genetic and epigenetic means. Moreover, the hypermethylation of E-cadherin was seen frequently in most tumor types, but mutations only frequently in a small number of specific subtypes[48]. In esophageal carcinoma, downregulation of E-cadherin is common and is associated with an increase in invasive and metastatic potential, but mutations of the gene are rare[49]. E-cadherin was methylated in 26 of 31 (84%) EAC specimens, 16 of 20 (80%) ESCC samples and 4 of 6 ESCC cell lines[49,50,51]. And treatment of E-cadherin-negative carcinoma cells with the demethylating agent, 5-aza-2'-deoxycytidine, induced re-expression of the gene[51]. These data suggest that epigenetic silencing via aberrant methylation of the E-cadherin promoter is the critical mechanism for inactivation of this gene in esophageal cancer.

### *The TSLC1 gene*

The TSLC1 (tumor suppressor in lung cancer) gene located on 11q23.2 was first characterized as a TSG in human non-small cell lung cancer(NSCLC) and termed TSLC1[52]. The tumor suppressor role of this gene has been demonstrated in the cell lines of NSCLC, hepatocellular carcinoma, pancreatic cancer and ESCC[52,53]. Loss of TSLC1 expression was observed in 75% of the ESCC cell lines and 50% of the primary tumors from ESCC patients[53]. Ito *et al*[53] examined the methylation status of six cytosine residues of CpG sites in a putative promoter sequence upstream from the TSLC1 translation initiation site by bisulfite sequencing in four cell lines, including KYSE270, which expressed TSLC1, and KYSE410, KYSE520, and KYSE960, which did not express it. They also found all of the cytosine residues in KYSE270 DNA were unmethylated, whereas all of the six cytosine residues in KYSE520 DNA and five residues in KYSE410 and KYSE960 DNA were methylated. Especially, the cytosine residues in KYSE520 DNA were all hypermethylated. However, the report about the status of the promoter methylation of TSLC1 gene in esophageal cancer tissue has not been available.

### *The RASSF1A gene*

Many known RAS effectors are oncoproteins on their own. Less is known about Ras effectors possessing tumor suppressor properties[54]. Recently, a new family of genes

encoding a putative Ras effector, the Ras-association domain family 1 (RASSF1) gene, has been identified within the critical lung and breast cancer deletion region at 3p21.3. The RASSF1 locus encodes several major transcripts by alternative promoter selection and alternative mRNA splicing: RASSF1A, RASSF1B and RASSF1C. Many studies have suggested that RASSF1A was a new putative TSG[54,55]. RASSF1A acts as a negative effector of Ras in a pro-apoptotic signaling pathway. Interestingly, mutational inactivation of this gene is very rare (< 2%), and the main mechanism of its inactivation is through promoter methylation and LOH[56]. The RASSF1A isoform is highly epigenetically inactivated in lung, breast, ovarian, kidney, prostate, thyroid, esophagus and several other carcinomas[54]. Hypermethylation of RASSF1A was detected in 73% of ESCC cell lines and 51% of primary ESCCs, whereas only 4.3% of RASSF1A hypermethylation were detected in corresponding noncancerous tissues[38]. There was a statistically significant correlation between the presence of hypermethylation and tumor stage[57]. Wong *et al*[58] also found that RASSF1A was partially methylated in 3/7 (43%) esophageal cancer cell lines; 22/64 (34%) ESCCs and 3/64 (4.7%) corresponding non-tumor samples; and was not methylated in 2 immortalized normal oesophageal epithelial cell lines and 6 normal esophageal epithelium samples. These findings suggest that epigenetic silencing of RASSF1A gene expression by promoter hypermethylation could play an important role in ESCC carcinogenesis.

Besides the above mentioned genes, there are hypermethylations of some other genes involving esophageal cancer, including hMLH1[59,60], VHL[38], TIMP3[42,61], DAP-kinase[42], pRb[62], ECRG4[63], Chfr[64], HLA class I[65], EYA4[66], CDH13[67], SFRP1[68] and PGP9.5[69]. Table 1 gives a summary of the profile of gene hypermethylation in human esophageal cancer.

From the above mentioned reports, we find that there is obvious different methylation frequency in a gene from different authors in some cases, which may be due to different assay methods and different specimen resources. And the geographical difference may be due to variable carcinogens in the different areas. Furthermore, there seems to be obvious difference of methylation frequency of some genes between ESCCs and EACs. For example, hypermethylation of the promoter region of the APC gene occurred in 48 of 52 (92%) patients with EAC, but in 16 of 32 (50%) patients with ESCC[41], which suggests that hypermethylation of the APC gene has distinct roles in ESCC and EAC.

**Table 1 Compilation of genes hypermethylated in esophageal cancer *n* (%)**

| Gene | Entity of pathology | Incidence of methylation | | Reference |
|---|---|---|---|---|
| | | Cancer tissue | (Adjacent) nonmalignant tissue | |
| $p14^{ARF}$ | ESCC | 6/40 (15) | ND | 29 |
| | EAC | 0/50 (0.0) | ND | 27 |
| p15 | ESCC | 6/34 (18) | ND | 26 |
| | ESCC | 5/40 (13) | ND | 29 |
| p16 | ESCC | 17/34 (50) | ND | 26 |
| | ESCC | 18/28 (64) | 0/30 (0.0) | 28 |
| | ESCC | 16/40 (40) | ND | 29 |
| | EAC | 27/50 (54) | ND | 27 |
| | EAC | 8/21 (38) | ND | 27 |
| | EAC | 16/41 (39) | 10/41 (24) | 42 |
| FHIT | ESCC | 25/36 (69) | ND | 33 |
| | ESCC | 85/257 (33) | ND | 35 |
| $RAR\beta_2$ | ESCC | 14/20 (70) | 2/17 (12) | 37 |
| | ESCC | 34/47 (73) | 18/47 (38) | 38 |
| APC | EAC | 48/52 (92) | 0/52 (0.0) | 41 |
| | ESCC | 16/32 (50) | 0/32 (0.0) | 41 |
| | EAC | 28/41 (68) | 3/41 (7.3) | 42 |
| | EAC | 39/50 (78) | ND | 27 |
| ER | EAC | 21/41 (51) | 5/41 (12) | 42 |
| MGMT | ESCC | 46/119 (39) | 0/21 (0.0) | 45 |
| | EAC | 24/41 (56) | 10/41 (24) | 42 |
| E-Cadherin | EAC | 26/31 (84) | ND | 49 |
| | EAC | 27/41 (66) | 5/41 (12) | 42 |
| | ESCC | 16/20 (80) | ND | 50 |
| TSLC1 | ESCC | 28/56 (50) | ND | 53 |
| RASSF1A | ESCC | 24/47 (51) | 2/47 (4.3) | 38 |
| | ESCC | 22/64 (34) | 3/64 (4.7) | 58 |
| hMLH1 | ESCC | 0/30 (0.0) | ND | 59 |
| | ESCC + EAC | 79/124 (64) | ND | 60 |
| VHL | ESCC | 6/47 (13) | 0/47 (0) | 39 |
| TIMP3 | EAC | 8/41 (19) | 0/41 (0.0) | 42 |
| | EAC | 71/79 (90) | 0/79 (0.0) | 61 |
| DAP-kinase | EAC | 8/41 (19) | 2/41 (4.9) | 42 |
| pRb | EAC | 10/30 (33) | ND | 62 |
| ECRG4 | ESCC | 12/15 (80 ) | 3/20 (15) | 63 |
| Chfr | ESCC + EAC | 7/43 (16) | ND | 64 |
| HLA class I | ESCC | 13/29 (45) | ND | 65 |
| EYA4 | EAC | 33/40 (83) | 2/58 (3.4) | 66 |
| CDH13 | ESCC+EAC | 5/37 (14) | ND | 67 |
| SFRP1 | EAC | 37/40 (93) | 3/30 (10) | 68 |
| PGP9.5 | ESCC | 21/50 (42) | ND | 69 |

ND: Not done.

## METHYLATION IN SERUM DNA FROM ESOPHAGEAL CANCER PATIENTS

Despite advances in diagnosis and treatment of various cancers, early detection and treatment of cancer remain a challenge. One potential early detection biomarker is DNA methylation of the promoter region of certain cancer-associated genes[70]. Genetic analysis has shown that cell-free circulating DNA in plasma or serum of cancer patients shares similar genetic alterations to those described in the corresponding tumor[70,71]. Numerous studies have demonstrated the presence of promoter hypermethylation of tumor suppressor genes in the serum DNA of patients with various cancers[72]. Hypermethylated APC DNA was observed in the plasma of 13 of 52 (25%) EACs and 2 of 32 (6.3%) ESCCs[41]. Hibi *et al*[73] found that aberrant promoter methylation of the p16 gene was detected in 31 of 38 (82%) ESCCs, and 7 of these 31 (23%) patients with a p16 alteration in the primary tumor had the same methylation changes in the corresponding serum DNA. This study yielded a promising result: a tumor associated DNA alteration could be detected in the serum of 18% of ESCC patients (7 of 38 patients) using p16 methylation as a target. Moreover, the clinical sensitivity of this assay can be potentially improved by incorporating other possibly methylated target genes, which have been

estimated in other tumor types. For example, Esteller *et al*[74] analyzed primary NSCLCs and serum from 22 patients for the methylation pattern of four TSGs (DAPK, GSTP1, p16, and MGMT). Methylation of at least one of these genes was detected in 68% of NSCLCs. Comparing primary tumors with methylation and matched serum samples, 73% of the matched serum samples were found to be methylated[74]. In addition, none of the sera from patients with tumors not demonstrating methylation were positive[74]. Therefore, combined detection of aberrant promoter hypermethylation of cancer-related genes in serum may be useful for esophageal cancer diagnosis or the detection of recurrence.

## PROGNOSTIC SIGNIFICANCE OF GENE HYPERMETHYLATION

In the past few years, numerous attempts have been made to establish a genetic technique for reliably predicting tumor prognosis, but these attempts have been hindered by two main problems. First, only a few genes are somatically mutated in solid tumors and, second, because cell populations of primary neoplasms are heterogeneous, no single marker can accurately predict the behavior of the tumor[13]. Fortunately, emerging evidence suggests a possible prognostic value of gene promoter hypermethylation[75]. Lee *et al*[35] reported that aberrant methylation of the FHIT promoter in ESCC was found to be significantly associated with a poor prognosis for stage 1-2 cases. Mandelker *et al*[69] reported that PGP9.5 methylation was an independent prognostic factor for ESCC survival ($P = 0.03$) . Kawakami *et al*[41] reported that high plasma levels of methylated APC DNA were statistically significantly associated with reduced EAC patient survival. Brock *et al*[42] analyzed the methylation status of seven genes (including APC, E-cadherin, MGMT, ER, p16, DAP-kinase and TIMP3) of 41 EAC samples and found that DNA methylation of some genes individually showed only trends toward diminished survival, whereas patients whose tumors had > 50% of their gene profile methylated had both significantly poorer survival and earlier tumor recurrence than those without positive methylation. The data suggest that combined detection of methylation status for multiple genes is an effective strategy for prediction of esophageal tumor behavior. Although some genes that are frequently inactivated by methylation and are of prognostic impact for esophageal cancer patients have already been found, additional genes need to be identified. Thus, patients with a worse prognosis could be selected. These patients might benefit from a more aggressive treatment strategy.

## ABERRANT DNA METHYLATION IS AN EARLY EVENT IN ESOPHAGEAL CARCINOGENESIS

In many tumors, it has been proved that aberrant DNA methylation frequently occurs in precancerous tissue as well as cancer tissue, and both factors, genetic and epigenetic, lie at the origin of carcinogenesis[76]. The relative contribution of each varies significantly in different human tumors[76]. Nie *et al*[77] compared hypermethylation of p16, p15, p14, HLA-A, -B, -C, hMLH1, E-cadherin, FHIT and VHL genes in precancerous esophageal tissues and found that in 48 biopsy samples with BCH or DYS, the most frequent hypermethylated genes were p16 (19%) and $p14^{ARF}$ (15%), and seventeen out of these 48 samples (35%) contained hypermethylation of at least one gene. In the resected tissues, 52% of the BCH and 81% of the tumors showed hypermethylation of at least one gene. Another study reported that 2 of 17 (12%) normal esophagus, 9 of 21 (43%)BCH, 7 of 12 (58%) DYS, and 14 of 20 (70%) ESCC samples had hypermethylation of the RAR$\beta_2$ promoter region[37]. As to progression of EAC, it has been reported that, methylation of the p16 promoter was detected in 18 of 22 (82%) EAC and 10 of 33 (30%) premalignant lesions, whereas no methylation of the p16 promoter was found in normal esophageal epithelia[78]. Hardie *et al*[30] reported hypermethylation of the p16 promoter was detected in 77% (14/18) of Barrett's epithelia, and in 85% (18/21) of EACs. These data suggest that aberrant DNA methylation participates early in the development of esophageal cancer. Recently, the lab of professor Yang CS reported that EGCG, the major polyphenol from green tea, inhibited DNMT activity and reactivated several methylation-silenced genes, including p16, RAR$\beta_2$, MGMT and hMLH1, in human esophageal cancer KYSE 510 cells, accompanied by the expression of mRNA of these genes[79]. The result suggests that methylation might be a new target of chemopreventive activity. In the last two decades, it has been proven that many drugs, such as tamoxifen, aspirin, COX-2 inhibitors, possess positive chemopreventive activity against esophageal cancer[2]. However, the exact mechanisms have not been elucidated so far. Therefore, it will be very attractive to examine the effect of these drugs on promoter methylation status of key genes in esophageal cancer cells, esophageal cancer tissue, and especially precancerous tissue of the esophagus.

## HYPERMETHYLATION AS A TARGET OF THERAPEUTIC INTERVENTION

It has been reported that demethylating agents 5-azacytidine and 5-aza-2'-deoxycytidine can restore the normal demethylated state of several types of TSGs and increase their expression in various cancers, including esophageal cancer, *in vitro* and *in vivo*[13,14,15,39,54]. Since methylation and transcriptional status are inversely correlated, the use of demethylating agents appears to be a promising option for the treatment of tumors. Methylation of genes in tumor cells could provide a tumor-specific target for new therapies[80,81,82]. In fact, these demethylating agents have exhibited significant activity in the treatment of patients with myelodysplastic syndrome, chronic myeloid leukaemia and acute myeloid leukaemia[83,84]. However, preliminary experience with these agents in solid tumors has been relatively low[85]. Esophageal tumor shows a high prevalence of TSG hypermethylation, and the above studies demonstrated that gene expression could be restored after treatment of esophageal tumor cells

with demethylating agents *in vitro*. However, up to date the clinical trial about demethylating agents in esophageal cancer is unavailable. Although it is too early to make any expectation about the effect of these drugs on esophageal cancer, this is a very promising concept and needs to be tested in clinical trials.

## CONCLUSION AND PERSPECTIVES

Esophageal carcinogenesis is a stepwise process of the accumulation of genetic and epigenetic abnormalities. It has become clear that promoter hypermethylation of TSGs is as important for this multistep process as genetic changes in the progression of esophageal carcinogenesis. The steadily growing list of genes inactivated by promoter hypermethylation in esophageal carcinoma provides not only new insights into the molecular basis of the diseases but also a long list of interesting candidate genes for the development of molecular markers which might contribute to the improvement of diagnosis and also prognosis. In addition, the fact that methylation can be reversed *in vitro* and the effect of the demethylating agent 5-aza-2'-deoxycytidine *in vitro* raise hope for new treatment strategies for esophageal cancer patients. Furthermore, understanding of the significance of aberrant DNA methylation in the precancerous stage may show that a new strategy, correction of aberrant DNA methylation, can prevent esophageal cancer in people with premalignant lesions, such as Barrett's esophagus, BCH and DYS.

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*REVIEW*

# Cancer gene therapy targeting angiogenesis: An updated review

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## Abstract

Since the relationship between angiogenesis and tumor growth was established by Folkman in 1971, scientists have made efforts exploring the possibilities in treating cancer by targeting angiogenesis. Inhibition of angiogenesis growth factors and administration of angiogenesis inhibitors are the basics of anti-angiogenesis therapy. Transfer of anti-angiogenesis genes has received attention recently not only because of the advancement of recombinant vectors, but also because of the localized and sustained expression of therapeutic gene product inside the tumor after gene transfer. This review provides the up-to-date information about the strategies and the vectors studied in the field of anti-angiogenesis cancer gene therapy.



**Key words:** Anti-angiogenesis; Tumor growth; Cancer gene therapy

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## INTRODUCTION

Angiogenesis is the formation of new blood vessels from pre-existing ones. Many developmental and pathological processes require angiogenesis[1]. As proposed by Folkman in 1971, angiogenesis is required for tumor growth[2]. Angiogenesis consists of several steps: endothelial cell (EC) proliferation, migration, basement membrane degradation, and new lumen organization[3]. This multi-step process is determined by a net balance between pro- and anti-angiogenesis regulators in the circulation blood, which are released from activated ECs, monocytes, smooth muscle cells and platelets[3].

The growth of tumor depends on new blood vessel growth and involves three steps: angiogenesis, vasculogenesis and intussusception[4]. Without angiogenesis, a solid tumor rarely grows larger than 2 to 3 mm[5]. As shown in Figure 1, ECs and tumor cells release angiogenesis regulators like vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF) and transforming growth factor (TGF) to mediate angiogenesis. The result is the development of invasive tumor. In addition to the presence of angiogenesis factors, activation of oncogene and loss of tumor suppressor gene are also essential for an angiogenesis phenotype that supports tumorigenicity[6]. As a result, anti-angiogenesis has been regarded as a target for cancer therapy.

There are already several extensive reviews on the development of anti-angiogenesis cancer gene therapy[3,7-10]. In the 2001 review, Liau *et al*[7] compared and contrasted the gene approach and recombinant protein approach. In the editorial written by Lau and Bicknel[9], the authors compared the delivery of the genes of anti-angiogenic factors with that of the therapeutic proteins. They suggested that the delivery of genes can allow a high local expression of the protein at the sites of active tumor growth[9]. El-Aneed pointed out in his review, which summarizes the strategies in cancer gene therapy, that the ease of accessing ECs of the blood vessels is one of the main advantages of gene delivery approach[10]. Figure 2 shows that the delivery of the anti-angiogenesis gene into tumor cells or ECs can inhibit tube formation, EC migration and proliferation. This can result in tumor necrosis. In this review, updated information on the development of cancer anti-angiogenesis gene therapy is discussed.

## ANTI-ANGIOGENESIS CANCER GENE THERPAY STRATEGIES

### *RNA interference*

RNA interference (RNAi) is the sequence-specific gene

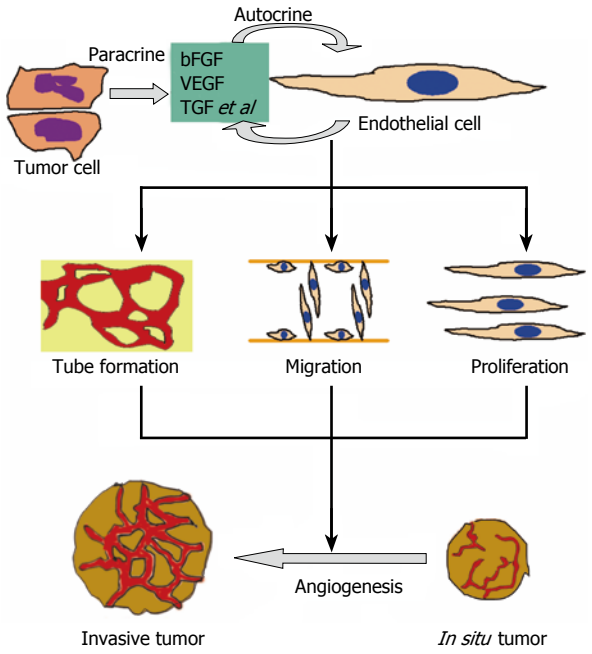


**Figure 1** Release of angiogenesis factors mediates the development of invasive tumor.

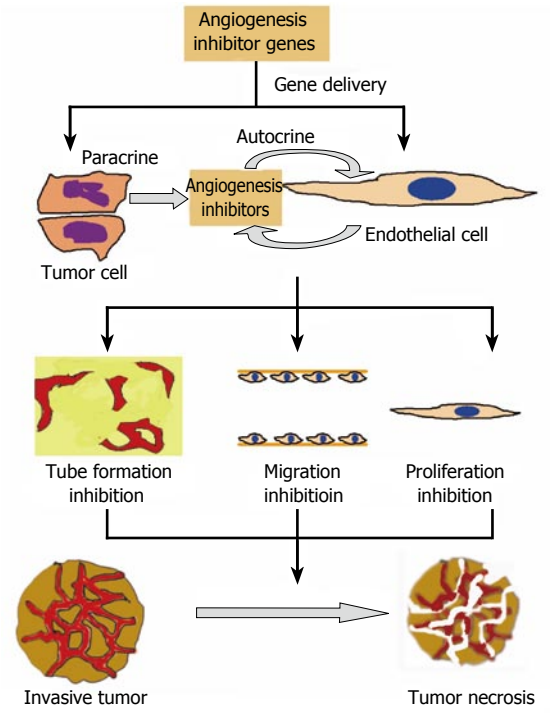


**Figure 2** Therapeutic actions of anti-angiogenesis gene therapy.

silencing induced by double-stranded RNA. Introduction of 21-23 small interfering RNAs (siRNAs) of the nucleotide can knock-out the expression of a particular gene[11]. A recent review written by Izquierdo[12] pointed out that siRNA against expression of vascular growth factor receptor (VGFR) can reduce tumor volume by blocking angiogenesis. Kwon and co-workers[13] described a method that can suppress the expression of VGFR-A at both transcriptional and post-transcriptional levels by a combination of zinc finger protein and siRNA. Gondi and co-workers[14] have demonstrated the potential application of RNAi in gene cancer therapy by inhibiting angiogenesis in both *in vivo* and *in vitro* human glioma cell models. Furthermore, it is possible to include more than one antiangiogenesis siRNA into a single retroviral vector because of the small size of siRNA, which could inhibit multiple pathways[12].

### *Antisense oligodeoxynucleotide*

Antisense oligodeoxynucleotide (ODN) is a synthetic molecule that blocks mRNA translation. The blockade of translation of mRNA of pro-angiogenesis factor genes can result in inhibition of tumor growth. Recently, ODN blocking of the expression of VEGF has been shown to be a promising cancer gene therapy. For example, Wang *et al*[15] have successfully reduced VEGF protein expression by 45% in human osteosarcoma cell line by transducing a eukaryotic expression plasmid containing antisense VEGF. Lipiodol is an effective treatment for unresectable liver cancer through transcatheter arterial embolization of the hepatic artery[16]. When VEGF antisense ODN is mixed with lipiodol, this combinational approach is better in inhibiting liver cancer growth, VEGF expression and microvessel density[16].

### *Expressing the genes of angiogenesis inhibitors*

Table 1 summarizes the genes of candidate angiogenesis inhibitors that have been studied recently. In the review published in the *Journal of Translational Medicine*[3], the authors made a thorough account of several candidates. To avoid overlapping of information, we only discuss those candidates that are not covered or recently have demonstrated significant advancement.

**Maspin:** The Maspin gene is a tumor suppressor gene which is under transcriptional control by p35 and DNA methyltransferase inhibitors. Its gene expression level decreases with malignancy and is lost in metastatic cells[17,18]. Transfection of maspin gene to nude mice could reduce the ability of cells to induce tumors and metastasis[19]. Recently, Watanabe *et al*[18] have shown that adeno-associated virus 2-mediated expression of human maspin can efficiently suppress tumor growth by inhibition of angiogenesis in prostate cancer.

**Human ribonuclease inhibitor:** Human ribonuclease inhibitor (hRI) is an acidic protein with a molecular weight of 50 kDa. It can inhibit the activity of pancreatic RNase (RNase A)[20]. It is proposed that hRI inhibits angiogenesis by forming a tight complex with its counterpart angiogenin (Ang) which is an angiogenesis factor[21]. Fu *et al*[20] demonstrated that hematopoietic cells carrying the *ri* gene can effectively inhibit tumor growth (by 47%) and reduce tumor microvessel density in mice. They concluded that hRI has the potential utility as a novel antiangiogenesis agent[20].

**Survivin:** Survivin has been identified as an anti-apoptosis gene over-expressed in cancer and lymphoma[22]. It has been shown that survivin is minimally expressed in endothelium of non-proliferating capillaries of normal skin, whereas it becomes massively up-regulated in newly formed blood vessels of granulation tissue *in vivo*. As a result, manipulation of survivin expression and function in endothelium may influence tumor angiogenesis[23]. Recently, a DNA vaccine targeting survivin and an adeno-associated

viral vector carrying survivin Cys84Ala mutant have been employed to demonstrate the anti-angiogenesis effect on lung tumor and colon cancer cells respectively[24,25].

**Soluble FMS-like tyrosine kinase receptor 1:** Soluble FMS-like tyrosine kinase receptor 1 (sFlt-1) has been identified as a receptor of vascular endothelial growth factor (VEGF)[26]. It functions by sequestering VEGF and forming inactive heterodimers with other membrane-spanning VEGF receptors both in *in vitro* and *in vivo*[26]. Intramuscular injection of recombinant adeno-associated virus (rAAV) vectors carrying the sFlt-1 gene into nude mice can protect against the human ovarian cancer cell line with increased disease-free survival[27].

**Interleukin-12:** In 2004 and 2005, Heinzerling *et al*[28] and Imagawa *et al*[29] showed that direct intratumoral injection of interleukin-12 (IL-12) gene produces a reduction in vessel density or angiogenesis in a murine head and neck cancer model and in patients with metastatic melanoma[28,29]. It is worth noting that a few cytokines, like IL-12, have recently been reported to participate in the regulation of the angiogenic switch[30]. These cytokines are related to inflammation. Whether there are relationships between angiogenesis and inflammation may be an interesting topic among scientists in the future.

**Pigment epithelium-derived factor:** Pigment epithelium-derived factor (PEDF) is a neurotrophic protein and belongs to the serine protease inhibitor (serpin) family[31,32]. It is believed to be a potent inhibitor of angiogenesis[33]. A full-length human PEDF expression vector has been used to transfect the glioma cell line U251, resulting in up-regulation and down-regulation of angiogenesis inhibitors and activators[34].

**Tissue inhibitors of metalloproteinase:** In 2004, adeno-associated virus-mediated gene transfer of tissue inhibitor of metalloproteinase (TIMP) to animal's tumor also showed that it can inhibit vascular tumor growth and angiogenesis[35].

**Angiostatin:** Angiostatin is a 38 kD kringle domain of plasminogen and is the most potent and well characterized body's angiogenesis inhibitor[3]. Recently, the effectiveness of co-administration of the mouse angiostatin kringle and the endostatin genes using cationic liposome has been investigated *in vitro* and *in vivo* by Kim *et al*[36].

**Melanoma differentiation-associated-7 gene or interleukin-24:** Melanoma differentiation-associated-7 (mda-7) gene is a novel melanoma differentiation-associated gene that modulates human melanoma differentiation, growth and progression. It was identified by subtractive hybridization in human melanoma cells by Jiang *et al*[37]. It has been demonstrated that the mda-7 gene functions as a multi-modality anti-cancer agent, possessing both pro-apoptotic and anti-angiogenic properties, and the adenovirus-mediated over-expression of mda-7 gene has the potential therapeutic effects in human lung cancer[38]. More recently, Nishikawa *et al*[39] performed a combination therapy on non-small-cell lung cancer (NSCLC) cell lines and showed that the combination of mda-7 gene therapy and radiotherapy may be a feasible and effective strategy for treatment of NSCLC.

**Fragments of hepatocyte growth factor:** NK4 is the N-terminal hairpin domain and subsequent four-kringle domains of hepatocyte growth factor (HGF). It was reported that HGF possesses anti-angiogenesis property[40]. A latest trial has been done using hydrodynamics-based gene delivery of naked NK4 plasmid into colon cancer cells in mice. HGF can efficiently express NK4, inhibit liver metastasis and subsequent invasive growth of colon cancer and prolong survival of mice[41]. In addition to NK4, recombinant kringle 1 domain of HGF (HGFK1) has been shown to inhibit bovine aortic endothelial cell proliferation stimulated by basic fibroblast growth factor (bFGF) in a dose-dependent manner[42]. These studies present the potency of the fragments of HGF in inhibiting angiogenesis.

**NC1 domains of collagen:** Endostatin (from collagen XVIII), restin (from collagen XV), arrestin (α1 chain of collagen IV) and canstatin (α2 chain of collagen IV) are all NC1 domains as reviewed by Tandle *et al*[3]. Recently it has been shown that vastatin, the NCI domain of collagen VIII (α1) possesses anti-angiogenesis ability in bovine aortic endothelial cells[43]. This provides another promising candidate for caner anti-angiogenesis gene therapy.

**Table 1 Genes of canadidate angiogenesis inhibitors**

| Candidate | Reference(s) |
|---|---|
| 16 kD prolactin fragment[1] | 3 |
| Angiostatin[1] | 3, 36 |
| Arresten[1] | 3 |
| Canstatin[1] | 3 |
| Endostatin[1] | 3 |
| Endothelial-monocyte activating polypeptide-Ⅱ (EMAP-Ⅱ)[1] | 3 |
| Fragments of hepatocyte growth factor (HGF) | |
| NK4 | 40, 41 |
| HGFK1 | 42 |
| Human rubonuclease inhibitor (hRI) | 20, 21 |
| Interferon-inducible protein-10 (IP-10)[1] | 3 |
| Interferons[1] | 3 |
| Interleukin-12 (IL-12)[1] | 28, 29 |
| Interleukin-18 (IL-18)[1] | 3 |
| Interleukin-24 (IL-24) | 37, 38, 39 |
| Maspin | 17, 18, 19 |
| p53[1] | 3 |
| Pigment epithelium-derived factor (PEDF) | 31, 32, 33, 34 |
| Platelet factor-4[1] | 3 |
| Restin[1] | 3 |
| Soluble FMS-like tyrosine kinase receptor 1 (sFlt-1) | 26, 27 |
| Survivin | 22, 23, 24, 25 |
| Thrombospondin-1 (THBS1)[1] | 3 |
| Tissue inhibitors of metalloproteinases (TIMPs)[1] | 3, 35 |
| Tumor necrosis factor alpha (TNF-α)[1] | 3 |
| Tumstatin[1] | 3 |
| Vastatin[1] | 43 |

[1]Candidates that have been covered in Tandle *et al*[3].

## GENE DELIVERY SYSTEMS

The viral vectors used for tumor vascular targeting therapy are summarized in a recent review[44]. Tandle *et al*[3] have also discussed some non-viral gene delivery vectors. Again, we will focus on those newly studied viral vectors showing advancement. Table 2 summarizes the vectors that are

**Table 2 Gene delivery vectors**

| Vector | Brief description | Reference (s) |
|---|---|---|
| Cationic liposomes | Spherical vesicle made of positively charged lipids, efficient uptake of DNA by the cell | 46, 47 |
| Low Voltage Electroporation | Entry of DNA into the cell whose membrane is permeabilized by electric field, efficient gene transfer is ensured | 48, 49 |
| Nanoparticles | Submicron-sized particle with the therapeutic agent situated within the matrix or on the surface | 3, 45 |
| Measles virus | Contains negative strand RNA molecule, can enter tumor cells without the defensive responses of the tumor | 44 |
| Herpes simplex virus | Dobule stranded DNA virus, wide host range, large transgene capacity, long-lasting effect | 3, 44 |
| Lentivirus | Derived from HIV, can achieve stable integration of the gene in non-dividing cells | 3, 44 |
| Retrovirus | Based on murine leukemia virus, foreign cDNA can be incorporated into host cell genome with high efficiency | 3, 44 |
| Replication-competent Retrovirus (RCR) | Modified retroviral vector that can replicate in solid tumor model so the transfer efficiency is enhanced | 55, 56 |
| Semliki Forest Virus (SFV) | One type of alphavirus, modification of its RNA genome yield a new expression vector that transfers transgene into tumor | 50, 51, 52, 53, 54 |
| Recombinant adenovirus (rAdv) | Double stranded DNA virus, can be produced in high titres and deliver transgene efficiently Special engineered adenovirus: oncolytic and gutless | 3, 57, 58, 59, 60, 61, 62, 63, 64 |
| Recombinant adeno-associated virus (rAAV) | Possess the advantages of rAdv and retrovirus, low level of immune response | 3, 25, 65, 66, 67, 68 |

recently used in cancer antiangiogenesis gene therapy.

**Nanoparticles:** Polymeric drug carriers are used to deliver low molecular mass drugs, oligonucleotides and peptides, which has attracted attention in recent years[3]. Due to their small sizes, nanoparticles penetrate into even small capillaries and are taken up by cells that can deliver targeted drugs to cells or tissues[3]. In 2005, Schiffelers *et al*[45] constructed self-assembling nanoparticles with siRNA as a means to target tumor neovasculature expressing integrins and to deliver siRNA which inhibits VEGF-R2 expression and thereby tumor angiogenesis. They pointed out that this mode of delivery overcomes the pharmacological hurdles of local administration of aqueous siRNA for cancer therapy.

**Cationic liposome:** The advantages of using a cationic liposome as a vehicle for drug delivery are the enhancement of delivery and expression of the transfected gene. The positive charge significantly increases the uptake of liposome by the endothelial cells of blood vessels in tumor tissues, which has made the cationic liposome useful for delivering tumor targeted drugs[46]. A recent successful case of angiogenesis inhibition using angiostatin and endostatin genes delivered by a cationic liposome has been reported. In addition, modified liposome targeting membrane type-1 matrix metalloproteinase (MT1-MMP) molecules expressed specifically on angiogenesis endothelium and tumor cells, enhances its binding to and accumulates ECs in tumor compared to unmodified liposome[47].

**Low voltage electroporation:** Electroporation is the formation of pores on the cell surface induced by electric pulse. Direct delivery of plasmid DNA into cells relies on electroporation. *In vivo* electroporation is a novel non-viral means of gene transfer and offers several advantages over viral means such as none of immunogenicity, ease of handling and high gene transfer efficiency[48]. Uesato and co-workers[49] have successfully demonstrated the anti-tumor effect of antiangiogenesis genes, mouse angiostatin and mouse endostatin, delivered to tumors by low-voltage electroporation in 26 models of mouse colon. They have also reported a decrease in microvessel density of tumors[49].

**Semliki forest virus:** A new expression vector system derived from semiliki forest virus (SFV) was introduced in 1994[50]. This system has been utilized in delivering glycoproteins in a recombinant vaccine study[51]. The vector has also been shown to be a candidate medium for human cancer gene therapy[52]. More recently, SFV vector carrying murine IL-12 gene demonstrated by Doppler ultrasonography, could cause B16 tumor regression through anti-angiogenesis[53]. After this, two IL-12 gene subunits cloned from mouse splenocytes and inserted into an enhanced SFV vector (pSFV10-E) could show complete tumor regression in mice[54].

**Replication-competent retroviruses:** Retroviruses are a class of virus which has a genome of a single stranded RNA molecule. Vectors derived from murine leukemia virus, a simple retrovirus, have been used in *in vivo* gene transfer in gene therapy. However, the limited efficiency of replication-defective retrovirus vector is a major obstacle in cancer gene therapy[55]. Logg's group in Los Angeles thus developed a replication-competent retrovirus (RCR) vector derived from murine leukemia virus[55]. This vector is able to replicate and transmit a transgene both in culture and in a solid tumor model *in vivo*. By taking advantages from RCR vectors, Sun *et al*[56] transduced RCR vectors carrying the human interferon-inducible protein-10 (IP-10) gene to tumor cells *in vivo* and *in vitro*, showing sustained production of IP-10 in culture and reduced angiogenesis in mice.

**Recombinant adenovirus:** Adenovirus has a double stranded DNA genome. Recombinant adenovirus (rAdv) vectors containing exogenous genes for *in vivo* transfer derived from adenovirus type 5 are made replication deficient by deletion of the E1 region[57]. rAdv is currently the most widely used gene delivery vector because it

enjoys several advantages like high delivery efficiency into both dividing and non-dividing cells, large ability to package foreign genes, easy to grow to high titers and to be purified, non-oncogenic and high expression of the transgenes[58]. In recent years, phase Ⅰ trials have been undertaken using adenoviral p53 (Adp53) for patients with ovarian cancer[59]. In China, phase Ⅰ and Ⅱ trials using recombinant Adp53 to treat laryngeal cancer (phase Ⅰ), head and neck squamous cell carcinoma (phase Ⅱ) and nasopharyngeal carcinoma (phase Ⅱ) have been undertaken extensively[60-62].

Oncolytic adenovirus is a specially engineered adenovirus which exhibits lytic property of virus replication[63]. This adenoviral system not only offers the advantage of high gene delivery efficiency, but also the ability to select infections of tumor cells[63]. As a result, an amplification effect of the therapeutic gene can be achieved through the lateral spread of the progeny vector[63].

The latest generation of adenoviral vector is the gutless adenovirus. It has become an attractive agent for gene therapy because of the reduction of *in vivo* immune response[64] and long-term sustained expression. However, because of the lack of all viral coding regions, the packaging of this virus requires the presence of helper virus which presents the possibility of contamination[64].

**Recombinant adeno-associated virus:** Recombinant adeno-associated virus (rAAV) has the advantages of broad host range, low level of immune response, and longevity of gene expression that enable the initiation of a number of clinical trials using this gene delivery system[65]. As reviewed recently, there are 8 well-defined serotypes (serotypes 1-5 and 7-9), and more than 100 variants[66]. The underlying mechanism of the selective tissue tropism of different serotypes remains elusive[66]. For anti-angiogenesis cancer gene therapy using rAAV, recent research examples are focusing on treating colon cancer (*in vitro* and *in vivo*), ovarian cancer (*in vivo*) and human glioblastoma (*in vitro*)[25,67,68].

## TUMOR SPECIFICITY AND GENE DELIVERY: LESSONS FROM CLINICAL TRIALS

While previous studies on gene targets are limited to pre-clinical stages, the recombinant proteins of some of these targets have entered clinical trials. Can we learn lessons from the trials to optimize the specificity and efficiency of the candidate gene therapeutics?

Recombinant endostatin is currently the most studied angiogenesis inhibitor in the clinical setting. The earliest phase Ⅰ trials were published in 2002 and 2003[69-72]. However, the results were disappointing. Two very recent reports stated that although the endostatin trials have confirmed the safety of endostatin as a pharmacological agent, it is difficult to establish the biologically effective dose of the recombinant protein[73,74]. To address the problem of effective dose of endostatin, Tjin Tham Sjin *et al*[75] recently demonstrated that adeno-associated viruses carrying canine endostatin can dose-dependently express transgene in the circulation after intramuscular injection in mice. Elevated levels of endostatin remain stable in the circulation for at least 4 mo[75]. Therefore, adeno-associated virus-mediated endostatin gene therapy appears to be a potential therapeutic regime with specific and sustained delivery efficiency.

IL-12 is another widely studied agent with anti-angiogenesis activity in clinical trials. Recombinant human IL-12 protein has entered phase Ⅰ and Ⅱ studies in Germany and United States, respectively[3]. Due to the occurrence of dose-limiting toxicity in some patients, the direction of study has switched to gene therapy approaches[3]. A phase Ⅰ trial involving an adenoviral vector encoding human IL-12 gene has been conducted[76], showing that dose-limited toxicity is significantly increased in tumor infiltration by effector immune cells. Despite the lower anti-tumor power of IL-12 gene therapy in human trials, the concept of stimulation of immune response by specific production of IL-12 inside a tumor is proved[77].

Recently, attention has been paid to combination therapy in which anti-angiogenesis treatment is combined with chemotherapy as well as radiotherapy[78]. Approaches like combination of endostatin and VGFR-2 tyrosine kinase inhibitor and even tri-combination of anti-angiogenesis, chemotherapy and radiotherapy have also been tested[79,80]. Co-targeting of tumor and tumor micro-environment can effectively suppress angiogenesis and tumor growth in the prostate cancer model[81]. A Chinese phase Ⅲ trial using recombinant endostatin in combination with chemotherapy in NSCLC has exhibited a significant increase in response rates and time to progression[73].

Specificity and safety of the vectors are the two main issues that should be addressed in the future. Development of vectors that exhibit superior safety and direct the therapeutic transgene to the right target position of the genome without any random insertion side effects would be a direction for studying human gene therapy against cancer.

## CONCLUSION

Targeting angiogenesis is a promising approach in suppressing tumor growth and metastasis. Due to the need for long term administration of the inhibitors, gene therapy has become an alternative which theoretically ensures a sustained availability of the anti-angiogenesis agents. Up till now, researches on anti-angiogenesis cancer gene therapy remain in pre-clinical stage. It is anticipated that when better vectors are developed and the molecular mechanisms of angiogenesis inhibitors against tumor growth are better understood, clinical trials will be undertaken in the future.

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# Comparison of gene expression profiles between primary tumor and metastatic lesions in gastric cancer patients using laser microdissection and cDNA microarray

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## Abstract

**AIM:** To study the differential gene expression profiles of target cells in primary gastric cancer and its metastatic lymph nodes using laser microdissection (LMD) in combination with cDNA microarray.

**METHODS:** Normal gastric tissue samples from 30 healthy individuals, 36 cancer tissue samples from primary gastric carcinoma and lymph node metastasis tissue samples from 58 patients during gastric cancer resection were obtained using LMD in combination with cDNA microarray independently. After P27-based amplification, aRNA from 36 of 58 patients (group 1) with lymph node metastasis and metastatic tissue specimens from the remaining 22 patients (group 2) were applied to cDNA microarray. Semiquantitative reverse transcriptase polymerase chain reaction (RT-PCR) and immunohistochemical assay verified the results of microarray in group 2 and further identified genes differentially expressed in the progression of gastric cancer.

**RESULTS:** The expression of 10 genes was up-regulated while the expression of 15 genes was down-regulated in 22 gastric carcinoma samples compared with that of genes in the normal controls. The results were confirmed at the level of mRNA and protein, and suggested that four genes (OPCML, RNASE1, YES1 and ACK1) could play a key role in the tumorigenesis and metastasis of gastric cancer. The expression pattern of 3 genes (OPCML, RNASE1 and YES1) was similar to tumor suppressor genes. For example, the expression level of these genes was the highest in normal gastric epithelium, which was decreased in primary carcinoma, and further decreased in metastatic lymph nodes. On the contrary, the expression pattern of gene ACK1 was similar to that of oncogene. Four genes were further identified as differentially expressed genes in the majority of the cases in the progression of gastric cancer.

**CONCLUSION:** LMD in combination with cDNA microarray provides a unique support foe the identification of early expression profiles of differential genes and the expression pattern of 3 genes (OPCML, RNASE1 and YES1) associated with the progression of gastric cancer. Further study is needed to reveal the molecular mechanism of lymph node metastasis in patients with gastric cancer.



**Key words:** Gastric cancer; cDNA microarray; Laser microdissection; Reverse transcriptase polymerase chain reaction; P27

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## INTRODUCTION

Gastric cancer is one of the leading causes of cancer death in the world, its clinical behavior depends on the potential metastasis of the tumor, and the prognosis of advanced gastric cancers remains very poor. Until now, several molecules have been reported to play an important role in gastrointestinal tumorigenesis and tumor metastasis[1-3], but the molecular mechanisms involved in tumor development and progression remain unclear in gastric cancer[1-3].

In this study, using the combined methods of laser microdissection (LMD), P27-based RNA amplification, and cDNA microarray, we evaluated the differentially expressed genes in primary carcinoma cells and lymph node metastatic cells in 36 of 58 patients. Moreover, we further identified four differentially expressed genes in the remaining 22 patients with progression of gastric cancer by semiquantitative reverse transcriptase polymerase chain reaction (RT-PCR), and the expression patterns of these

four genes were similar to those of tumor suppressor genes or oncogenes.

It has been widely accepted that many malignant tumors contain heterogeneous subpopulations of cells. This heterogeneity exhibits a wide range of genetic, biochemical and immunologic characteristics. It is likely that specific tumor cells or colonies within larger heterogeneous tumor specimens are the forerunners of distant metastases[4]. Therefore, many biologic differences exist in tumor cells of primary carcinoma and metastatic lesions. Furthermore, interaction of tumor cells with their living environment may add more differences to these tumor cells[5]. As a result, tumor metastasis-related genes can be identified by comparing their gene expression profiles.

LMD and cDNA microarray are two of the new emerging techniques in the post-genomic era. LMD is an innovative technique which offers researchers a simple, reliable, rapid and accurate tool for precise and contamination-free procurement of cell groups from tissue sections under direct visualization[6,7].

Large-scale analysis of gene expression with cDNA microarray allows us to evaluate the gene-expression profiles of hundreds to tens of thousands of genes in a single experiment[8]. This techniques is a powerful tool for analyzing the expression of genes which may be correlated with pathological phenotypes of various diseases. However, the expression profile of a specific cell type may be primarily masked or even lost because of the bulky surrounding cells. Therefore, LMD in combination with cDNA microarray can provide a unique opportunity to study gene expression of subpopulations of cells in their native tissue environment *in vivo*.

## MATERIALS AND METHODS

### Patients with gastric cancer and their pathological samples

Fifty-eight advanced gastric adenocarcinoma (TNM stage Ⅲ-Ⅳ) patients with lymph node metastasis diagnosed by postoperative pathology were investigated in this study. There were 30 male and 28 female patients, whose ages ranged from 45 to 68 years with an average age of 58.7 ± 3.46 years (Table 1). Histologically, 38 patients had moderately differentiated adenocarcinoma and 20 had poorly differentiated adenocarcinoma. All patients underwent gastrectomy with regional lymph nodes dissected and informed consent to participate in this study was obtained from each patient. Tissue blocks of normal gastric epithelium (> 5 cm away from the edge of the tumor), primary tumors and corresponding metastatic lymph nodes were obtained within 30 min after removal from the patient. Each block was cut into 2 pieces, one for routine pathologic diagnosis, and the other for molecular analysis. The latter samples were frozen in liquid nitrogen immediately and stored at -260℃ until use.

**Table 1 Characteristics of the studied patients ($n$ = 58)**

| Characteristics | $n$ (%) |
|---|---|
| Age (yr) | |
| Range | 48-68 |
| mean ± SD | 58.7 ± 3.46 |
| Median | 59 |
| Mode | 59 |
| Gender | |
| Male | 30 (52) |
| Female | 28 (48) |
| Tumor location | |
| Gastric antrum | 38 (66) |
| Gastric body | 10 (17) |
| Gastric cardia | 10 (17) |
| Anemia | |
| Yes | 48 (83) |
| No | 10 (17) |
| Abdominal pain | |
| Yes | 27 (47) |
| No | 31 (53) |
| Weight los | |
| Yes | 34 (59) |
| No | 24 (41) |
| Occult blood test | |
| Positive | 43 (74) |
| Negative | 25 (26) |

### Laser microdissection and RNA extraction

Before sectioning, tissue blocks were embedded in Tissue Tek OCT compound medium (VWR Scientific Products, San Diego, CA, USA) in a cryostat. Then serial 8-micron thick sections were prepared and mounted onto a foiled slide and stored at -70℃ until use.

Frozen section slides were stained just before laser microdissection on ice. Briefly, the slides were fixed in 70% ethanol for 30 s and stained with 0.1% toluidine blue (TBO) for 15 s, followed by dehydration in 75%, 95% and 100% ethanol respectively for 5 s and dehydration in xylene for 5 min. Once airdried, the sections were laser microdissected with a LMD system (Leica Microsystems, Wetzlar, Germany) and the target cells were selectively collected. Next, total RNA was extracted from the interest cells independently with the RNA-lyase Micro kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. The integrity of the total RNA was measured by Lab-on-chip (Agilent, Palo Alto, USA).

### cDNA microarray

We carried out cDNA microarray analysis in 58 metastatic gastric cancer tissue samples of lymph node metastatic gastric cancer, 36 tissue samples of primary gastric cancer and 30 tissue samples of normal gastric epithelium, all technical services were provided by Shanghai Biochip Corporation (Shanghai, China). Two hundred ng of total RNA was amplified for array analysis, 100 ng of total RNA was not amplified for later certification. After P27-based RNA amplification, aliquots (2.5-microgram) of aRNA from primary carcinoma tissues and their corresponding metastatic lymph nodes were labeled with Cy3-dCTP and Cy5-dCTP, respectively. The labeled probes were hybridized with Human cDNA Chip version 2.0 (SBC-R-HC-100-20, Shanghai, China) containing 13 824 genes (including 10 positive controls and 6 negative ones), and the signals were detected by the Agilent scanner (Agilent, Palo Alto, USA). We set the cutoff values for signal intensities, i.e., the signal to noise ratios of Cy3 or Cy5

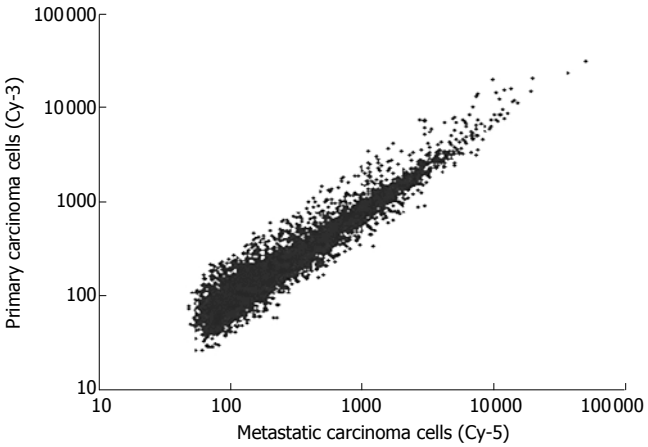


**Figure 1** Scatter plots of cDNA microarray analysis. Primary carcinoma cells and lymph node metastatic cells from 58 patients (TNM stage Ⅲ-Ⅳ) with gastric cancer were labeled by Cy-3 and Cy-5 respectively, and hybridized to the cDNA microarray using laser microdissection in combination with microarray.

must be greater than 2. Genes with Cy3:Cy5 ratios > 4 or < 0.25 in the remaining 22 patients were defined as up- or down-regulated genes.

### *Semiquantitative RT-PCR*

Nonamplified total RNA (100 ng) was reverse transcribed into cDNA using Superscript Ⅱ reverse transcriptase (Invitrogen, USA) at 42℃ for 60 min and at 70℃ for 15 min. Each single-stranded cDNA was diluted for subsequent PCR amplification and the content of cDNA was semiquantitatively normalized by housekeeping gene β-actin. PCR conditions for different genes included an initial denaturation at 94℃ for 3 min, followed by 30-35 cycles of denaturation at 94℃ for 30 s, annealing at 94℃ for 30 s, and elongation at 72℃ for 1 min (The primer sequences, annealing temperatures and cycles of each gene are available on request). Amplified PCR products were visualized by electrophoresis on 1% agarose gel containing ethidium bromide.

### *Immunohistochemical assay*

The slides were incubated with rabbit anti-OPCML antibody (BOSTER, USA) at 1:50 dilution. The sections were then stained with KIT SA1028 (BOSTER, USA) according to the manufacturer's instructions. The tissue was counterstained with hematoxylin.

### *Laser microdissection*

Target cells in each sample were successfully laser microdissected. Consequently, about $6 \times 10^6$-$8 \times 10^6$ cells were collected for total RNA extraction, 200-400 ng total RNA was obtained. The integrity of each sample was proven by Lab-on-chip.

### *Statistical analysis*

All data were expressed as mean ± SD. Statistical significance between the two groups was determined by Student's *t*-test using GraphPad Prism version 3.02 for Windows (GraphPad Software Inc., San Diego, USA). $P < 0.05$ was considered statistically significant.

**Table 2 Differentially expressed genes detected by cDNA microarray**

| GeneBank | Description | $C_y3:C_y5$[2] |
|---|---|---|
| NM_006500 | MCAM (melanoma adhesion molecule) | 10.79[1] |
| NM_002545 | OPCML (opioid binding protein/cell adhesion molecule-like) | 10.56[1] |
| NM_002933 | RNASE1 (ribonuclease, RNase A family, 1) | 6.8[1] |
| NM_001993 | F3 (coagulation factor Ⅲ) | 6.29 |
| NM_005433 | YES1(v-yes-1 Yamaguchi sarcoma viral oncogene homolog 1 | 5.916[1] |
| NM_016525 | UBAP (ubiquitin associated protein) | 5.876 |
| NM_001428 | ENO1 (enolase 1) | 5.692[1] |
| S68616 | SLC9A1 (Na+/H+ exchanger NHE-1 isoform) | 5.484 |
| NM_003254 | TIMP1 (tissue inhibitor of metalloproteinase 1) | 5.29[1] |
| NM_005903 | MADH5 (mothers against decapentaplegic, homolog 5) | 5.083 |
| U82828 | ATM (ataxia telangiectasia) | 0.205[1] |
| NM_006343 | MERTK (c-mer proto-oncogene tyrosine kinase) | 0.19 |
| NM_002985 | SCYA5 (small inducible cytokineA5) | 0.19[1] |
| NM_005348 | HSPCA (heat shock 90kD protein 1, alpha) | 0.164 |
| NM_003968 | UBE1C (ubiquitin-activating enzyme E1C) | 0.161 |
| NM_004374 | COX6C (cytochrome c oxidase subunit VIc) | 0.131 |
| NM_002990 | SCYA22 (small inducible cytokine subfamily A, member22) | 0.129 |
| NM_005781 | ACK1 (activated p21cdc42Hs kinase) | 0.128[1] |
| NM_005139 | ANXA3 (annexin A3) | 0.124 |
| AF053630 | SERPINB1(serine proteinase inhibitor, clade B, member 1) | 0.123 |
| NM_012090 | MACF1 (microtubule-actin crosslinking factor 1) | 0.098 |
| XM_042551 | CAMK2A (calcium/calmodulin-dependent protein kinase) | 0.0645 |
| NM_000909 | NPY1R (neuropeptide Y receptor Y1) | 0.0252[1] |
| NM_015230 | CENTD1 (centaurin, delta 1) | 0.024 |
| NM_004958 | FRAP1 (FK506 binding protein 12-rapamycin associated protein 1) | 0.0205 |

[1]Up-regulated genes were confirmed by semiquantitative RT-PCR in another group of 15 cases; [2]Down-regulated genes and aRNA from primary carcinoma tissues and their corresponding metastatic lymph nodes were labeled with Cy3-dCTP and Cy5-dCTP, respectively.

## RESULTS

After $P^{27}$-based amplification, we evaluated the expression profiles of tumor cells of primary gastric cancer and their corresponding metastatic lymph nodes in 36 patients. Scatter plots of cDNA microarray analysis are shown in Figure 1. Amplified aRNA from primary gastric carcinoma cells (Cy3) and metastatic carcinoma cells (Cy5) were labeled and hybridized to the cDNA microarray.

Analysis of the cDNA microarray data showed that 49 genes (including 31 with unknown function) were over-expressed (Cy3:Cy5 > 4) in primary carcinoma cells from 36 patients. On the other hand, 37 genes (including 9 with unknown function) were significantly suppressed (Cy3:Cy5 < 0.25) in primary carcinoma cells from 36 patients. The up-regulated genes were related to cell adhesion, cytoskeleton, cell defense and metabolism. Meanwhile, the down-regulated genes included those associated with cell development, cell cycle, signal transduction, adhesion, cell

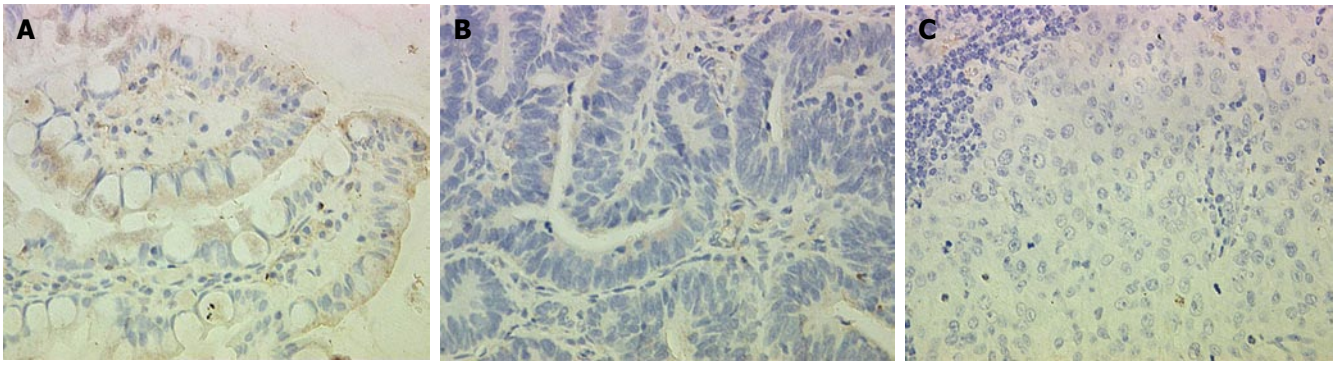


**Figure 2** Immunohistochemical staining of OPCML. **A**: Normal gastric epithelium showing abundant and strong immunoreactivity; **B**: Primary gastric cancer demonstrating weak immunoreactivity; **C**: lymph node metastastasis showing rare immunoreactivity (× 400).

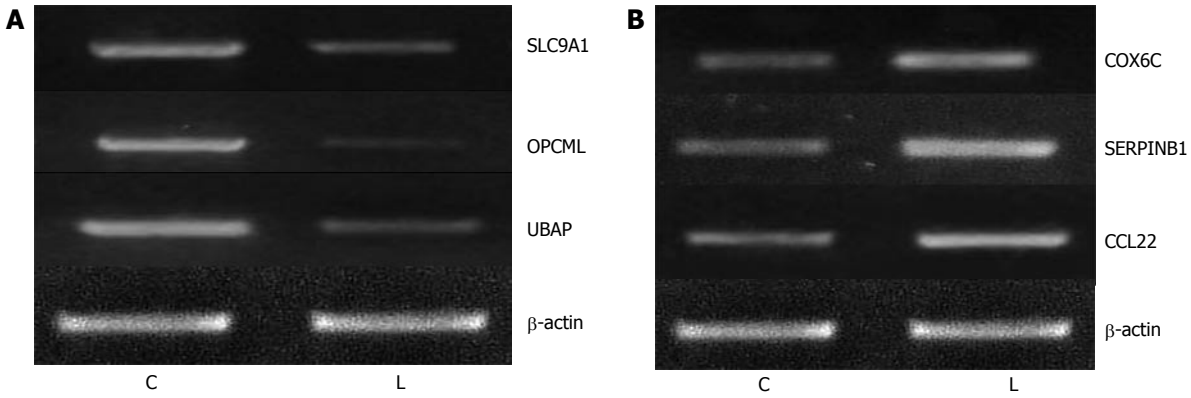


**Figure 3** Validation of microarray data by semiquantitative RT-PCR. **A**: Expression levels of 3 genes (SLC9A1, OPCML and UBAP) was up-regulated in primary carcinoma; **B**: Expression of 3 genes (COX6C, SERPINB1 and CCL22) was down-regulated in primary carcinoma. C: A sample from the primary carcinoma cells; L: A sample from the metastatic gastric carcinoma cells.

defense, gene expression and cell metabolism (Table 2).

### *Confirmation of microarray findings*

To examine the reliability of microarray data, we confirmed our data at the level of mRNA and protein. First, we selected 3 up-regulated genes (SLC9A1, OPCML and UBAP) and 3 down-regulated genes (COX6C, SERPINB1 and CCL22) to measure their expression levels by semiquantitative RT-PCR. To obtain truly comparable results, we used the unamplified total RNA (from the same batch used for array hybridizations) as the template. The results were very similar to the microarray data on these genes.

To confirm our data at the protein level, we performed immunohistochemical analysis of OPCML. The results were in parallel with the differential expression pattern detected by cDNA microarray and RT-PCR (Figure 2).

Overall, the above results demonstrated that the samples obtained by P27-based amplification well reflected the status of the original RNA in a proportional manner, and supported the reliability of our expression data (Figure 3).

### *Expression of differential genes in progression of gastric cancer*

By semiquantitative RT-PCR, we further evaluated the expression levels of 25 selected genes, including 10 up-regulated and 15 down-regulated genes, in microdissected normal gastric epithelial cells from 30 healthy individuals, primary gastric cancer cells from 36 patients and lymph node metastatic cells from the remaining 22 patients. First, we investigated the expression levels of these genes in primary gastric cancer and lymph node metastatic cells. The results showed that the expression of 12 genes had the same pattern in > 50% (12/22) patients as revealed by cDNA microarray.

We further measured the expression levels of these 12 genes in paired normal gastric epithelium and primary carcinoma samples from the same 22 patients. We found that the expression pattern of 3 genes (OPCML, RNASE1 and YES1) was similar to that of tumor suppressor genes in > 50% (8/15) patients. For example, the expression level of these genes was the highest in normal gastric epithelium, which was decreased in primary carcinoma and further decreased compared to that in primary gastric cancer patients with lymph node metastasis and normal controls ($P < 0.05$). Meanwhile, the expression level of ACK1 demonstrated the opposite tendency, the pattern of which was similar to that of oncogene in > 50% (12/22) patients (Figure 4). The expression pattern of OPCML was also confirmed at the protein level by immunohistochemical staining (Figure 2).

## DISCUSSION

cDNA microarrays allow an effective investigation of functional genomics. However, the existence of bulky surrounding cells produces much useless noise information because of its high sensitivity[9,10]. Therefore, selection of cancer cells using LMD is of indispensable value in combination with the cDNA microarray. The LMD system used in this study integrates a UV laser of 337 nm wavelength with an upright microscope. The ultraviolet laser microbeam causes dissection by local photolysis of the supporter membrane and tissue section due to the high photon density of the microbeam rather than by local heating or coagulation. The cut sample falls down into PCR tubes placed underneath by gravity without any mechanical contact or further destroying energy and the integrity of the extracted mRNA is maximally kept.

Metastasis of cancer is a highly selective sequential step which favors the survival of a subpopulation of metastatic cells preexisting within the primary tumor mass to produce clinically relevant metastases. The metastatic cells exhibit a complex phenotype that is regulated by transient or permanent changes in different genes at the DNA and/or mRNA level(s). This has also been proved in gastric cancer by other researchers[8,9,11].

Differential expression profiles of laser microdissected primary gastric cancer cells and lymph node metastatic cells were discovered using cDNA microarray consisting of 13824 genes, demonstrating that analysis of gene-expression profiles can be performed using LMD, P27-based RNA amplification and cDNA microarray[1,11]. Although the majority of these genes have been implicated in various aspects of tumor biology, few are associated with gastric cancer.

Among the above genes, some may be differentially expressed because of different living environments[5,12-17]. Therefore, to further confirm and screen the results of cDNA microarray, we measured the expression levels of 25 selected genes in 22 patients by semiquantitative RT-PCR. These target cells were collected by LMD, and the normal gastric epithelium was included. As a result, we identified 4 genes, the expression level of which was different not only between primary carcinoma and metastatic lymph nodes (the same results as cDNA microarray), but also between normal gastric epithelium and primary tumor, suggesting that these four genes play a key role in the tumorigenesis and metastasis of gastric cancer. The expression pattern of 3 genes (OPCML, RNASE1 and YES1) is similar to that of tumor suppressor genes. For example, the expression of these genes is the highest in normal gastric epithelium, which is decreased in primary carcinoma, and further decreased in metastatic lymph nodes. OPCML encodes a member of the IgLON subfamily in the immunoglobulin protein superfamily and acts as a GPI-anchored protein. It was reported that OPCML has tumor-suppressor function in epithelial ovarian cancer[11,18-20], which is in accordance with our findings in gastric cancer. Interestingly, YES1 is the cellular homolog of a virus oncogene associated with esophageal tumorigenesis[12,21-24], which is contrary to our results. The RNAS gene encodes a member of the pancreatic-type of secretory ribonuclease, a subset of the ribonuclease A superfamily, and has no relationship with human cancers.

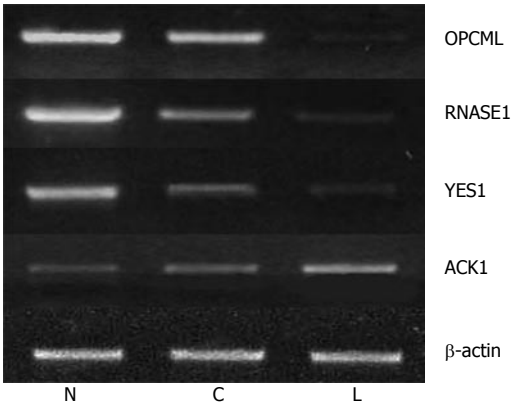


**Figure 4** Identification of differentially expressed genes in progrssive gastric cancer by semiquantitative RT-PCR. N: A sample from normal gastric epithelium; C: Signifies a sample from the primary carcinoma cells; and L: A sample from the metastatic carcinoma cells.

In this study, the expression pattern of gene ACK1 was different from that of the above mentioned genes, but similar to that of oncogenes. ACK1 encodes a tyrosine kinase that binds to Cdc42Hs in its GTP-bound form and inhibits intrinsic and GTPase-activating protein (GAP)-stimulated GTPase activity of Cdc42Hs. It is directly linked to a tyrosine phosphorylation signal transduction pathway, but its effect on tumor progression has not been reported[12-17].

Both tumor suppressor gene and oncogene are important target molecules in clinical diagnosis and treatment of malignant gastric tumors. The different expressions of these four genes have not been reported during the progression of gastric cancer.

In conclusion, analysis of gene expression profiles by LMD, P27-based amplification, and cDNA microarray can provide useful information for clarifying the mechanism underlying the development and metastasis of gastric cancer[25-29], not only revealing the differentially expressed genes in progression of gastric cancer, but also providing information for identifying novel diagnostic and therapeutic targets.

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# Efficacy and safety of thalidomide in patients with hepatocellular carcinoma

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## Abstract

**AIM:** To evaluate which patients with hepatocellular carcinoma (HCC) are most likely to respond to thalidomide treatment.

**METHODS:** From July 2002 to July 2004, patients with HCC who received thalidomide treatment, were enrolled. We extracted relevant data from the patients' medical records, including history and type of hepatitis, comorbidity, serum α-fetoprotein (α-FP) level, volumetric changes in tumor, length of survival, and the dose, duration, side effects of thalidomide treatment. The tumor response was evaluated. On the basis of these data, the patients were divided into two groups: those with either partial response or stable disease (PR + SD group) and those with progressive disease (PD group).

**RESULTS:** Two of 42 (5%) patients had a partial tumor response after treatment with thalidomide, 200 mg/d, and 9 (21%) had stable disease. Patients in the PR + SD group all had cirrhosis. Comparing patients with and without cirrhosis, the former were more likely to respond to thalidomide therapy (PR + SD: 100% *vs* PD: 64.5%, $P = 0.041 < 0.05$). Thalidomide was significantly more likely to be effective in tumors smaller than 5 cm (PR + SD: 63.6% *vs* PD: 25.8%, $P = 0.034 < 0.05$). Compared with patients with progressive disease (PD), patients in the PR + SD group had a higher total dose of thalidomide (13669.4 ± 8446.0 mg *vs* 22022.7 ± 11461.4 mg, $P = 0.023 < 0.05$) and a longer survival (181.0 ± 107.1 d *vs* 304.4 ± 167.1 d, $P = 0.047 < 0.05$). Patients with comorbid disease had a significantly greater incidence of adverse reactions than those without (93.8% *vs* 60.0%, $P = 0.021 < 0.05$). The average number of adverse reactions in each person with a comorbid condition was twice as high as in those without other diseases (2.2 ± 1.3 *vs* 1.1 ± 1.2; $P = 0.022 < 0.05$).

**CONCLUSION:** Thalidomide therapy is most likely to be effective in patients with early stage small HCC, especially in those with other underlying diseases. A low dose (200 mg/d) of thalidomide is recommended to continue the treatment long enough to make it more effective.



**Key words:** Thalidomide; Hepatocellular carcinoma; Antiangiogenic agents; Adverse events

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## INTRODUCTION

Hepatocellular carcinoma (HCC) is the fifth most prevalent cancer in the world. About one million people die of it each year[1,2]. Particularly in Taiwan, it poses a tremendous threat to citizens' health and lives. HCC has been the leading cause of cancer death in Taiwan for the past two years, leading to about 6000 to 7000 deaths each year.

Potentially curative therapies for HCC include surgical resection, liver transplantation, and percutaneous ablation. However, HCC is often diagnosed at an advanced stage when curative therapy is likely to be of little value. Thus, most patients are candidates for palliative treatment, which includes transarterial embolization (with or without chemotherapy), hormonal therapy, interferon therapy, *etc.* Transarterial embolization has been shown to improve survival[1]. The response rate to systemic chemotherapy is generally less than 30%[1], and patients often tolerate it poorly. A further difficulty is that about 80% of patients with HCC also have cirrhosis, which severely limits the utility of both surgery and chemotherapy. Therefore, we urgently need new therapeutic approaches for HCC.

In 1971, Dr. Judah Folkman suggested using anti-angiogenic agents to inhibit tumors, a proposal which has made a new form of anticancer treatment possible. Cancer cells whose blood supply is disrupted are starved of nutrients and oxygen, leading to atrophy and death. Endothelial cells play a key role in angiogenesis, forming the external theca of new blood vessels and possessing a strong ability to reproduce and migrate. Proteins known to activate endothelial cells include vascular endothelial growth factor (VEGF) and basic fibroblast growth factor

(BFGF), which are regarded as important contributors to the growth of new blood vessels. HCC is a hypervascular tumor, with high concentrations of VEGF and BFGF in tumor cells and blood[3,4]. It is therefore reasonable to infer that anti-angiogenesis may inhibit the growth of HCC.

A number of anti-angiogenic agents have been developed, some of which are in phase Ⅱ or Ⅲ clinical trials. Thalidomide is such a drug. It was widely used in the 1950s as a sedative and anti-emetic agent but was found to be teratogenic. Its use has been therefore prohibited in Europe and America. However, in recent years, thalidomide has been found to have many pharmacological properties, including inhibition of angiogenesis and inflammation and regulation of immunity. Thalidomide apparently blocks factors such as VEGF and BFGF of cancer cells[5,6], thereby inhibiting angiogenesis. In some cancers strongly dependent on angiogenesis, such as Kaposi's sarcoma and metastatic prostate cancer, thalidomide has been used with some success[7,8]. HCC, also strongly dependent on angiogenesis, is treated with thalidomide in Taiwan.

As with any newly proposed therapy, it is important to identify those patients most likely to benefit from the treatment while minimizing the risk of adverse effects. This understanding may also facilitate conservation of medical resources when the drug is unlikely to help. We designed this study to evaluate the efficacy and adverse effects of thalidomide in HCC by retrospectively reviewing the results in a series of patients in order to identify those most likely to benefit from the treatment while avoiding serious adverse effects.

## MATERIALS AND METHODS

### Patients

From July 2002 to July 2004, 159 patients with HCC were treated with thalidomide. All the patients were informed of the necessity of contraception and the benefits and risks of thalidomide treatment. They gave their informed consent to receive it by the Medicine Advisory Committee of Mackay Memorial Medical Center and the Department of Public Health in Taiwan. HCC was diagnosed by histologic examination, imaging findings (abdominal sonography and computed tomography), or a serum α-fetoprotein (α-FP) level greater than 400 ng/mL. The disease was staged by the pathologic tumor-node-metastasis (pTNM) system, the Okuda system for HCC, and the Cancer of the Liver Italian Program (CLIP) scoring system[9-11]. We extracted relevant data from the patients' medical records, including age, gender, history and type of hepatitis, comorbidity, duration of HCC, serum α-FP level, volumetric changes in tumor, length of survival, and the dose, duration, side effects of thalidomide treatment. Patients were excluded from analysis if they took thalidomide for less than one month, had other therapy after thalidomide, or if the data were inadequate in the record to define the stage or treatment response.

### Evaluation of tumor response

The tumor response was evaluated according to the imaging findings one month before and after administration of thalidomide according to the new version of the Response Evaluation Criteria in Solid Tumor (RECIST) Guidelines[12], as well as α-FP levels before and after thalidomide treatment. On the basis of these data, the patients were divided into two groups: those with either partial response or stable disease (PR + SD group) and those with progressive disease (PD group).

### Thalidomide therapy

Thalidomide (50 mg tablet marketed as Thado®, Taiwan Tung Yang Biopharm Co. Ltd., Taipei) in a dose of 50 to 200 mg/d, was give twice daily in the morning and evening. The dose was adjusted according to the clinical response and side effects. Thalidomide-related toxicity was classified by the Common Terminology Criteria for Adverse Events v 3.0 (CTCAE) established by the National Cancer Institute (NCI)[13].

### Statistical analysis

The clinical characteristics, dose of thalidomide, treatment duration, and length of survival of the two groups were compared by using the Mann Whitney *U* test and Fisher's exact test. The relation between comorbidity and adverse reactions was examined by Fisher's exact test. $P < 0.05$ was considered statistically significant. The overall survival was calculated by the Kaplan-Meier method from the time of the first dose of thalidomide to the patient's death or the last follow-up. The statistical software package used was SPSS (version 10).

## RESULTS

### Patients

Of the 159 patients with HCC treated with thalidomide, 57 took thalidomide for less than one month, and 35 were given other treatment in addition to thalidomide. The data for 25 patients were incomplete, leaving records for 42 patients that were available for analysis. The clinical characteristics are shown in Table 1. Thirty-one (74%) patients were older than 60 years. Cirrhosis was present in 31, and 32 (76%) had comorbid conditions. The prevalence of hepatitis B (15/42) and C (11/42) was similar. Twenty-one (50%) of the patients had TNM stage ⅢA, and the majority had their liver function classified as Child-Pugh A or Okuda Ⅱ. The most frequent CLIP score was 3 (15/42).

### Tumor Response

Of the 42 patients, 2 had a partial tumor response to thalidomide at a dose of 200mg/day and a decrease in α-FP level. They both had TNM stage ⅢA disease, Okuda phase Ⅰ or Ⅱ, and a CLIP score of 3 or 4. Another 9 patients had stable disease, 4 of whom also had a fall in serum α-FP level. The remaining 31 patients had disease that continued to progress after thalidomide. These results were comparable to those of other studies[14-19] (Table 2), none of which showed an objective tumor response rate above 10%. The mean time to partial response in our patients was 34 d. The median survival of all 42 patients was 319 d (range, 144 to 494 d), the one-year survival was 14%. The median survival of the PR + SD group was 524 d (range, 218 to 830 d) (Figure 1).

**Table 1 Clinical characteristics of 42 patients with HCC**

| Characteristic | *n* | yr | % |
|---|---|---|---|
| Age | | | |
| Median | | 67 | |
| Range | | 32-84 | |
| Sex | | | |
| Male | 11 | | 26 |
| Female | 31 | | 74 |
| Comorbidity | | | |
| Absent | 10 | | 24 |
| Present | 32 | | 76 |
| Cirrhosis | | | |
| Absent | 11 | | 26 |
| Present | 31 | | 74 |
| Type of hepatitis | | | |
| Hepatitis B | 15 | | 36 |
| Hepatitis C | 11 | | 26 |
| Hepatitis B + hepatitis C | 1 | | 2 |
| Hepatitis B + hepatitis D | 1 | | 2 |
| No record | 14 | | 33 |
| TNM stage | | | |
| Ⅰ | 2 | | 5 |
| Ⅱ | 6 | | 14 |
| ⅢA | 21 | | 50 |
| ⅢB | 2 | | 5 |
| ⅣA | 7 | | 17 |
| ⅣB | 4 | | 10 |
| Child-Pugh classification | | | |
| Grade A | 25 | | 60 |
| Grade B | 14 | | 33 |
| Grade C | 3 | | 7 |
| Okuda stage | | | |
| Ⅰ | 11 | | 26 |
| Ⅱ | 27 | | 64 |
| Ⅲ | 4 | | 10 |
| CLIP score | | | |
| 0 | 2 | | 5 |
| 1 | 10 | | 24 |
| 2 | 7 | | 17 |
| 3 | 15 | | 36 |
| 4 | 4 | | 10 |
| 5 | 4 | | 10 |
| 6 | 0 | | 0 |
| α-fetoprotein | | | |
| > 400 ng/mL | 21 | | 50 |
| ⩽ 400 ng/mL | 21 | | 50 |
| Pre thalidomide therapy | | | |
| TAE[1] | 26 | | 62 |
| PEI + TAE[1] | 3 | | 7 |
| Radiation + TAE[1] | 2 | | 5 |
| Surgery + PEI[2] | 1 | | 2 |
| Chemotherapy | 1 | | 2 |
| No therapy | 9 | | 21 |

[1]Transarterial embolization; [2]Percutaneous ethanol injection.

**Table 2 Response rates of HCC to thalidomide**

| Author | *n* | Tumor response, *n* (%) | | | | |
|---|---|---|---|---|---|---|
| | | CR[1] | PR[2] | SD[3] | PD[4] | CR[1] + PR[2] + SD[3] |
| Kong *et al*[14] | 11 | 0 | 1 (9) | 4 (36) | 6 (55) | 5 (46) |
| Lin *at al*[15] | 27 | 0 | 1 (4) | 1 (4) | 25 (93) | 2 (7) |
| Feun *et al*[16] | 7 | 0 | 0 | 0 | 7 (100) | 0 |
| Schwartz *et al*[17] | 30 | 1 (3) | 1 (3) | 9 (30) | 19 (63) | 11 (37) |
| Hsu *et al*[18] | 63 | 1 (2) | 3 (5) | 20 (32) | 39 (62) | 24 (38) |
| Wang *et al*[19] | 99 | 0 | 6 (6) | - | - - | |
| Chiou *et al* | 42 | 0 | 2 (5) | 9 (21) | 31 (74) | 11 (26) |

[1]Complete response; [2]Partial response; [3]Stable disease; [4]Progressive disease.

**Table 3 Comparison of patients with partial response or stable disease with patients with progressive disease**

| Characteristic | PR[1] + SD[2] *n* | PD[3] *n* | *P* |
|---|---|---|---|
| Sex | | | 1.0 |
| Male | 8 | 23 | |
| Female | 3 | 8 | |
| Age | | | 0.234 |
| > 60 yr | 10 | 21 | |
| ⩽ 60 yr | 1 | 10 | |
| Cirrhosis | | | 0.041[a] |
| Absent | 0 | 11 | |
| Present | 11 | 20 | |
| Hepatitis | | | |
| Hepatitis B | 4 | 13 | 1.0 |
| Hepatitis C | 3 | 9 | 1.0 |
| TNM stage | | | |
| Ⅰ + Ⅱ | 3 | 4 | 1.0 |
| ⅢA + ⅢB | 4 | 19 | 0.180 |
| ⅣA + ⅣB | 4 | 8 | 1.0 |
| Child-Pugh classification | | | |
| Grade A | 5 | 21 | 0.280 |
| Grade B | 6 | 8 | 0.136 |
| Grade C | 0 | 2 | 1.0 |
| Okuda stage | | | |
| Ⅰ | 3 | 8 | 1.0 |
| Ⅱ | 8 | 19 | 0.717 |
| Ⅲ | 0 | 4 | 0.558 |
| CLIP score | | | |
| 0-2 | 7 | 13 | 0.298 |
| 3-6 | 4 | 18 | 0.298 |
| α-fetoprotein | | | 0.081 |
| > 400 ng/mL | 3 | 19 | |
| < 400 ng/mL | 8 | 12 | |
| Tumor size | | | 0.034[a] |
| ⩽ 5 cm | 7 | 8 | |
| > 5 cm | 4 | 23 | |

[1]Partial response; [2]Stable disease; [3]Progressive disease; [a]$P < 0.05$ between the two groups.

Table 3 shows that all patients in the PR + SD group had cirrhosis, suggesting that cirrhosis might influence the efficacy of thalidomide. Patients in this group were more likely to have a CLIP score of 0 to 2 (7/11) or an α-FP level of < 400 ng/mL (8/11). Response to thalidomide was significantly more likely when the tumor was < 5 cm (PR + SD: 63.6% *vs* PD: 25.8%, $P = 0.034 < 0.05$). Other clinical characteristics did not differ significantly between the groups.

The duration of thalidomide therapy was longer in the PR + SD group (Table 4). Patients in the PR + SD group had a significantly higher total dose and a significantly longer survival than those in the PD group.

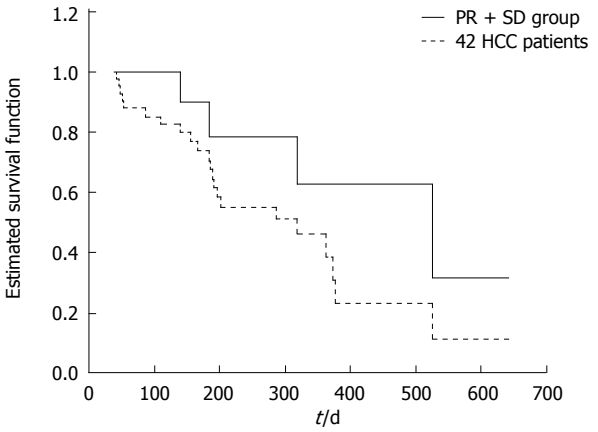


**Figure 1** Survival curves in patients with HCC on thalidomide.

**Table 4 Comparison of thalidomide treatment and survival between the two groups**

| | PR[1] + SD[2] | PD[3] | *P* |
|---|---|---|---|
| Duration (d) | 167.1 ± 98.9 | 107.7 ± 71.7 | 0.069 |
| Total dose (mg) | 22022.7 ± 11461.4 | 13669.4 ± 8446.0 | 0.023[a] |
| Survival (d) | 304.4 ± 167.1 | 181.0 ± 107.1 | 0.047[c] |

[1]Partial response; [2]Stable disease; [3]Progressive disease; [a]$P < 0.05$, [c]$P < 0.05$ *vs* duration, between the two groups.

**Table 5 Thalidomide-related adverse events in patients with HCC**

| | Grade of severity (*n*) | | | | | Total |
|---|---|---|---|---|---|---|
| Adverse event | G[1] | G[2] | G[3] | G[4] | G[5] | *n* (%) |
| Constipation | 2 | 22 | 0 | 0 | 0 | 24 (57) |
| Skin rash | 6 | 8 | 3 | 0 | 0 | 17 (41) |
| Low leg edema | 1 | 10 | 0 | 0 | 0 | 11 (26) |
| Dizziness | 3 | 5 | 1 | 0 | 0 | 9 (21) |
| Lethargy | 3 | 2 | 2 | 0 | 0 | 7 (17) |
| Nausea/vomiting | 1 | 5 | 0 | 0 | 0 | 6 (14) |
| Neurosensory | 3 | 0 | 0 | 0 | 0 | 3 (7) |
| Dyspnea | 0 | 2 | 0 | 0 | 0 | 2 (5) |
| Leukopenia | 1 | 0 | 0 | 0 | 0 | 1 (2) |
| Thrombocytopenic purpura | 0 | 1 | 0 | 0 | 0 | 1 (2) |
| Insomnia | 0 | 1 | 0 | 0 | 0 | 1 (2) |
| Alopecia | 1 | 0 | 0 | 0 | 0 | 1 (2) |

[1]mild adverse event; [2]Moderate adverse event; [3]Severe adverse event; [4]Life-threatening or disabling adverse event; [5]Death related to adverse event.

### *Thalidomide therapy*

Twenty patients began their treatment with a dose of 200 mg/d and the remaining 22100-150 mg/d initially. Ten patients had their dose escalated to 200 mg according to treatment response, while three patients had their dose reduced because of thalidomide- induced drowsiness, dizziness and constipation, and 2 discontinued it entirely. Most adverse reactions were slight or moderate, only 6 (14%) events (skin rash in 3, dizziness in 1, and lethargy in 2) were severe. The most common adverse reactions were constipation and skin rash, with an incidence of 57% and 41%, respectively (Table 5).

**Table 6 Thalidomide-related adverse events and comorbidity**

| Adverse drug events per patient | Comorbidity (*n*) | |
|---|---|---|
| | Absent | Present |
| 1 | 3 | 10 |
| 2 | 1 | 7 |
| 3 | 2 | 6 |
| 4 | 0 | 7 |

**Table 7 Correlation between thalidomide-related adverse events and comorbidity**

| | Comorbidity (*n*) | | *P* |
|---|---|---|---|
| | Absent | Present | |
| Without adverse events | 4 | 2 | 0.021 |
| With adverse drug events[a] | 6 | 30 | |
| Adverse events per patient[c] | 1.1 ± 1.2 | 2.2 ± 1.3 | 0.022 |

[a]$P < 0.05$, [c]$P < 0.05$ *vs* without adverse events, between groups of with comorbidity and without comorbidity.

Tables 6 and 7 show the relationship between thalidomide-induced adverse reactions and comorbidity. Adverse reactions were more likely to occur in patients with an other underlying diseases (93.8% *vs* 60.0%, $P = 0.021 < 0.05$). The average number of adverse reactions in each person with a comorbid condition was twice as high as in those without other diseases (2.2 ± 1.3 *vs* 1.1 ± 1.2, $P = 0.022 < 0.05$).

## DISCUSSION

In our series, the partial response rate of HCC to thalidomide at 100 to 200 mg/d was 5%, 21% of patients who had stabilization of their disease after treatment with thalidomide. These results generally agree with those of other researchers. In our study, two thirds of the patients were older than 60 and had cirrhosis or other comorbid conditions. Most of them had TNM stage ⅢA disease or were in Okuda class Ⅱ.

The pharmacological mechanisms of thalidomide include anti-angiogenic, immunomodulatory and anti-inflammatory effects. The drug inhibits angiogenesis in tumors by blocking the activity of VEGF and BFGF[20]. The immunomodulatory effects include induction of TNF-α mRNA degradation, thereby inhibiting the synthesis of TNF-α[21]. It also downregulates lymphocyte surface molecules, lowering the CD4:CD8 peripheral lymphocyte ratio. It can inhibit chemotaxis of neutrophils, interleukin-6 and interleukin-12, and stimulate the synthesis of interleukin-2[22]. In addition, it inhibits the activity of IκB kinase, thus blocking the activity of nuclear factor-κB[23].

Because of its many pharmacological actions, thalidomide is now widely used in the treatment of a number of diseases. In 1999, it was approved by the FDA to treat erythema nodosum leprosum. It has been used for aphthous ulcers in HIV-positive patients, graft versus

host disease after bone marrow grafting, tuberculosis, sarcoidosis, inflammatory bowel disease, rheumatoid arthritis, as well as for some cancers such as multiple myeloma, renal cell carcinoma, brain tumors, prostate cancer, melanoma and Kaposi sarcoma[19,23]. The response of multiple myelomas to thalidomide (600 to 800 mg daily) is about 30%, and increases to 60% when combined with dexamethasone. The therapeutic effect of thalidomide in HCC is thus not as satisfactory as in multiple myeloma. The reported response is 40% in Kaposi sarcoma and 15%-27% in prostate cancer[23].

In our study, the plasma concentrations of VEGF and BFGF in multiple myeloma remained unchanged irrespective of whether the disease responded to thalidomide. It is likely that there are mechanisms other than the inhibition of angiogenesis that are responsible for the drug's efficacy in multiple myeloma. For example, there are decreased adhesion of multiple myeloma cells to stromal cells, reduced secretion of cytokines, and activation of caspase-8, leading to death of multiple myeloma cells[24].

Because of the different structures and functions of blood vessels in different tissues and tumors at different sites and the genetic instability and biologic heterogeneity of tumor cells, new blood vessels in tumors may be quite diverse. There are therefore considerable complexity and unpredictability of tumor microvasculature[25]. This may help explain why thalidomide has different effects on different malignancies. The relatively poor response of HCC compared to multiple myeloma may be related to the differences in angiogenesis.

In our series, only about one fifth of the patients had their disease stabilized after thalidomide therapy, indicating that thalidomide helps only a few patients with advanced HCC, and rarely results in a decrease in tumor size. The median survival in the PR + SD group was 524 d (range, 218-830 d) compared with 319 d (range, 144-494 d) of all 42 patients. The one-year survival was only 14%, suggesting that thalidomide is not very effective in prolonging the life of HCC patients.

Our results did show that patients with cirrhosis tolerated thalidomide well and even had a somewhat better response to treatment. The reason why response in those with tumors smaller than 5 cm is better may be that thalidomide can prominently inhibit formation of new blood vessels rather than disrupt existing mature vessels. Larger tumors with mature vasculature may therefore be relatively resistant to the action of thalidomide. The maximum therapeutic effect may thus be seen in smaller tumors with newer vessels.

In the PR + SD group 64% of patients had a CLIP score of 0 to 2, compared with 42% in the PD group. Similarly more patients in the PR + SD group (73%) had an α-FP level of < 400 ng/mL compared with the PD group (39%). The PR + SD group also took a significantly higher total dose of thalidomide (22022.7 ± 11461.4 mg *vs* 13 669.4 ± 8446.0 mg, $P = 0.023$) and had a significantly longer survival (304.4 ± 167.1 d *vs* 181.0 ± 107.1 d; $P = 0.047$). These results indicate that treatment of earlier stage HCC with thalidomide may leave enough time for the drug to produce a significant therapeutic effect.

A commonly-reported adverse effect in patients treated with thalidomide for multiple myeloma is venous thromboembolism, particularly when combined with doxorubicin or with dexamethasone[27]. None of the patients in our series had thromboembolism, perhaps because we used smaller doses than the commonly-used doses for multiple myeloma. The most common adverse reactions in our series were constipation (57%) and skin rash (41%). Severe reactions were uncommon (14%), and only 5 patients required a dose adjustment or discontinuation of treatment. Three patients developed slight peripheral neuropathy, and one of them had a dose adjustment from 200 mg to 100 mg. Thalidomide-related peripheral neuropathy may occur in patients on long-term treatment and is irreversible. Therefore, if it occurs, the drug should be stopped or the dose is reduced. Generally, the adverse reactions caused by thalidomide are dose- and duration-related[27]. We also found a significant positive correlation between adverse reactions and comorbidity ($P = 0.021$) of diabetes mellitus, hypertension, ischemic heart disease, peptic ulcer, chronic obstructive lung disease and benign prostatic hyperplasia. In considering therapy for patients with HCC, the higher risk of adverse reactions to thalidomide in the presence of other diseases should be taken into account.

Our study is limited by the fact that it was retrospective. It would be difficult to collect enough patients with HCC in similar stages for comparison in a prospective study. We also were only able to analyze the data from a relatively small number of patients, which might skew the results to some extent. However, thalidomide is unlikely to be effective in a large proportion of patients with HCC. This makes it even more important to identify those who are likely to benefit from thalidomide treatment.

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# Proposal of a new and simple staging system of colorectal liver metastasis

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## Abstract

**AIM:** To create a new, simple and useful staging system for colorectal liver metastasis analogous to the Tumor Node Metastasis classification system of International Union Against Cancer.

**METHODS:** A retrospective review was undertaken of 81 consecutive patients who underwent partial hepatectomy for colorectal liver metastases (group 1). Clinical and pathological features of both primary and metastatic liver cancers were entered into a multivariate analysis to determine independent variables helpful in accurately predicting long-term prognosis after hepatectomy. Using selected variables, we created a new staging system like TNM classification. The usefulness of the new staging system was examined in a series of 92 patients from another hospital (group 2).

**RESULTS:** Multivariate analysis showed that 81 patients in group 1 had significant multiple hepatic tumors with the largest tumor being more than 5 cm in diameter, resectable extrahepatic distant metastases, and independent prognostic factors for poor survival after hepatectomy. Using these three variables, we created a new staging system to classify patients with colorectal liver metastases. Finally, our new staging system classified the patients both in group 1 and in group 2.

**CONCLUSION:** Our new staging system of colorectal liver metastasis is simple and useful for staging patients.



**Key words:** Colorectal liver metastasis; Staging system; Prognostic factor

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## INTRODUCTION

It is well accepted that hepatic resection of colorectal liver metastases is a beneficial clinical procedure in that it reportedly improves 5-year survival rates of affected patients by as high as 30%-50% following curative hepatic resection[1-14]. Although this procedure is performed worldwide by a number of liver surgeons, it is often hazardous to compare results from different studies due to the fact that there is no universally accepted classification system for staging colorectal hepatic metastatic diseases. Several recent papers have offered classification systems based on a variety of variables of colorectal liver metastasis[1-6], including the number of metastatic nodules[1,4-6], the size of metastases[4,6], the sites of unilobar or bilobar involvement[1,4], the extent of liver involvement ($\leqslant$ 25%, and $\leqslant$ 50%)[1,3], the chronology of synchronous or metachronous disease[1,5,6], the invasion to major vessels or bile ducts[2], the presence of extrahepatic metastasis[2-4], the performance status and serum alkaline phosphatase[3]. However, it is uncertain whether these classification systems are accepted and adopted outside the confines of proposing institutions. In the present study, we aimed to create a new, simple and useful classification system for colorectal liver metastases analogous to the Tumor Node Metastasis (TNM) classification system of International Union Against Cancer (UICC).

## MATERIALS AND METHODS

### Patients

**Group 1:** Between January 1, 1981 and March 31, 1997, 83 consecutive patients underwent partial hepatectomy for colorectal liver metastases at the First Department of Surgery (presently the Department of Surgical Oncology), Tokyo University Hospital. During the post-operative period, two patients died in the hospital. One died of secondary aspiration pneumonia and the other died of severe intra-abdominal sepsis (mortality rate: 3.1%). The remaining 81 patients were followed up either until death or their last outpatient visit up to December 31, 2002. The follow-up period ranged from 4 to 197 mo with a median of 53.0 mo. The demographic characteristics and tumor-

related features, which were statistically analyzed later, are summarized in Table 1.

**Group 2:** Between January 1, 1989 and December 31, 2003, 95 consecutive patients underwent partial hepatectomy for colorectal liver metastases at the Second Department of Surgery, Teikyo University Hospital. During the post-operative period, three patients died in the hospital. Two died of hepatic failure due to massive hepatectomy and the other died of severe intra-abdominal sepsis (mortality rate: 3.2%). The remaining 92 patients were followed up either until death or their last outpatient visit up to December 31, 2004. The follow-up period ranged from 4 to 110 mo with a median of 39.0 mo.

Every hepatectomy was considered curative since surgeons were confident of the complete macroscopic resection of hepatic tumors at the time of surgery. Hepatectomy was performed even in the presence of extrahepatic metastases if surgeons were reasonably assured of the complete macroscopic resection of extrahepatic metastases as well. After discharge from the hospital, the patients were closely monitored either at the outpatient clinic or at the affiliated institutions. Measurement of serum carcinoembryonic antigen (CEA) levels and ultrasonography were performed during post-operative visits at least once every two months in an effort to detect early recurrence. In addition, computed tomography was performed approximately twice a year. Almost all cases of cancer recurrence were diagnosed by these investigative tests. If the diagnosis was unclear, angiography and/or needle biopsy was performed, under ultrasonic guidance in an effort to confirm or rule out recurrence of the disease.

### Prognostic factors

Eleven factors that were expected to influence the long-term prognosis were evaluated for statistical significance. These factors could only be determined preoperatively or during surgery (therapeutic factors were excluded and not considered). Factors that were considered included gender, age at hepatectomy (< 60 or ≥ 60 years), chronology of synchronous or metachronous hepatic metastases, and post-operative disease-free interval ≤ 1 year, the number of solitary or multiple metastatic nodule hepatic metastases, the maximum diameter of hepatic metastases (≤ 5 cm), unilobar or bilobar hepatic involvement, resectable extrahepatic metastasis (pulmonary metastases, localized peritoneal metastases, or hepatoduodenal lymph node metastases, each was completely resected), serum CEA levels at hepatectomy (less or higher than 10 times the upper level of normal), serosal exposure to the primary colorectal tumor, and regional lymph node metastases of the primary colorectal tumor.

### Statistical analysis

Survival rates after hepatectomy were calculated using data obtained from patients by the Kaplan-Meier method. Only deaths attributable to recurrent cancer were treated as deaths due to disease. Patients who died of secondary or other causes without recurrence were treated as censored.

Prognostic variables concerned with cancer-related survival rate were entered into multivariate analysis. The Cox stepwise analysis proportional hazard regression model was used to select independent and significant prognostic variables. Stepwise variable selection was performed at a value of $P < 0.20$ level of significance. $P < 0.05$ was considered statistically significant.

**Table 1 Prognostic factors entered into multivariate analysis based on proposed clinical and histopathological features**

| Variable | Patients (*n*) |
|---|---|
| Gender (male/female) | 61/20 |
| Age at hepatectomy (< 60/≥ 60) | 32/49 |
| Chronology of hepatic metastasis (synchronous/metachronous) | 41/40 |
| Disease free interval after colectomy (≤ 1 yr/> 1 yr) | 56/25 |
| Extrahepatic distant metastases (no/yes) | 72/9 |
| CEA (≤ 10 times of normal value/> 10 times of normal value) | 57/24 |
| Primary lesion | |
| Depth of invasion (up to subserosa/more) | 57/24 |
| Lymphnodemetastasi (no/yes) | 39/42 |
| Hepatic metastasis | |
| Number (single/multiple) | 45/36 |
| Maximum diameter (≤ 5 cm/> 5 cm) | 59/22 |
| Lobe involved (unilobar/bilobar) | 64/17 |

## RESULTS

### Group 1

**Significant prognostic factors:** The overall 1-, 2-, 3-, 4-, and 5-year cancer-related survival rates after surgical resection in the 81 patients were 89.6%, 67.7%, 56.0%, 54.2%, and 49.6%, respectively (Figure 1A). The results of the multivariate analysis of the variables expected to influence cancer-related survival rate after surgical resection are provided in Table 1. Only variables selected by the stepwise analysis at a value of $P < 0.20$ level of significance, using the Cox proportional hazard regression model, are also shown in Table 2. Multiple tumors, tumor over 5 cm in diameter, and resectable extrahepatic metastases were significant and independent variables influenced cancer-related survival rate ($P < 0.05$). On the other hand, serosal exposure and regional lymph node metastases of the primary colorectal tumor, and recurrent hepatic metastases within one year after resection of the primary colorectal cancer including synchronous hepatic metastases, were the factors selected by stepwise analysis as the possible indication of poor prognosis, but they were not statistically significant ($P > 0.05$ or $P < 0.20$).

**Classification of patients and survival:** In devising our classification system, we considered three variables selected by the multivariate analysis, including the number and size of hepatic metastases (H-factor), and the presence of extrahepatic metastases (M-factor), which were resected completely. Results were shown as follows: H1: single metastasis with diameter ≤ 5 cm; H2: single metastasis with diameter > 5 cm or multiple metastases with diameter ≤ 5 cm; H3: multiple metastases with diameter > 5 cm; M0: extrahepatic metastasis (-); M1: extrahepatic metastasis (+, resectable). Staging system (A): stage I: H1 and M0; stage Ⅱ: H2 and M0; stage Ⅲ: H3 and M0; stage Ⅳ: H1-3 and M1; staging system (B): stage I: H1&M0; stage Ⅱ: H1 and M1 or H2 and M0; stage Ⅲ: H2 and M1 or H3 and M0; stage Ⅳ: H3 and M1

**Table 2 Regression statistics for the stepwise cox proportional hazard model[1]**

| Variable | Parameter | *P* | Hazard Ratio (95% CI) |
|---|---|---|---|
| Diameter > 5 cm | 1.35657 | 0.0013 | 3.883 (1.703-8.852) |
| Extrahep. Met (+). | 1.19430 | 0.0133 | 3.301 (1.282-8.502) |
| Number ⩾ 2 | 0.85412 | 0.0265 | 2.349 (1.105-4.997) |
| D.F.interval < 1 yr | 0.67838 | 0.1602 | 1.971 (0.765-5.080) |
| *n* (+) of primary | 0.60034 | 0.1352 | 1.823 (0.829-4.007) |
| ⩾ se (+) of primary | 0.54877 | 0.1676 | 1.731 (0.794-3.774) |

[1]Only those variables selected by the stepwise analysis, at the *P*-value of 0.20 level of significance, are shown. *n* = 81 (Tokyo Univ. 1981-1997). Age ⩾ 60, gender, synchronous, bilobar invasion, CEA; *P* > 0.2.

**Staging system (A):** The survival curves of patients in group 1 based on the staging system (A) are shown in Figure 1B. They were statistically significant ($P = 0.0057$). However, the survival curve of patients with stage Ⅳ cancer seemed to be better than that of those with stage Ⅲ cancer. The 5-year survival rates of patients with cancer in stage I ($n = 26$), stage Ⅱ ($n = 38$), stage Ⅲ ($n = 8$), and stage Ⅳ ($n = 9$) were 74.8%, 49.2%, 15.6%, and 25.0%, respectively. Their median survival time was 52, 18 and 19 mo, respectively, with that unsettled in stage I patients.

**Staging system (B):** The survival curves of patients in group 1 based on the staging system (B) are shown in Figure 1C, and the findings were statistically significant ($P = 0.0003$). The 5-year survival rates of patients with cancer in stage I ($n = 26$), stage Ⅱ ($n = 43$), and stage Ⅲ ($n = 12$) were 74.8%, 49.8%, and 9.5%, respectively. Their median survival time was 52, 52, and 18 mo, respectively. By coincidence, there were no patients with stage Ⅳ disease in this group.

### *Group 2*

The overall 1-, 2-, 3-, 4-, and 5-year cancer-related survival rates after surgical resection in the 92 patients were 82.2%, 65.2%, 51.6%, 42.5%, and 40.0%, respectively (Figure 2A).

**Staging system (A):** The survival curves of 92 patients in group 2 based on the staging system (A) are shown in Figure 2B. They were statistically significant ($P < 0.0001$). However, the survival curve of patients with stage Ⅳ cancer seemed to be better than that of those with stage Ⅲ cancer. The 5-year survival rates of patients with cancer in stage I ($n = 24$), stage Ⅱ ($n = 44$), stage Ⅲ ($n = 8$), and stage Ⅳ ($n = 15$) were 75.0%, 44.5%, 0%, and 0%, respectively. Their median survival time was 46, 13.5, and 20 mo, respectively, with that unsettled in stage I patients.

**Staging system (B):** The survival curves of 92 patients in group 2 based on the staging system (B) are shown in Figure 2C, and findings were statistically significant ($P < 0.0001$). The 5-year survival rates of patients with cancer in stage I ($n = 24$), stage Ⅱ ($n = 48$), stage Ⅲ ($n = 16$), and stage Ⅳ ($n = 3$) were 75.0%, 43.0%, 0%, and 0%, respectively. Their median survival time was 41, 12, and 10 mo, respectively, with that unsettled in stage I patients.

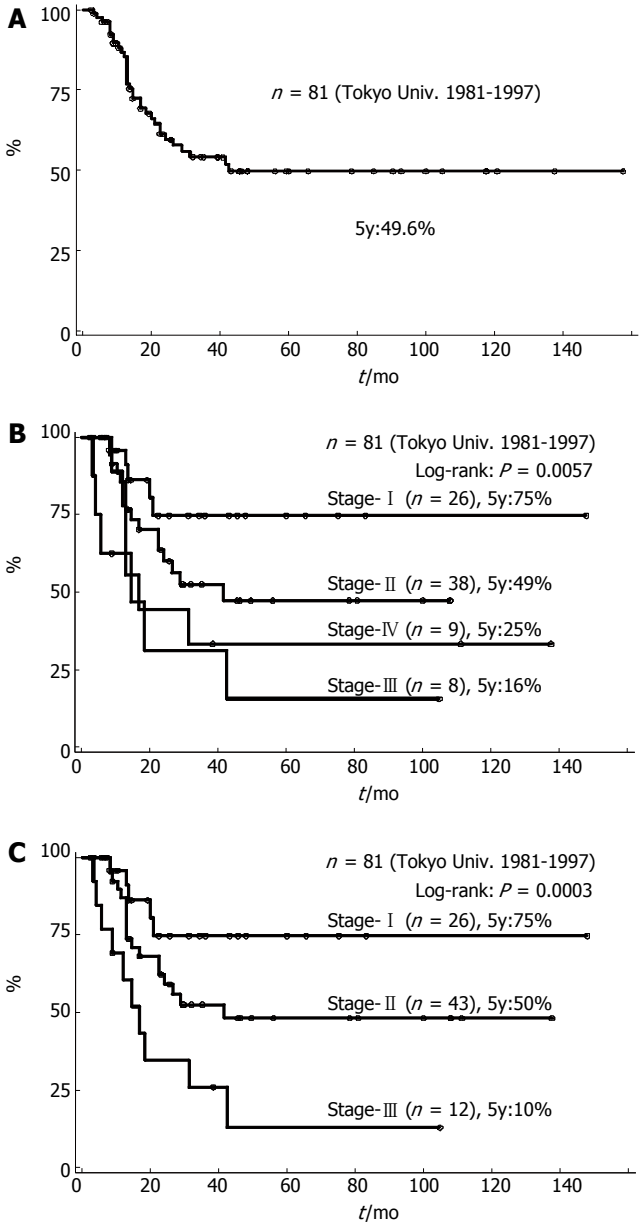


**Figure 1** Kaplan-Meier cancer-related survival curve after hepatic resection for patients with colorectal liver metastases in group 1. **A**: Staging system (A) (log-rank test, $P = 0.0057$); **B**: staging system (B); **C**: stage Ⅰ through sage Ⅲ (log-rank test, $P = 0.0003$ ).

## DISCUSSION

At present, there is no universally accepted classification system for patients with colorectal liver metastasis. Our first step in this investigation was to determine the important clinical and pathological variables that significantly influence prognosis following surgical resection of colorectal liver metastases. Many studies have identified factors which are thought to represent important prognostic determinants, including age at hepatectomy[4,7], sex[23], stage of the primary tumor[2,4,5,7,8,15,20,22,23,28,29] (including regional lymph node metastases and depth of invasion), synchronous or metachronous hepatic metastases[4,7,10,12,14-16,31], the number[4,7,10,11,17,22-25,31] and size[6,7,12,13,15,17,23,26-28] of hepatic metastases, the distribution of hepatic metastases[4,10,20,30], serum carcinoembryonic antigen (CEA) levels[7,11,29,31], extrahepatic metastases[2,8-10,12,14,22,23,26], type of hepatectomy[15], surgical margins[4,9,11,13,15-17,23,24,26], and adjuvant chemotherapy[12,32,33]. Therapeutic factors, such

**Table 3 Our proposal staging system for colorectal liver metastases**

| Staging system |
|---|
| H-factor; |
| H1: Solitary&Diameter ≤ 5 cm |
| H2: Solitary&Diameter > 5 cm/Multiple&Diameter ≤ 5 cm |
| H3: Multiple&Diameter > 5 cm |
| M-factor; |
| M0: Extrahepatic metastasis (-) |
| M1: Extrahepatic metastasis (+): resectable |
| Staging; |
| Ⅰ: H1&M0 |
| Ⅱ: H2&M0/H1&M1 |
| Ⅲ: H3&M0/H2&M1 |
| Ⅳ: H3&M1 |
| [IVb: any H & unresectable extraheptic metastasis (+)] |

as type of hepatectomy, surgical margin, and adjuvant chemotherapy, were excluded in the present statistical analysis, because these therapeutic factors are usually not considered in the classification system. At last three independent and significant determinants, such as multiple tumors, tumor larger than 5 cm in diameter, and macroscopically resected extrahepatic metastasis, were selected in the present study for devising a staging system.

To make a staging system, we considered the number and size of hepatic metastases as hepatic factors (H-factors) while the resected extrahepatic metastases as metastatic factor (M-factor). At first we considered M-factor even if extrahepatic metastases were resected, as an advanced state of disease, e.g. stage Ⅳ in the staging system (A). However, this classification did not seem logical since the prognosis of stage Ⅳ cancer patients was better than that of stage Ⅲ cancer patients. We therefore considered M-factor as one of the risk factors similar to H-factor variable in devising the staging system (B). Following this method, the patients were clearly classified according to their disease stage in group 1 and group 2. This result was reasonable because the risk ratio of M-factor was similar to that of H-factor variables, such as the number and size of hepatic metastases (3.301 *vs* 3.883/2.349) in the present study. Therefore, we propose a new staging system as shown in Table 3. It has not been our policy to perform hepatectomy for patients with unresectable extrahepatic metastases, such as diffuse lung metastases, diffuse peritoneal dissemination, and extensive lymph node metastases. In the present study, we included only the patients with extrahepatic metastases, who underwent hepatectomy as well as complete macroscopic resection of extrahepatic metastases. Therefore, patients with unresectable extrahepatic metastases should be classified as more advanced stages.

In conclusion, we have identified three important and independent prognostic determinants, namely multiple tumors, tumor larger than 5 cm in diameter, and resectable extrahepatic metastasis, using multivariate analysis from a retrospective review of patients who underwent surgical resection for colorectal liver metastases. At last we propose a new and simple staging system with these three determinants to classify the patients according to the long-term outcome after hepatectomy. We hope that it can promote a prospective study on the efficacy of some other therapies such as adjuvant chemotherapy. Although the present study population sample is small, our staging system is simpler and more useful than any other previous classification systems. Further investigation utilizing a larger patient population is necessary.

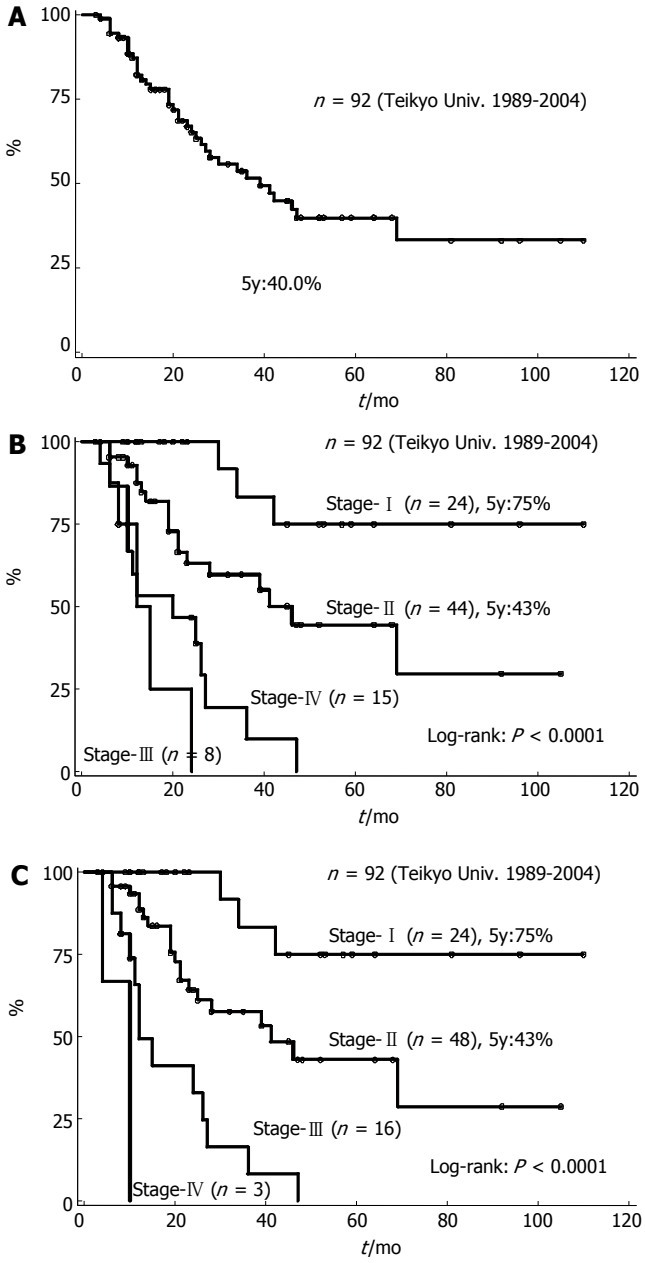


**Figure 2** Kaplan-Meier cancer-related survival curve after hepatic resection for patients with colorectal liver metastases in group 2. **A**: Staging system (log-rank test, $P < 0.0001$); **B**: staging system; **C**: stage Ⅰ through stage Ⅲ (log-rank test, $P < 0.0001$).

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*BASIC RESEARCH*

# Expression patterns and action analysis of genes associated with drug-induced liver diseases during rat liver regeneration

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## Abstract

**AIM:** To study the action of the genes associated with drug-induced liver diseases at the gene transcriptional level during liver regeneration (LR) in rats.

**METHODS:** The genes associated with drug-induced liver diseases were obtained by collecting the data from databases and literature, and the gene expression changes in the regenerating liver were checked by the Rat Genome 230 2.0 array.

**RESULTS:** The initial and total expression numbers of genes occurring in phases of 0.5-4 h after partial hepatectomy (PH), 4-6 h after PH (G0/G1 transition), 6-66 h after PH (cell proliferation), 66-168 h after PH (cell differentiation and structure-function reconstruction) were 21, 3, 9, 2 and 21, 9, 19, 18, respectively. It is illustrated that the associated genes were mainly triggered at the initial stage of LR and worked at different phases. According to their expression similarity, these genes were classified into 5 types: only up-regulated (12 genes), predominantly up-regulated (4 genes), only down-regulated (11 genes), predominantly down-regulated (3 genes), and approximately up-/down-regulated (2 genes). The total times of their up- and down-expression were 130 and 79, respectively, demonstrating that expression of most of the genes was increased during LR, while a few decreased. The cell physiological and biochemical activities during LR were staggered according to the time relevance and were diverse and complicated in gene expression patterns.

**CONCLUSION:** Drug metabolic capacity in regenerating liver was enhanced. Thirty-two genes play important roles during liver regeneration in rats.



## INTRODUCTION

The liver has a very strong capacity to regenerate[1]. Liver cells proliferate rapidly to compensate for lost liver tissues after liver injury or drug stimulus, which is called liver regeneration (LR)[2]. The LR process is usually categorized based on hepatic physiological activities divided into four stages: initiation phase [0.5-4 h after partial hepatectomy (PH)], transition from G0 to G1 (4-6 h after PH), cell proliferation (6-66 h after PH), cell differentiation and reorganization of the structure-function (66-168 h after PH)[3]. The process involves hepatic cell activation, dedifferentiation, proliferation and its regulation, redifferentiation, structure-functional reorganization[4].

Liver is a vital organ of drug metabolism[5]. Disorder of drug metabolism in liver could cause drug-induced liver diseases[6]. It is indicated that 182 genes are associated with drug-induced liver diseases. In addition, there are gene-gene, protein-protein, gene-regulator, and protein-regulator interactions. It is hardly possible to highlight the role of the genes in LR unless gene expression profiles is analyzed with high-throughput[7,8]. Therefore, we used the Rat Genome 230 2.0 array containing 84 genes associated with drug-induced liver diseases to detect gene expression changes after PH, finding that 32 of them were associated with LR, and analyzed these genes expression changes, patterns and actions during LR primarily.

## MATERIALS AND METHODS

### *Regenerating liver preparation*

Healthy SD rats weighing 200-250 g were obtained from the Animal Center of Henan Normal University. The rats were separated into groups at random and each group included 6 rats (male:female = 1:1). PH was performed according to Higgins and Anderson[9], the left and middle

lobes of the liver were removed. Rats were killed by cervical vertebra dislocation at 0.5, 1, 2, 4, 6, 8, 12, 16, 18, 24, 30, 36, 42, 48, 54, 60, 66, 72, 96, 120, 144 and 168 h after PH and the regenerating livers were observed at corresponding time points. The livers were rinsed three times in PBS at 4℃, then 100-200 mg liver from the middle parts of the right lobe, six samples of each group were collected, wrer mixed with 1-2 g (0.1-0.2 g × 6) total liver tissues, and stored at -80℃. The sham-operation (SO) groups were treated the same with the PH group except that the liver lobes were unremoved. The laws of animal protection of China were enforced strictly.

### RNA isolation and purification

Total RNA was isolated from frozen livers according to the manual of the Trizol kit (Invittrogen)[10] and purified based on the RNeasy mini-kit (Qiagen)[11]. Total RNA samples exhibited a 2:1 ratio of 28S to 18S rRNA intensities by agarose electrophoresis (180V, 0.5h). Total RNA concentration and purity were estimated by optical density measurement at 260/280 nm[12].

### cDNA, cRNA synthesis and purification

As a template, 1-8 μg total RNA was used for cDNA synthesis. cDNA and cRNA synthesis was proceeded by the established method of Affymetrix[13]. cRNA labeled with biotin was synthesized using 12 μL of the above synthesized cDNA as the template, and cDNA and cRNA were purified[13]. Concentration, purity and quality of cDNA and cRNA were measured by the same method mentioned above[12].

### cRNA fragmentation and microarray detection

Fifteen μL (1 μg/μL) cRNA incubated with 5 × fragmentation buffer at 94℃ for 35 min was digested into 35-200 bp fragments. The hybridization buffer was added to the prehybridized Rat Genome 230 2.0 microarray produced by Affymetrix, then hybridization was carried out for 16 h at 45℃ on a rotary mixer at 60 rpm. The microarray was washed and stained by GeneChip fluidics station 450 (Affymetrix Inc., USA). The chips were scanned by GeneChip Scan 3000 (Affymetrix Inc., USA), and the signal values of gene expression were observed[14].

### Microarray data analysis

The normalized signal values, signal detections (P, A, M) and experiment/control (Ri) were obtained by quantifying and normalizing the signal values using GCOS1.2[14].

### Normalization of microarray data

To minimize error in the microarray analysis, each analysis was performed three times by Rat Genome 230 2.0 microarray. Results with a total ratio was maximal ($R^m$) and when the average of three housekeeping genes (β-actin, hexokinase and glyceraldehyde-3-phosphate dehydrogenase) approached 1.0 ($R^h$), it was taken as a reference. The modified data were generated by applying a correction factor ($R^m/R^h$) multiplying the ratio of every gene in $R^h$ at each time point. To remove spurious gene expression changes resulting from errors in the microarray analysis, the gene expression profiles at 0-4 h, 6-12 h and 12-24 h after PH were reorganized by NAP software (normalization analysis program) according to the cell cycle progression of the regenerating hepatocytes. Data statistics and cluster analysis were done using GeneMath, GeneSpring, Microsoft Excel software[14-16].

### Identification of genes associated with liver regeneration

The nomenclature of a liver disease (e.g. drug-induced liver diseases) was adopted from the GENEONTOLOGY database (www.geneontology.org), and input into the databases at NCBI (www. ncbi.nlm.nih.gov) and RGD (rgd. mcw.edu) to identify the rat, mouse and human genes associated with the above liver diseases. Then the genes associated with the drug-induced liver diseases were collated. The results of this analysis were codified, and compared with the results obtained for human and mouse searches in order to identify the difference of human and mouse genes from rats. In comparison to these genes with the analysis output of the Rat Genome 230 2.0 array, the genes, showing a greater than twofold change in expression level as meaningful expression changes[17] , were referred to as rat homologous genes or rat specific genes associated with drug-induced liver diseases. Genes, which displayed reproducible results with three independent analyses with the chip and showed a greater than twofold change in expression level in at least one time point during liver regeneration with significant difference ($0.01 \leqslant P < 0.05$) or extremely significant difference ($P \leqslant 0.01$) between PH and SO, were referred to as associated with liver regeneration.

## RESULTS

### Expression changes of genes associated with drug-induced liver diseases during LR

According to the data from databases at NCBI, GENEMAP, KEGG and BIOCARTA, 182 genes were associated with drug-induced liver diseases. Among them, 84 genes were contained in the Rat Genome 230 2.0 array. Thirty-two of them revealed meaningful changes in expression at least at one time point after PH. There was significant difference or extremely significant difference in expression between PH and SO, and reproducible results were obtained with three analyses with Rat Genome 230 2.0 array, suggesting that the genes were associated with LR (Table 1). The analysis indicated that 12 genes were up-, 11 genes down-, and 9 were up/down-regulated during liver regeneration. Total expression times of up- and down-expressed genes were 130 and 79, respectively (Figure 1A). At the initial stage of liver regeneration (0.5-4 h after PH), 13 genes displayed up-, 7 genes down-, 1 gene up/down-regulation; at the transition phase from $G_0$ and $G_1$ (4-6 h after PH), 10 genes revealed up-, 2 genes down-regulation; at cell proliferation phase (6-66 h after PH), 12 genes showed up-, 8 genes down-, 5 genes up/down-regulation; and at cell differentiation and structure-function reorganization phase (66-168 h after PH), 9 genes displayed up-, 10 genes down-, 5 genes up/down-regulation (Figure 1B).

**Table 1 Expression abundance of 32 genes associated with drug-induced liver diseases during liver regeneration**

| Gene | Recovery time (h) after partial hepatectomy (PH) | | | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Abbr. | 0 | 0.5 | 1 | 2 | 4 | 6 | 8 | 12 | 16 | 18 | 24 | 30 | 36 | 42 | 48 | 54 | 60 | 66 | 72 | 96 | 120 | 144 | 168 |
| 1 Cholestasis | | | | | | | | | | | | | | | | | | | | | | | |
| Ace | 1 | 0.80 | **0.48** | 1.29 | 0.88 | 1.39 | 0.82 | 1.14 | 1.16 | 1.00 | 0.88 | 0.93 | 1.18 | 1.03 | 1.18 | 1.03 | 1.39 | 0.56 | 0.71 | 0.78 | 0.75 | 0.56 | **0.47** |
| Akt1 | 1 | 0.81 | 0.76 | 0.98 | 0.86 | 1.01 | 0.87 | 1.11 | 1.12 | 0.62 | 0.71 | *3.91* | 0.65 | *2.60* | 0.53 | 0.57 | 0.68 | 1.10 | 0.97 | 1.17 | 1.02 | 0.94 | 1.01 |
| Apoe | 1 | 0.93 | 1.24 | 0.87 | 0.93 | 1.07 | 0.95 | 0.76 | **0.15** | 0.93 | 1.08 | **0.13** | 0.93 | **0.17** | 1.00 | 1.00 | 0.93 | 1.07 | 1.20 | **0.33** | 1.41 | 1.23 | 1.07 |
| Bdnf | 1 | 0.97 | **0.46** | 1.98 | 1.43 | 1.04 | 1.04 | *2.00* | *2.08* | 0.63 | 0.58 | *2.12* | 0.52 | 1.43 | **0.45** | 1.25 | *2.50* | 1.06 | 1.32 | *2.56* | **0.39** | 0.52 | **0.50** |
| Cyp2d6 | 1 | 0.76 | **0.33** | 1.62 | 1.00 | 1.15 | 1.35 | 1.62 | 1.14 | 1.15 | 1.24 | 0.60 | 0.57 | 0.74 | 0.81 | 0.81 | 0.66 | 1.73 | 0.65 | 1.07 | 0.66 | **0.44** | **0.50** |
| Ephx1 | 1 | 1.52 | 1.53 | 1.32 | 0.93 | 1.00 | 0.63 | **0.41** | 0.86 | 0.76 | 0.62 | 0.97 | *2.46* | 1.12 | *2.14* | 1.74 | *2.00* | *2.82* | *2.77* | 0.54 | 1.74 | 1.62 | 1.62 |
| Il6 | 1 | 1.87 | 1.08 | *3.25* | *3.25* | *3.03* | *3.81* | 1.32 | 0.99 | *3.25* | 1.87 | 0.91 | **0.50** | 1.28 | *2.83* | 1.87 | *2.30* | 0.87 | **0.28** | *6.06* | 0.81 | 1.52 | 0.93 |
| Mmp9 | 1 | *2.05* | *2.16* | *2.10* | *2.07* | 1.37 | *2.81* | *2.75* | *2.13* | 1.12 | 1.58 | 1.27 | 1.83 | *5.89* | 1.00 | 1.20 | 1.15 | 1.16 | 1.21 | *9.54* | *2.22* | **0.48** | *2.03* |
| Slc22a1 | 1 | 0.62 | 0.88 | 0.76 | 0.66 | 0.66 | 0.59 | 0.71 | 0.86 | 0.81 | 0.82 | 1.20 | 0.71 | 1.47 | **0.41** | 0.71 | 0.62 | 1.31 | 1.05 | 1.23 | 0.81 | 0.76 | 0.71 |
| Slc22a2 | 1 | 1.23 | 1.01 | **0.50** | 0.93 | 0.87 | **0.39** | **0.38** | **0.33** | **0.38** | **0.33** | **0.08** | 0.87 | 0.69 | 1.07 | 1.07 | 1.62 | **0.18** | 0.98 | **0.38** | 0.66 | 1.15 | 1.32 |
| 2 Fatty liver | | | | | | | | | | | | | | | | | | | | | | | |
| Esr1 | 1 | 1.07 | 0.62 | *3.48* | *4.92* | *4.92* | 1.17 | 1.41 | 1.31 | *2.14* | 1.08 | 1.29 | 1.23 | 1.37 | 1.74 | 2.64 | 4.29 | 1.51 | 1.48 | 1.23 | *4.59* | *6.06* | *2.30* |
| Fabp1 | 1 | 0.87 | 1.16 | 0.81 | 0.81 | 1.00 | 0.77 | 0.66 | 1.40 | 0.87 | 0.94 | 1.29 | 0.87 | 1.12 | 0.87 | 0.93 | 0.93 | 1.07 | 1.29 | **0.50** | 1.23 | 1.00 | 0.93 |
| Hiat1 | 1 | 0.93 | 1.43 | 1.07 | 1.07 | 1.32 | 1.90 | 1.15 | 1.14 | 1.15 | 1.33 | **0.10** | 1.00 | **0.14** | 1.41 | 1.07 | 0.93 | 1.41 | 1.20 | **0.15** | 1.32 | 1.32 | 1.32 |
| Hsd11b1 | 1 | *4.92* | *6.13* | *2.64* | *4.92* | *2.83* | *3.81* | 1.07 | 0.99 | *2.14* | *3.50* | 1.04 | 0.87 | 0.97 | *2.83* | 1.87 | *2.46* | 0.93 | *2.77* | 0.93 | *3.73* | *5.28* | *4.92* |
| Il5 | 1 | 1.15 | 0.67 | 1.00 | 0.76 | 0.76 | 1.26 | 1.52 | 0.86 | *2.83* | 1.52 | 0.85 | 1.32 | 1.28 | *3.03* | *3.25* | *3.48* | 1.51 | 1.70 | 1.07 | 0.93 | 1.32 | 1.32 |
| Mthfr | 1 | **0.47** | **0.44** | 1.87 | *3.25* | *2.64* | *3.19* | *3.73* | 1.50 | **0.50** | **0.38** | 1.48 | 0.66 | 1.69 | 0.66 | 0.71 | 0.87 | **0.46** | **0.40** | *2.00* | 0.57 | 0.66 | **0.50** |
| Tnf | 1 | 0.87 | 0.62 | 1.23 | 1.41 | 1.15 | 1.17 | 0.93 | 0.93 | 0.71 | 0.66 | 0.79 | 1.41 | 0.85 | 1.62 | 1.07 | 1.15 | 0.93 | 0.52 | 1.23 | *3.25* | 0.81 | 0.71 |
| Trp63 | 1 | *3.00* | *2.44* | *2.40* | 1.06 | 0.97 | 1.70 | *2.03* | 1.58 | 1.81 | 1.35 | 0.84 | 1.19 | 1.33 | 1.55 | 2.12 | 1.62 | 1.23 | 1.12 | 1.11 | 0.74 | 1.19 | 0.90 |
| Ugt1a1 | 1 | 0.93 | 1.43 | 1.28 | 1.23 | 1.15 | 0.99 | 0.71 | *2.06* | 1.04 | 0.88 | 1.36 | 1.04 | 1.25 | 0.95 | 0.95 | 1.08 | 1.51 | 1.34 | 0.69 | 1.42 | 1.12 | 1.08 |
| 3 Liver ischemic injury | | | | | | | | | | | | | | | | | | | | | | | |
| Ces2 | 1 | 0.81 | 1.08 | 1.32 | 1.32 | 1.23 | 1.44 | 1.00 | *2.81* | 1.23 | 1.42 | 0.85 | 1.62 | *2.23* | 1.74 | 1.62 | 1.52 | *3.24* | *2.77* | 1.23 | 1.87 | 1.41 | 1.52 |
| Ces3 | 1 | 1.74 | *3.29* | *2.46* | *3.03* | *3.03* | *2.88* | 1.32 | 0.70 | 0.87 | 1.00 | 0.64 | **0.44** | 0.60 | **0.27** | **0.44** | 0.57 | **0.33** | 1.48 | 0.71 | 1.23 | 1.62 | 1.62 |
| Creb1 | 1 | 0.93 | 1.01 | 0.93 | **0.50** | 1.00 | 0.55 | 0.66 | 1.06 | 1.23 | 0.94 | 0.91 | 0.87 | 0.91 | 0.87 | 1.41 | 1.15 | 1.14 | 0.85 | 0.93 | 1.23 | 1.07 | 1.00 |
| Fos | 1 | *28.43* | *16.11* | *8.54* | *3.07* | *4.82* | *2.07* | *3.72* | *3.50* | *2.83* | *2.63* | *3.21* | *1.77* | *2.03* | *2.76* | 1.65 | 1.69 | 1.90 | 1.71 | 1.12 | *2.44* | 0.88 | 1.00 |
| Per1 | 1 | *3.03* | *3.49* | *4.29* | *3.91* | *3.73* | *3.09* | 0.93 | 1.61 | **0.38** | 0.71 | 1.38 | **0.44** | 1.81 | **0.44** | **0.33** | **0.47** | 0.75 | 0.79 | 1.15 | *3.48* | *4.00* | *3.48* |
| 4 Drug hepatitis | | | | | | | | | | | | | | | | | | | | | | | |
| Bcl2 | 1 | 0.82 | 0.64 | 1.12 | 1.00 | 1.19 | 1.17 | 1.15 | 0.85 | **0.33** | **0.37** | 1.80 | **0.32** | 0.79 | **0.44** | **0.39** | **0.46** | 0.81 | **0.38** | 0.84 | 0.50 | 0.68 | 0.64 |
| Cyp2d6 | 1 | 0.76 | **0.33** | 1.62 | 1.00 | 1.15 | 1.35 | 1.62 | 1.14 | 1.15 | 1.24 | 0.60 | 0.57 | 0.74 | 0.81 | 0.81 | 0.66 | 1.73 | 0.65 | 1.07 | 0.66 | **0.44** | **0.50** |
| Egr1 | 1 | *17.15* | *18.59* | *13.93* | *2.30* | *2.00* | *2.68* | *3.48* | 0.86 | 1.62 | *2.84* | 0.64 | *3.03* | 0.56 | *2.83* | *3.25* | *3.73* | *4.58* | *2.25* | 0.66 | *3.03* | 1.00 | 0.93 |
| Esr1 | 1 | 1.07 | 0.62 | *3.48* | *4.92* | *4.92* | 1.17 | 1.41 | 1.31 | *2.14* | 1.08 | 1.29 | 1.23 | 1.37 | 1.74 | *2.64* | *4.29* | 1.51 | 1.48 | 1.23 | *4.59* | *6.06* | *2.30* |
| Gstm1 | 1 | 1.32 | *2.17* | 1.32 | 1.52 | 1.52 | 1.26 | 0.66 | 1.61 | 1.23 | 1.42 | 1.12 | 1.23 | 1.69 | 1.41 | 1.52 | 1.52 | 1.73 | *2.10* | 1.07 | 1.62 | 1.52 | 1.32 |
| Il5 | 1 | 1.15 | 0.67 | 1.00 | 0.76 | 0.76 | 1.26 | 1.52 | 0.86 | *2.83* | 1.52 | 0.85 | 1.32 | 1.28 | *3.03* | *3.25* | *3.48* | 1.51 | 1.70 | 1.07 | 0.93 | 1.32 | 1.32 |
| Mthfr | 1 | **0.47** | **0.44** | 1.87 | *3.25* | *2.64* | *3.19* | *3.73* | 1.50 | **0.50** | **0.38** | 1.48 | 0.66 | 1.69 | 0.66 | 0.71 | 0.87 | **0.46** | **0.40** | *2.00* | 0.57 | 0.66 | **0.50** |
| Nat2 | 1 | 1.00 | 1.89 | 1.07 | *2.30* | 1.32 | 1.09 | 1.41 | 0.99 | *3.25* | *4.02* | 1.29 | 1.07 | 1.04 | *3.48* | *2.83* | *3.25* | *2.63* | 1.70 | 1.41 | *3.48* | 1.74 | *2.46* |
| Pten | 1 | 0.66 | 1.08 | 0.93 | **0.50** | **0.47** | 0.72 | 0.76 | 0.86 | 1.23 | 1.33 | 0.79 | 1.41 | 1.04 | 1.74 | 1.32 | 1.23 | 1.62 | 1.12 | 1.41 | 1.52 | 1.32 | 1.23 |
| Ptgs2 | 1 | 0.57 | 0.90 | *2.14* | 1.23 | 1.41 | 1.17 | 1.41 | 1.31 | 0.62 | 0.66 | 0.85 | **0.12** | 1.12 | **0.47** | 0.71 | 1.15 | **0.31** | 0.69 | 1.00 | **0.44** | **0.33** | **0.47** |
| Trp63 | 1 | *3.00* | *2.44* | *2.40* | 1.06 | 0.97 | 1.70 | 2.03 | 1.58 | 1.81 | 1.35 | 0.84 | 1.19 | 1.33 | 1.55 | *2.12* | 1.62 | 1.23 | 1.12 | 1.11 | 0.74 | 1.19 | 0.90 |
| 5 Granulomatous disease of the liver | | | | | | | | | | | | | | | | | | | | | | | |
| Cyp2d6 | 1 | 0.76 | **0.33** | 1.62 | 1.00 | 1.15 | 1.35 | 1.62 | 1.14 | 1.15 | 1.24 | 0.60 | 0.57 | 0.74 | 0.81 | 0.81 | 0.66 | 1.73 | 0.65 | 1.07 | 0.66 | **0.44** | **0.50** |
| 6 Peliosis hepatis | | | | | | | | | | | | | | | | | | | | | | | |
| Erbb2 | 1 | **0.50** | **0.10** | 1.74 | 0.66 | 0.62 | **0.34** | 0.62 | 1.40 | **0.29** | **0.17** | 1.12 | **0.41** | 1.47 | **0.50** | **0.41** | **0.47** | **0.15** | **0.15** | 1.15 | **0.25** | **0.23** | **0.31** |
| Sult1a1 | 1 | 1.62 | *2.17* | 1.41 | 1.52 | 1.41 | 1.26 | 0.76 | **0.22** | 1.00 | 1.24 | 1.12 | 1.15 | **0.49** | 1.87 | 1.32 | 1.41 | 1.62 | 1.70 | 0.71 | 1.74 | 1.62 | 1.62 |

Italic numbers: Genes are up-regulated more than twofold; Bold numbers: Genes are down-regulated more than twofold.

### *Initial expression time of genes associated with drug-induced liver diseases during LR*

At each time point of LR, the numbers of initial up-, down-regulation and total up-, down-regulation genes were in the sequence: both 6 and 2 at 0.5 h; 3, 3 and 9, 5 at 1 h; 3, 1 and 10, 1 at 2 h; 1, 2 and 10, 2 at 4 h; 0, 0 and 8, 1 at 6 h; 0, 0 and 8, 2 at 8 h; 0, 1 and 6, 2 at 12 h; 2, 1 and 5, 3 at 16 h; 1, 1 and 6, 5 at 18 h; 0, 0 and 4, 4 at 24 h; 1, 1 and 3, 3 at 30 h; 0, 0 and 2, 6 at 36 h; 0, 0 and 4, 3 at 42 h; 0, 1 and 7, 7 at 48 h; 0, 0 and 5, 4 at 54 h; 0, 0 and 8, 3 at 60 h; 0, 0 and 4, 5 at 66 h; 0, 0 and 5, 4 at 72 h; 0, 1 and 4, 4 at 96 h; 1, 0 and 8, 3 at 120 h; 0, 0 and 3, 4 at 144 h , 0, 0 and 5, 6 at 168 h (Figure 2).

### *Expression similarity and time relevance of genes associated with drug-induced liver diseases during LR*

Thirty-two genes mentioned above during LR could be characterized based on their similarity in expression as follows: only up-, predominantly up-, only down-, predominantly down-, and up-/down-regulated, involving 13, 4, 11, 3 and 2 genes, respectively (Figure 3). They could also be classified according to the time relevance into 15 groups, including 0.5 and 1 h, 2 h, 4 and 6 h, 8 h, 12 h, 16 h, 18 and 24 h, 30 h, 36 h, 42 and 96 h, 48 h, 54 h and 60 h, 66 and 72 h, 120 h, 144 and 168 h. Their times of up- and down-regulation genes were respectively 15 and 7, 10 and 1, 18 and 3，8 and 2, 6 and 2，6 and 3, 10 and 9, 3 and 3, 6 and 2, 8 and 7, 7 and 7, 13 and 7, 9 and 9, 8 and 3, 8 and 10 (Figure 3).

### *Expression patterns of genes associated with drug-induced liver diseases during LR*

Thirty-two genes mentioned above during LR might be

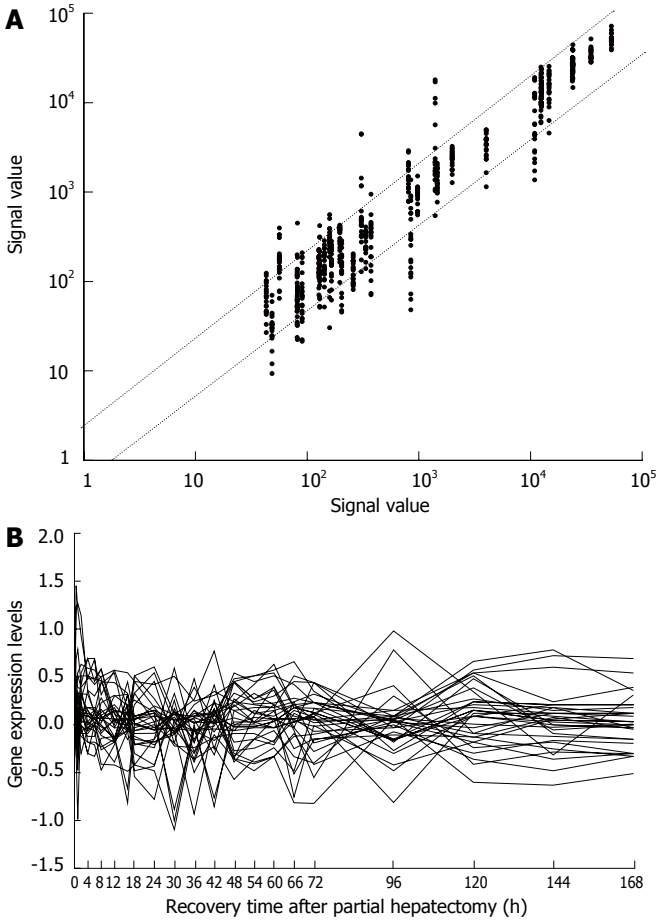


**Figure 1** Expression profiles of 32 genes associated with drug-induced liver diseases during liver regeneration. **A**: The abundance and frequency of gene expression, each point represents the signal value of one gene at corresponding time point. The dots above bias represent the genes up-regulated by more than twofold, those under bias represent the genes down-regulated by more than twofold, and the ones between biases represent the genes meaningless alteration in expression. The farer the genes from the bias, the greater the folds of gene change; **B**: The expression changes of genes associated with LR.

categorized according to the changes in expression into 20 types of patterns: (1) up-regulation at one time point, i.e. at 16, 120 h after PH, (Figure 4A), 2 genes; (2) up- at two time point, i.e. at 1 and 72 h, 30 and 42 h, (Figure 4B), 2 genes; (3) up- at one time point/phase, i.e. at 18 and 48-72 h (Figure 4C), 1 gene; (4) up- at one time point/two phases (Figure 4C), 1 gene; (5) up- at one time point/three phases (Figure 4C), 1 gene; (6) up- at two time points/one phase (Figure 4D), 2 genes; (7) up- at three time points/two phases (Figure 4E), 2 genes; (8) up- at three time points/phases (Figure 4D), 1 gene; (9) down- at one time point, at 0.5, 48 or 96 h (Figure 4F), 3 genes; (10) down- at two time points, i.e. at 1 and 168 h (Figure 4G), 1 gene; (11) down- at three time points(Figure 4G), 1 gene; (12) down- at more time points (Figure 4G), 1 gene; (13) down- at one phase, i.e. at 1-6 h (Figure 4H), 1 gene; (14) down- at one time point/phase, i.e. at 1 and 144-168 h (Figure 4H), 1 gene; (15) down- at two time points/phases (Figure 4H), 1 gene; (16) down- at two time points/four phases (Figure 4H), 1 gene; (17) down- at three time points/one phase (Figure 4H), 1 gene; (18) predominantly up- (Figure 4I), 4 genes; (19) predominantly down- (Figure 4J), 3 genes; (20) up/down- approximately (Figure 4K), 2 genes.

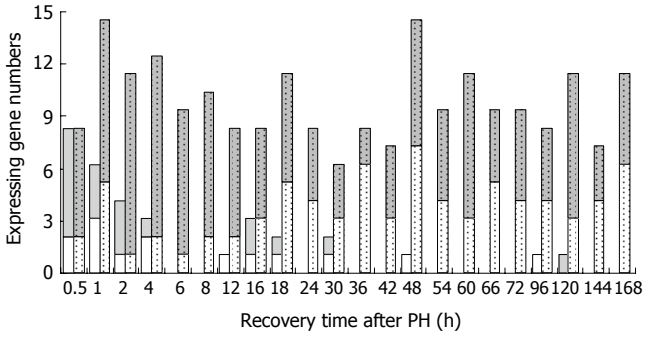


**Figure 2** The initial and total expression profiles of 32 genes associated with drug-induced liver diseases at each time point of liver regeneration. Blank bars: Initial expressing gene number; Dotted bars: Total expressing; Grey bars: Up-regulated; White bars: Down-regulated.

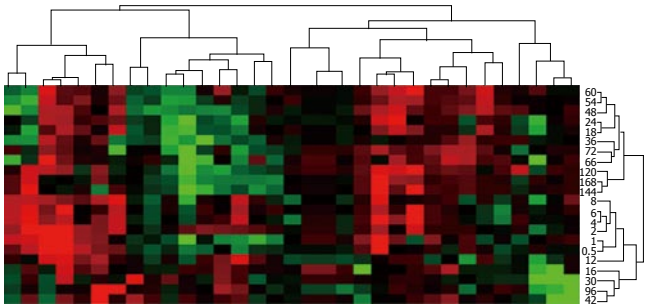

**Figure 3** Expression similarity and time relevance clusters of 32 genes associated with drug-induced liver diseases during liver regeneration. Detection data of Rat Genome 230 2.0 array were analyzed by H-clustering. Red: Up-regulation genes; Green: Down-regulation; Black: No-sense in expression change; The upper and right trees respectively show expression cluster and time series cluster.

## DISCUSSION

In this paper, the roles of 84 genes associated with drug-induced liver diseases during liver regeneration were analyzed. Of the 36 genes associated with drug-induced abnormality of cell proliferation and apoptosis, cocaine addiction-associated cAMP responsive element binding protein 1 (CREB1)[18] and estradiol-induced interleukin 6 (IL6)[19] were related to liver regeneration initiation[20]. Cocaine-induced V-fos FBJ murine osteosarcoma viral oncogene homolog (FOS)[21], troglitazone-induced early growth response 1 (EGR-1)[22], prostaglandin-endoperoxide synthase 2 (PTGS2) repressed by cyclophosphamide[23], estradiol-activated Akt (v–akt) murine thymoma viral oncogene homolog 1 (AKT1)[24] and estradiol-induced brain derived neurotrophic factor (BDNF)[25] all promote cell growth or cell division[26,27]. Valproic acid-restrained estrogen receptor 1 (ESR1)[28] inhibits cell division[29]. Period homolog 1 (Drosophila) (PER1) promotes apoptosis[30]. Cyclophosphamide-induced B-cell leukemia/lymphoma 2 (BCL2) restrains apoptosis[31]. Diethylstilbestrol-restrained transformation related protein 63 (TRP63)[32] is associated with differentiation[33]. Estradiol-induced matrix metallopeptidase 9 (MMP9)[34] is involved in the breakdown of extracellular matrix. Indomethacin-induced phosphatase and tensin homolog (PTEN)[35] blocks tumor cell proliferation and migration[36].

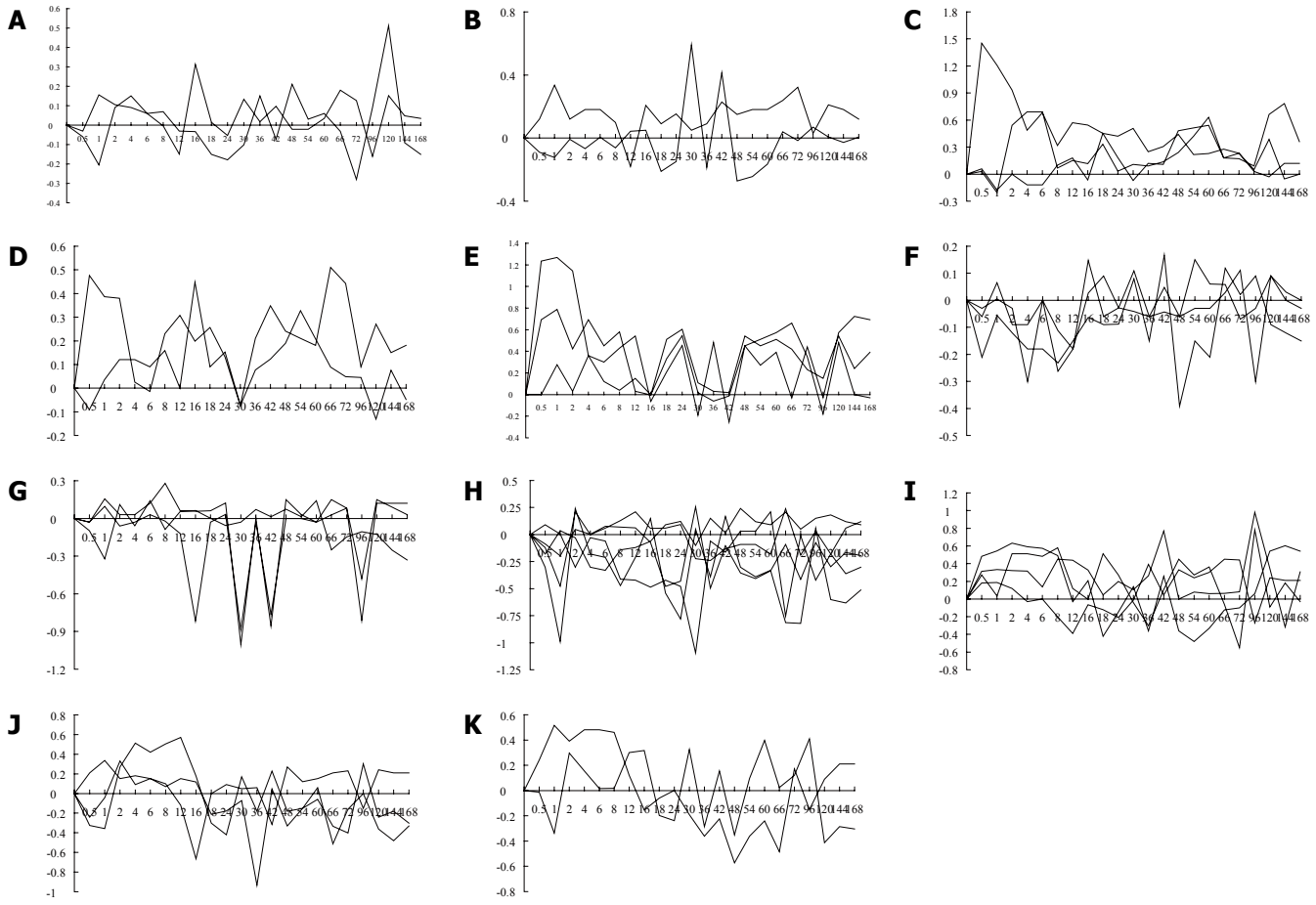


**Figure 4** Expression patterns of 32 genes associated with drug-induced liver diseases during liver regeneration. These genes exhibit 20 types of expression patterns. **A-E**: Up-regulation in expression; **F-H**: Down-regulation; **I-J**: Up/down-regulation mixed. X-axis represents recovery time after PH (h), Y-axis shows logarithm ratio of the signal values of genes at each time point to the control.

Angiotensin I converting enzyme (ACE), whose activity is inhibited by captopril[57], participates in the control of blood pressure. The sameness or similarity in some time points, and the difference in other points of meaningful expression changes of these genes during LR may indicate that they regulate the mass of regenerating liver together.

Of the 21 genes associated with drug-induced disorder of lipid metabolism or amino-acid metabolism, estradiol-induced apolipoprotein E (APOE)[38] and fatty acid binding protein 1, liver (FABP1) play a part in the metabolism and transport of lipid[39,40]. One of the tamoxifens' target proteins: epoxide hydrolase 1, microsomal (EPHX1)[41], and tetracyclin-induced tumor necrosis factor (TNF)[42] participate in lipid metabolism, and 5, 10-methylenetetrahydrofolate reductase (MTHFR)[43] plays a role in methionine biosynthesis. That meaningful expression changes of these genes are the same or similar in some time points, then different in other points during LR perhaps regulate the metabolism of lipid and/or amino-acid together.

Of the 27 genes associated with drug metabolism disorder, six genes including solute carrier family 22 member 1, 2, (SLC22A1, SLC22A2), UDP glucuronosyltransferase 1 family A1 (UGT1A1), glutathione S-transferase M1 (GSTM1), amitriptyline-restrained cytochrome P450 family 2 subfamily D 6 (CYP2D6)[44] and sulfotransferase family cytosolic 1A phenol-preferring member 1 (SULT1A1) are involved in drug metabolism[45-47]. Hippocampus abundant gene transcript 1 (HIAT1) is responsible for transmembrane of tetracyclin[48]. N-acetyltransferase 2 (NAT2) catalyzes decomposition of isoniazid. Hydroxysteroid (11-β) dehydrogenase 1 (HSD11B1) can inactivate cortisol[49]. Carboxylesterase 2, 3 (CES2, CES3) catalyze the hydrolysis of fatty acids and cocaine[50]. Interleukin 5 (IL5) is associated with corticosteroid resistance[51] and inflammation[52]. V-erb-b2 erythroblastic leukemia viral oncogene homolog 2 (ERBB2) can impede the function of tamoxifen[53]. The expression changes of the genes mentioned above were the same or similar at some time points and different at other time points during LR, speculating that they promote drug metabolism together.

In conclusion, some genes associated with drug-induced liver diseases are up-regulated and the others are down-regulated during liver regeneration. In liver regeneration, some drug-induced liver diseases-related genes regulate the liver cell number by adjusting cell proliferation and apoptosis, some control lipid metabolism or amino acid metabolism, and others participate and modulate drug metabolism, demonstrating that they are closely in line with liver regeneration. We will use northern blotting, protein array, RNA interference etc. to further confirm the above results at the cell level in the future.

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*CLINICAL RESEARCH*

# Meta-analysis on inoperable pancreatic cancer: A comparison between gemcitabine-based combination therapy and gemcitabine alone

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## Abstract

**AIM:** To compare gemcitabine-based combination therapy and gemcitabine (GEM) alone in patients with advanced pancreatic cancer (APCa) through meta-analysis.

**METHODS:** MEDLINE and EMBASE searches were supplemented by information from trial registers of randomized controlled trials (RCTs) for GEM-based combination therapy and GEM alone for APCa. A quantitative meta-analysis was carried out by two reviewers based on the inclusion criteria from all available RCTs. The meta-analysis involved overall survival (OS), objective remission rate (ORR), clinical benefit rate (CBR), time to progress/progress free survival (TTP/PFS) and toxicity.

**RESULTS:** The meta-analysis included 22 RCTs. There was significant improvement in the GEM combination group with regard to the 6-mo survival rate (RD = 0.04, 95% CI 0.01-0.06, $P$ = 0.008), 1-year survival rate (RD = 0.03, 95% CI 0.01-0.05, $P$ = 0.01), ORR (RD = 0.04, 95% CI 0.01-0.07, $P$ = 0.02), CBR (RD = 0.10, 95% CI 0.02-0.17, $P$ = 0.01) and 6-mo TTP/PFS (RD = 0.07, 95% CI 0.04-0.10, $P$ < 0.00001). However, the Grade 3-4 toxicity set by WHO was higher for the GEM combination group for neutropenia (RD = 0.05, 95% CI 0.01-0.10, $P$ = 0.02), thrombocytopenia (RD = 0.05, 95% CI 0.02-0.08, $P$ = 0.002) and vomiting/nausea (RD = 0.03, 95% CI 0.00-0.05, $P$ = 0.02).

**CONCLUSION:** GEM-based combination therapy may improve the overall survival and palliation in optimal patients with APCa as compared with GEM alone.



**Key words:** Chemotherapy; Pancreatic cancer; Meta-analysis; Gemcitabine

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## INTRODUCTION

Gemcitabine (GEM) monotherapy currently is considered as a standard treatment for patients with advanced pancreatic cancer (APCa). However, patients treated with GEM alone have poor prognoses, and their overall median survival (OS) was only 5.65 mo[1]. Attempts have been made to increase the objective remission rate (ORR) and survival of APCa patients, in particular, by exploring the effects of the combined GEM with other drugs. In many phase Ⅱ studies, GEM combinations have improved ORR and OS. Based on these results, many prospective, randomized phase Ⅲ trials comparing GEM used in combination and alone have been carried out. But these trials had different results and the population enrolled is small. Therefore, the NCCN guidelines (National Comprehensive Cancer Network, v.2.2006) indicate that GEM-based combination therapy may be an optimal treatment for APCa patients with a good performance status, including GEM + cisplatin (DDP), GEM + oxaliplatin, GEM + capecitabine, GEM + erlotinib and so on. But these guidelines were based on low level evidence including clinical experience (category 2A). The role of GEM-based combination therapy for the treatment of APCa is still unclear. We therefore, conducted a systematic review and quantitative meta-analysis to evaluate the available evidence from the relevant randomized trials.

## MATERIALS AND METHODS

### *Literature search*

We carried out a comprehensive search of literature

with MEDLINE (1966-2006), EMBASE (1966-2006), CBMdisc (1981-2006), ASCO Abstracts (1995-2005) and EBM Reviews (Cochrane Database of Systematic Reviews 1st Quarter, 2006) ACP Journal Club (1991-2006), (Database of Abstracts of Reviews of Effects 1st Quarter 2006), Cochrane Central Register of Controll ed Trials ( 1st Quarter, 2006 ), using the terms: 'pancreas', 'pancreatic cancer', 'pancreatic carcinoma, 'pancreatic adenocarcinoma', 'pancreatic neoplasms', 'gemzar', ' gemcitabine' (no limit to language). Date of last search: April 26, 2006.

### Selection criteria

**Study design:** Trials should be prospective, properly randomized and well designed, which were matched for age, stage, performance status, *etc*.

**Study population:** Patients with APCa, as well as those with locally advanced, or metastatic disease, were included in the study. Patients eligible for the study were required to have histologically or cytologically proved pancreatic cancer. Furthermore, they should have a baseline Karnofsky performance status of $\geqslant$ 50% (or Eastern Cooperative Oncology Group performance status < 2) and adequate hematological, renal, cardiac and hepatic function. Patients with estimated life expectancy of at least 12 wk, should have received no chemotherapy, radiotherapy and other antitumor therapy in the 6 mo prior to the study entry.

**Intervention:** The treatment group received GEM-based combination therapy, and the control group received GEM alone.

**Outcome:** The primary outcome measurement was OS, followed by ORR and toxicity. The follow-up rate should be above 95%.

**Data collection and analysis:** Two reviewers assessed the abstracts identified from the defined sources. Both reviewers independently selected trials for inclusion according to prior agreement regarding the study population and the intervention. If one of the reviewers concluded an abstract to be eligible, the full text of article was retrieved and reviewed in detail by both reviewers. Missing data from the primary study reports was requested from the investigators. If the same trial appeared on different publications, the final data of the trial was chosen. Methodologic quality of the trials was assessed using a validated scale (range, 0 to 5) applied to items that influence the intervention efficacy. It was reported by Jadad *et al*[2] that the scale consisted of items pertinent to randomization, masking, dropouts, and withdrawals. The following information was obtained from each trial: year of publication, number of patients, performance status, chemotherapy regimen, objective response rate, overall survival, progress free survival, clinical benefit, toxicity, etc. For response assessment, we used trials that included patients with measurable or assessable diseases, and that were analyzed mainly with WHO's criteria. Toxicity profiles were reported according to the WHO's criteria.

### Statistical analysis

The primary end point was a 6-mo survival rate after randomization. The other end points were 1-year survival rate, ORR, CBR, 6-mo TTP/PFS rate and adverse effects. All variables were defined as dichotomous data (e.g., 6-mo survival rate used variables as follows: the alive or dead at 6 mo after randomization). We standardized the therapeutic results by obtaining the risk difference (RD) between the GEM combination group and the GEM alone group. Publication bias was examined using a funnel plot[3]. We looked for heterogeneity among the trials based on Cochran's $\chi^2$ test. All analyses were performed strictly with RevMan software (version 8.2, Cochrane). *P* value less than 0.05 was considered as significant in difference.

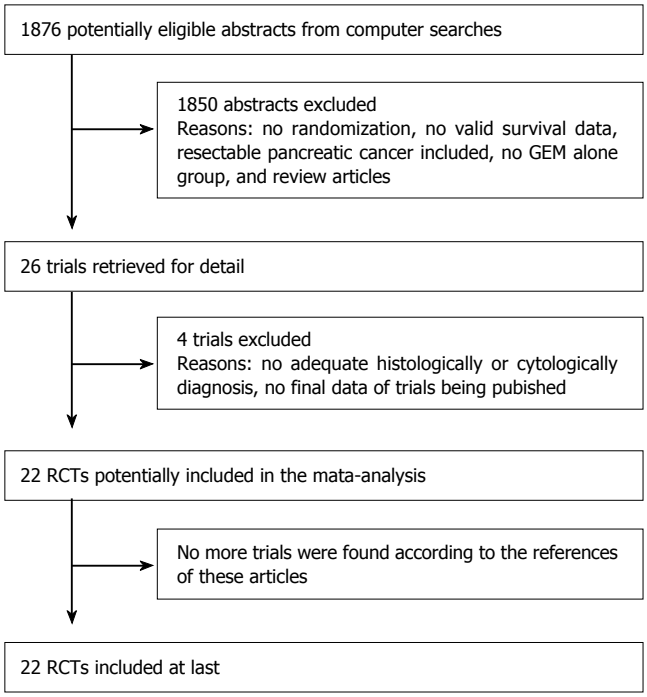


**Figure 1** The flow chart. GEM: gemcitabine; RCTs: randomized controlled trials.

## RESULTS

### Trial flow

The flow chart of our study is shown in Figure 1. Because the trial reported by Degen *et al*[4] involved some patients diagnosed by imageology, we excluded this trial from our analysis. Of the 26 trials, three reported by Ohkawa *et al*[5], Richards *et al*[6], and Shapiro *et al*[7], were excluded because of no final data. Both reviewers finally agreed to include 22 RCTs involving 5473 APCa patients in the meta-analysis.

### Characteristics of selected trials

These prospective randomized controlled studies are summarized in Table 1. All selected trials for inclusion strictly according to prior selection criteria, were prospective, randomized and well designed, and the clinical characteristics were matched for age, stage, performance status, and so on. All studies reviewed were considered high in quality, for they achieved a score of 3 or higher in the assessment scale of Jadad's study design. Patients eligible for these studies had histologically or cytologically

**Table 1 Randomized controlled trials (GEM combination *vs* GEM alone)**

| Studies | Intervention | Patients | OS (d) | 6-mo survival (%) | 1-yr survival (%) | 6-mo TTP/PFS/ TTF rate (%) | ORR (CR + PR) % | CBR | Jadad score |
|---|---|---|---|---|---|---|---|---|---|
| Scheithauer 2003[8] | Gem | 42 | 246 | 59.4 | 37.2 | 24.6 (PFS) | 6/42 | 10/30 | 3 |
| | Gem + Capecitabine | 41 | 285 | 67.7 | 31.8 | 36.9 | 7/41 | 15/31 | |
| Colucci 2002[9] | Gem | 54 | 140 | 31.5 | 11 | 18 (TTP) | 5/48 | 21/43 | 3 |
| | Gem + DDP | 53 | 210 | 47 | 11.3 | 28 | 14/45 | 20/38 | |
| Wang XY 2002[10] | Gem | 20 | - | 81.3 | 31.3 | - | 1/16 | 14/16 | 3 |
| | Gem + DDP | 22 | - | 61.6 | 11.1 | - | 2/18 | 14/20 | |
| Gansauge 2002[11] | Gem | 28 | 144 | 32 | 11 | - | 1/28 | - | 3 |
| | Gem + NSC-631570 | 28 | 279 | 64 | 29 | - | 6/28 | - | |
| Berlin 2002[12] | Gem | 162 | 162 | 42 | 15.5 | 32/160 (PFS) | 9/162 | - | 3 |
| | Gem + 5-FU | 160 | 201 | 55 | 21.9 | 41/158 | 11/160 | - | |
| Bramhall 2002[13] | Gem + placebo | 119 | 164 | 43 | 17 | 23 (TTF) | 14/88 | - | 5 |
| | Gem + marimastat | 120 | 165.5 | 47 | 18 | 29 | 11/97 | - | |
| Cutsem 2004[14] | Gem + placebo | 347 | 182 | 49 | 24 | - | 28/347 | - | 5 |
| | | 341 | 193 | 53 | 27 | - | 20/341 | - | |
| Louvet 2005[15] | Gem | 156 | 213 | 60.4 | 27.8 | 27.4 (PFS) | 27/156 | 26.9 | 3 |
| | Gem[1] + Oxaliplatin | 157 | 270 | 68 | 34.7 | 43 | 42/157 | 38.2 | |
| Reilly 2004[16] | Gem | 174 | 186 | 51 | 21 | 27 (TTP) | 9/127 | - | 3 |
| | Gem + DX-8951f | 175 | 201 | 54 | 23 | 39 | 12/147 | - | |
| Richards 2004[17] | Gem | 282 | 189 | 50.9 | 20.1 | 27.6 (PFS) | 20/220 | - | 3 |
| | Gem + Pemetrexed | 283 | 186 | 50.9 | 21.4 | 32.1 | 42/230 | - | |
| Li CP 2004[18] | Gem | 25 | 138 | 20.3 | 13.6 | 11.8 (TTP) | 3/25 | 9/25 | 3 |
| | Gem + DDP | 21 | 168 | 31.1 | 6.3 | 11.8 | 2/21 | 6/21 | |
| Reni 2004[19] | Gem | 47 | - | 63.9 | 21.3 | 12.9 (PFS) | 4/47 | 5/20 | 3 |
| | Gem + 5-FU + DDP + EPI | 52 | - | 64.6 | 38.5 | 37.4 | 20/52 | 15/23 | |
| Viret 2004[20] | Gem | 41 | 201 | 58.3 | 25.1 | 10 (TTF) | 2/41 | - | 3 |
| | Gem + DDP | 42 | 241 | 55.5 | 32.4 | 14 | 3/42 | - | |
| Rocha Lima 2004[21] | Gem | 180 | 198 | 52.9 | 22 | 21.9 (TTP) | 8/180 | - | 3 |
| | Gem + Irinotecan | 180 | 189 | 50.7 | 21 | 30.6 | 29/180 | - | |
| Costanzo 2001[22] | Gem | 49 | 217 | 59 | 14.5 | - | 4/48 | - | 3 |
| | Gem + 5-FU | 44 | 210 | 59 | 23.3 | - | 5/43 | - | |
| Heinemann 2003[23] | Gem | 97 | 180 | 48.6 | 22.5 | 25.6 (TTP) | 8/93 | - | 3 |
| | Gem + DDP | 95 | 228 | 59.4 | 27.5 | 39.3 | 10/92 | - | |
| Kulke 2004[24] | Gem[2] | 45 | - | 24/45 | - | - | - | - | 3 |
| | Gem + DDP | 45 | - | 23/45 | - | - | - | - | |
| | Gem + Docetaxel | 49 | - | 22/49 | - | - | - | - | |
| | Gem + Irinotecan | 44 | - | 21/44 | - | - | - | - | |
| Richards 2002[25] | Gem + Placebo | 88 | 213 | 62.9 | 20.4 | 25.9 (TTF) | 5/63 | - | 5 |
| | Gem + CI-994 | 86 | 191 | 60.8 | 18.5 | 16.7 | 1/61 | - | |
| Moore 2005[26] | Gem + Erlotinib | 285 | 191 | 58 | 24 | 32 (PFS) | 23/268 | - | 5 |
| | Gem + placebo | 284 | 177 | 49 | 17 | 25 | 21/262 | - | |
| Stathopoulos 2005[27] | Gem | 70 | 195 | 50 | 21.82 | - | 7/70 | - | 3 |
| | Gem + Irinotecan | 60 | 192 | 60 | 24.29 | - | 9/60 | - | |
| Riess 2005[28] | Gem | 236 | 186 | 53 | 20 | 30 (TTP) | 17/236 | - | 3 |
| | Gem + 5-FU/CF | 230 | 175.5 | 49 | 20 | 29 | 11/230 | - | |
| Herrmann 2005[29] | Gem | 157 | 219 | 62 | 27 | 42 (PFS) | 12/152[3] | - | 3 |
| | Gem + Capecitabine | 159 | 252 | 60 | 31 | 42 | 15/148 | - | |

[1]Gemcitabine 1 g/m$^2$ as a 100-min infusion; [2]Gemcitabine 1500 mg/m$^2$ at a fixed dose rate of 10 mg/m$^2$ per minute; [3]RECIST criteria.

proved pancreatic cancer, with same baseline data and without evidence of selection bias. Of the 22 trials, seven were randomized phase Ⅱ trials, and the others were randomized phase Ⅲ trials. The 6-mo survival rate was extracted from each of the 22 trials, and objective remission rates were recorded in most of the trials. Only a few trials provided CBR, PFS, TTP and TTF (time of treatment failure).

### Overall survival

The 5473 randomized patients from 22 RCTs, 2772 in the GEM combination group and 2701 in the GEM alone group, were included in the meta-analysis. The result of the test for heterogeneity of the therapeutic effect was not significant ($P = 0.19$). Therefore, we selected a fixed effect model. There was a significant improvement in 4% of the GEM combination group in 6-mo survival rate (95% CI 0.01-0.06, $P = 0.008$). The results of the meta-analysis in 6-mo survival rate are presented in Figure 2.

With the same technique, 5292 patients from 21 RCTs were analyzed. In the GEM combination group, a 3% improvement was made in 1-year survival rate as compared with the GEM alone group, and this difference being statistically significant (95% CI 0.01-0.05, $P = 0.01$).

The 4912 randomized patients from 21 RCTs, 2461 in the GEM combination group and 2451 in the GEM alone group, were included in the meta-analysis. The result of the test for heterogeneity of the therapeutic effect

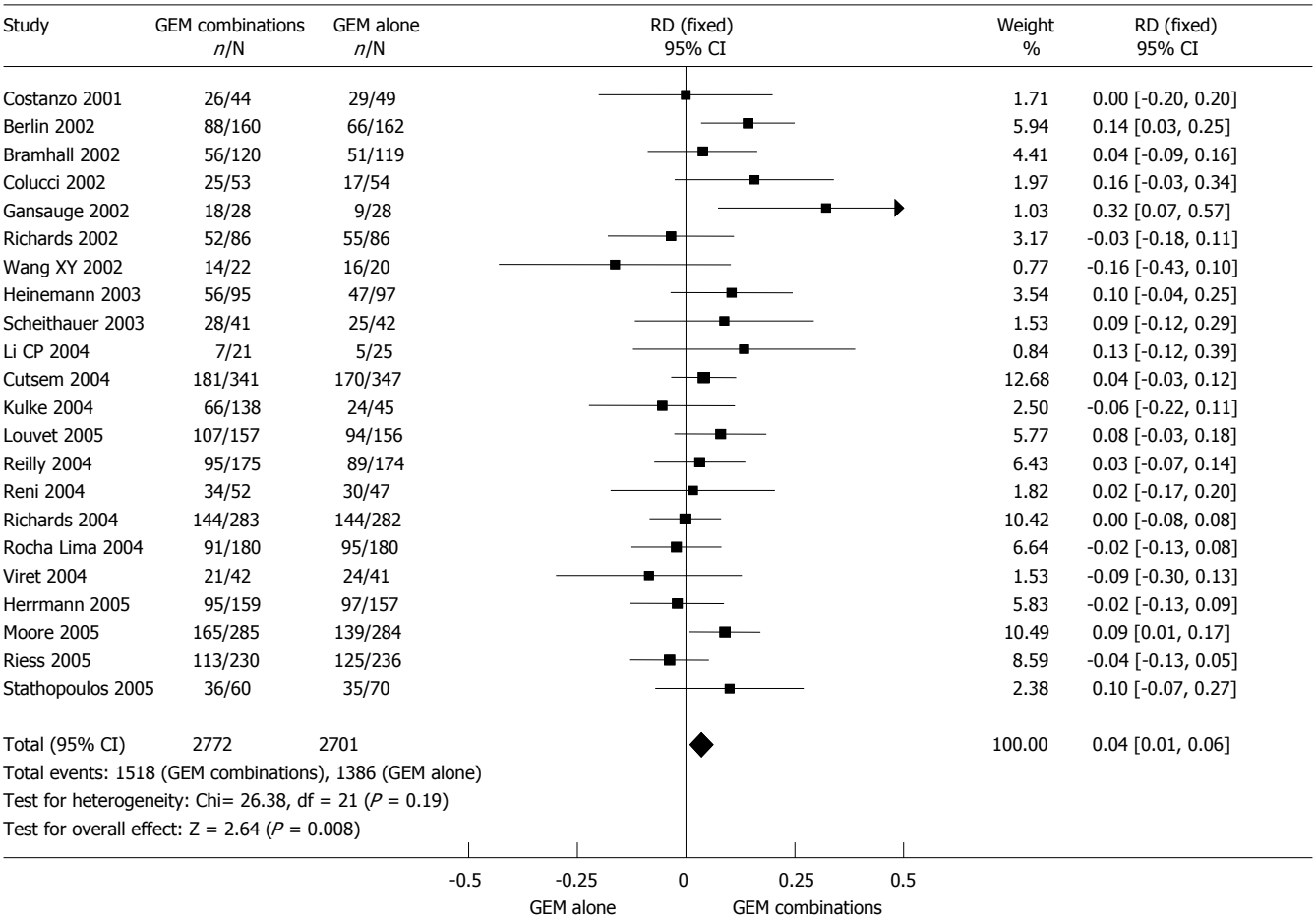


**Figure 2** Fixed effect model on RD of 6-mo survival rate.

was significant ($P < 0.0001$). A random effect model was adopted. There was a significant improvement in 4% of the GEM combination group in ORR (95% CI 0.01-0.07, $P = 0.02$). The outcome of the meta-analysis in ORR is presented in Figure 3.

The 580 randomized patients from 6 RCTs, 290 in the GEM combination group and 290 in the GEM alone group, were included in the meta-analysis. The result of the test for heterogeneity of the therapeutic effect was not significant ($P = 0.05$).

A fixed effect model was used. There was a significant improvement in 10% of the GEM combination group in CBR (95% CI 0.02-0.17, $P = 0.01$). The outcome of the meta-analysis in CBR is shown in Figure 4.

### Six-month TTP/PFS rate

TTP/PFS was defined as the period from randomization to documented disease progression for TTP or to disease progression or death for PFS. In almost all of the trials, patients recruited with good performance status died of disease progression, so TTP was very close to PFS. Therefore, we can analyze TTP and PFS together.

The 3783 randomized patients from 13 RCTs, 1889 in the GEM combination group and 1894 in the GEM alone group, were included in the meta-analysis. The result of the test for heterogeneity of the therapeutic effect was not significant ($P = 0.20$). A fixed effect model was used. Significant improvement was found in 7% of GEM combination group in 6-mo TTP/PFS rate (95% CI 0.04-0.10, $P < 0.00001$). The meta-analysis in TTP/PFS is presented in Figure 5.

### Toxic effects of chemotherapy

Toxic effects of 21 RCTs are summarized in Table 2 (only Grade 3-4 toxic effects were recorded). Main toxic effects were analyzed. Grade 3-4 toxicity was higher in GEM combination group for neutropenia (RD = 5%, 95% CI 0.01-0.10, $P = 0.02$), thrombocytopenia (RD = 5%, 95% CI 0.02-0.08, $P = 0.002$) and vomiting/nausea (RD = 3%, 95% CI 0.00-0.05, $P = 0.02$), all reached significant difference.

### Assessment for publication bias

Figures 6 and 7 represent funnel plots that test for publication bias. Funnel plots for the 6-mo survival rate (Figure 6) and 1-year survival rate (Figure 7) supported the lack of evidence for publication bias.

### Subgroup analysis

Table 3 shows the subgroup analyses in 6-mo survival rate. It revealed that only the combined chemotherapy consisting of GEM plus a new targeted drug yielded a 6% higher survival rate as compared with chemotherapy of GEM alone.

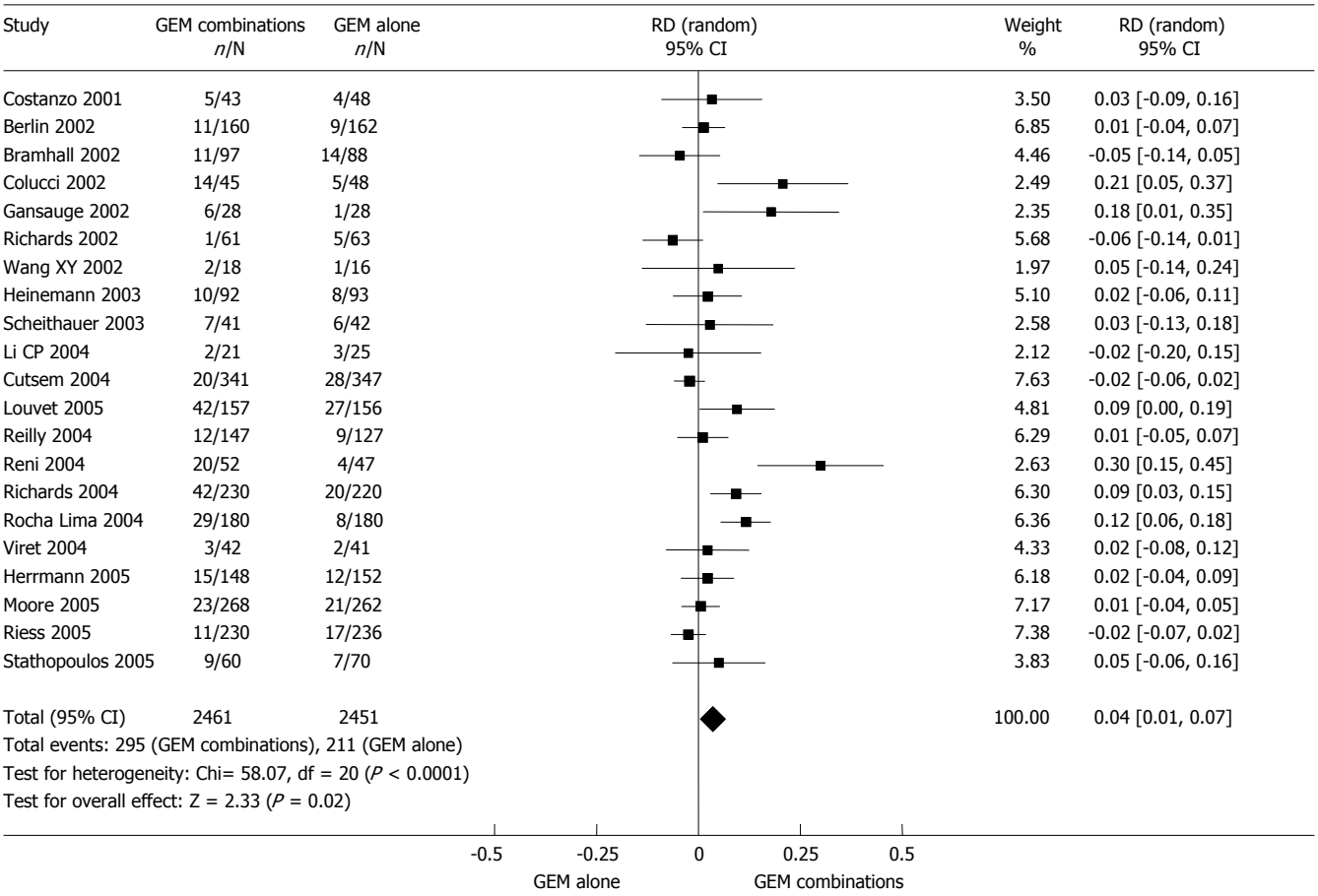


**Figure 3** Random effect model on RD of ORR.

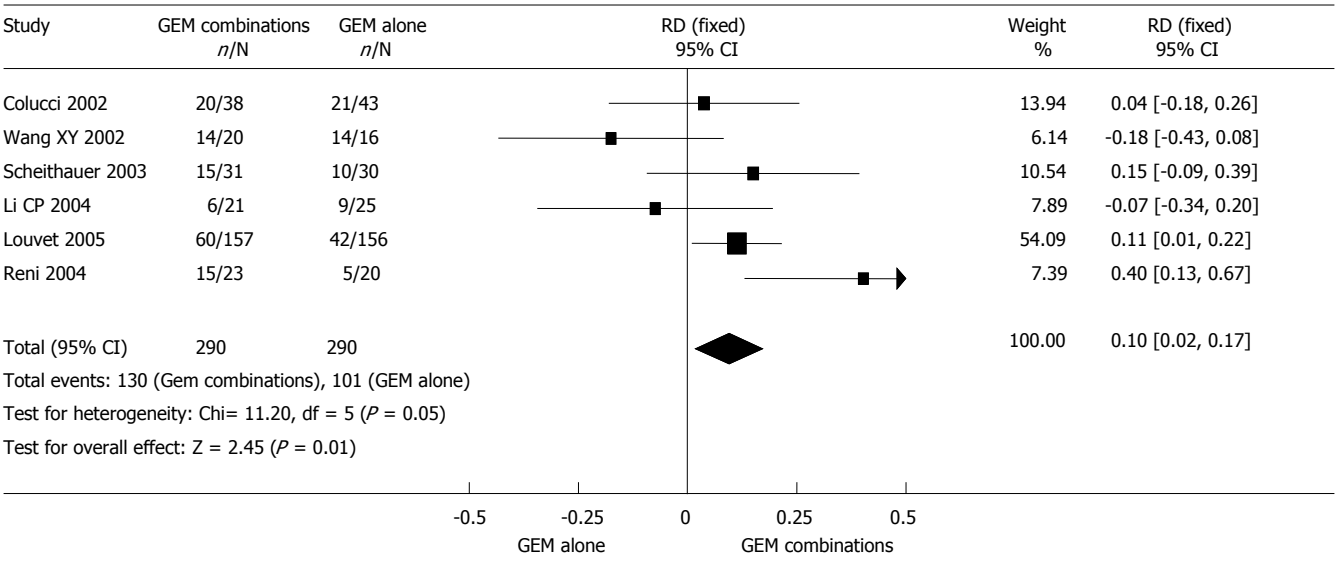


**Figure 4** Fixed effect model on RD of CBR.

## DISCUSSION

To improve the clinical results of GEM, Phase Ⅱ-Ⅲ trials have been made recently to evaluate the efficacy of combination of GEM with other drugs which were shown to be synergistic *in vitro*, such as 5-fluorouracil (5-FU), DDP, topotecan, *etc*[30,31]. Many trials demonstrated that combined GEM chemotherapy improved ORR and PFS compared with GEM alone, and a few trials reported significant OS advantage (Table 1).

The present meta-analysis shows that GEM combination produced a significant survival advantage as compared with GEM alone in patients with APCa. GEM combination was also found superior to GEM alone in

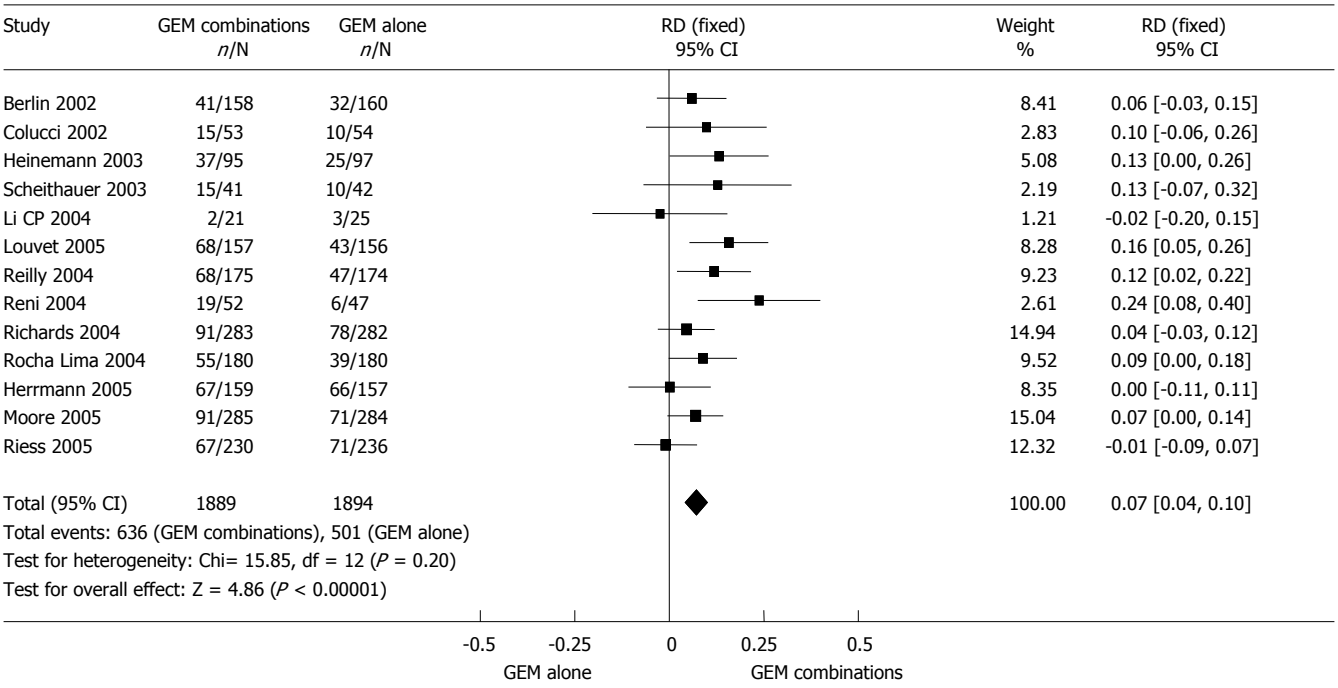


**Figure 5** Fixed effect model on RD of 6-mo TTP/PFS rate.

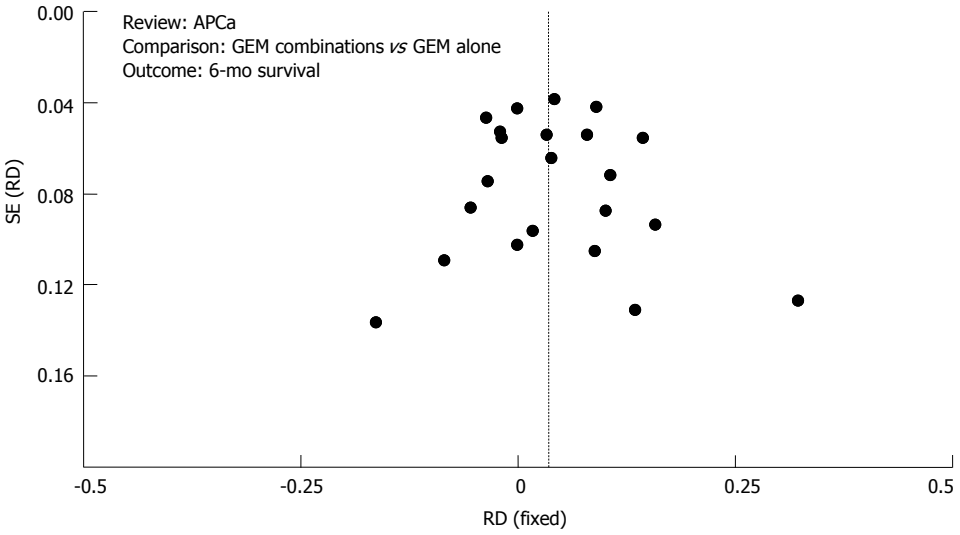


**Figure 6** Funnel plots for 6-mo survival rate.

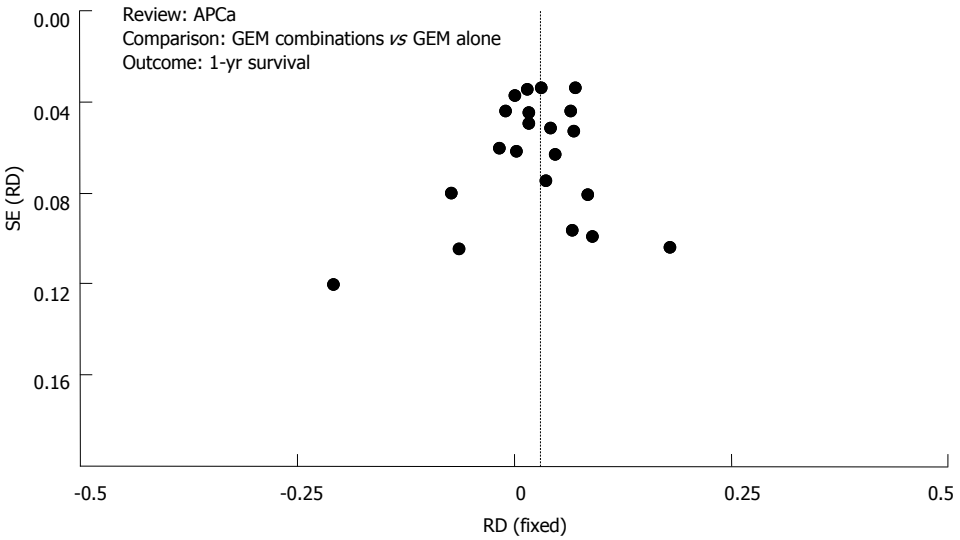


**Figure 7** Funnel plots for 1-yr survival rate.

**Table 2 Toxic effects recorded from randomized controlled trials (Grade 3-4 toxic effects)**

| Studies | Intervention | Neutrophils | Platelets | Anemia | Infection | Nausea/vomit | Mucositis | Diarrhea |
|---|---|---|---|---|---|---|---|---|
| Scheithauer2003[8] | Gem | 3/39 | 1/39 | 0 | 0 | 0 | 0 | 0 |
| | Gem + Capecitabine | 4/40 | 0 | 2/40 | 0 | 0 | 0 | 2/40 |
| Colucci 2002[9] | Gem | 5/53 | 1/53 | 2/53 | - | 1/53 | 1/53 | 0 |
| | Gem + DDP | 9/51 | 1/51 | 3/51 | - | 1/51 | 0 | 2/51 |
| Wang XY 2002[10] | Gem | 5/19 | 7/19 | 2/19 | - | 0 | - | - |
| | Gem + DDP | 7/21 | 8/21 | 9/21 | - | 2/21 | - | - |
| Gansauge2002[11] | Gem | - | - | - | - | 3/28 | - | 1/28 |
| | Gem + NSC-631570 | - | - | - | - | 1/18 | - | 0 |
| Berlin 2002[12] | Gem | 8/158 | 17/158 | 16/158 | - | 30/158 | 3/158 | /158 |
| | Gem + 5-FU | 11/158 | 30/158 | 16/158 | - | 29/158 | 2/158 | /158 |
| Bramhall 2002[13] | Gem + placebo | 9/119 | - | 7/119 | 12/119 | 16/119 | - | - |
| | Gem + marimastat | 3/120 | - | 3/120 | 11/120 | 13/120 | - | - |
| Cutsem 2004[14] | Gem + placebo | 103/342 | 41/342 | 55/342 | - | 58/342 | - | 10/342 |
| | Gem + R115777 | 132/331 | 50/331 | 66/331 | - | 46/331 | - | 13/331 |
| Louvet 2005[15] | Gem | 2/156 | 5/156 | 16/156 | - | 12/156 | - | 2/156 |
| | Gem + Oxaliplatin | 2/157 | 22/157 | 10/157 | - | 30/157 | - | 9/157 |
| Reilly 2004[16] | Gem | 24/157 | 7/157 | 13/157 | - | 17/157 | - | 2/157 |
| | Gem + DX-8951f | 50/168 | 28/168 | 11/168 | - | 33/168 | - | 6/168 |
| Richards 2004[17] | Gem | 35/273 | 17/273 | 8/273 | - | 18/273 | - | 2/273 |
| | Gem + Pemetrexed | 123/273 | 49/273 | 38/273 | - | 18/273 | - | 8/273 |
| Li CP 2004[18] | Gem | 2/25 | 1/25 | 2/25 | - | - | - | - |
| | Gem + DDP | 4/21 | 5/21 | 2/21 | - | - | - | - |
| Reni 2004[19] | Gem | 9/47 | 1/47 | 2/47 | - | 4/47 | 1/47 | - |
| | Gem + 5-FU + DDP + EPI | 22/52 | 15/52 | 2/52 | - | 3/52 | 2/52 | - |
| Viret 2004[20] | Gem | 16/40 | 5/40 | 11/40 | - | 3/40 | - | - |
| | Gem + DDP | 23/41 | 14/41 | 14/41 | - | 9/41 | - | - |
| Rocha Lima 2004[21] | Gem | 54/169 | 24/169 | 22/169 | - | 31/169 | - | 3/169 |
| | Gem + Irinotecan | 65/173 | 34/173 | 27/173 | - | 53/173 | - | 33/173 |
| Costanzo 2001[22] | Gem | 1/49 | 0 | 3/49 | - | 0 | 0 | 0 |
| | Gem + 5-FU | 1/41 | 1/41 | 3/41 | - | 1/41 | 2/41 | 0 |
| Heinemann 2003[23] | Gem | 8/97 | 10/97 | 10/97 | 2/97 | 6/97 | 2/97 | 4/97 |
| | Gem + DDP | 10/95 | 4/95 | 13/95 | 1/95 | 21/95 | 4/95 | 3/95 |
| Kulke 2004[24] | Gem | 27/58 | 15/58 | 6/58 | 6/58 | 13/58 | - | 1/58 |
| | Gem + DDP | 29/62 | 27/62 | 11/62 | 2/62 | 24/62 | - | 0/62 |
| | Gem + Docetaxel | 19/65 | 7/65 | 8/65 | 8/65 | 10/65 | - | 5/65 |
| | Gem + Irinotecan | 12/60 | 9/60 | 4/60 | 4/60 | 17/60 | - | 10/60 |
| Moore2005[26] | Gem + Erlotinib | 71/282 | 28/282 | 34/282 | 45/282 | 20/282 | <1 | 17/282 |
| | Gem + placebo | 73/280 | 34/280 | 34/280 | 39/280 | 20/280 | 0 | 6/280 |
| Stathopoulos 2005[27] | Gem | 8/70 | 0/70 | 2/70 | 0 | 1/70 | 0 | 2/70 |
| | Gem + Irinotecan | 10/60 | 2/60 | 2/60 | 0 | 1/60 | 0 | 2/60 |
| Riess 2005[28] | Gem | 27/225 | 15/225 | 15/225 | 19/225 | 16/225 | - | 9/225 |
| | Gem + 5-FU/CF | 26/220 | 28/220 | 18/220 | 12/220 | 30/220 | - | 8/220 |
| Herrmann 2005[29] | Gem | 30/153 | 7/153 | 9/153 | - | 5/153 | 1/153 | 3/153 |
| | Gem + Capecitabine | 34/155 | 8/155 | 9/155 | - | 8/155 | 0/155 | 8/155 |

5-FU: 5-fluororacil; EPI: Epirubicin.

terms of ORR, CBR and 6-mo TTP/PFS. Although most of the selected RCTs showed no significant survival advantage in the GEM combination group, many trials demonstrated slight survival benefit. Physicians should carefully interpret these results when they apply them in clinical practice because GEM combined with other regimens might lead to reversed therapeutic effects.

Straightforward conclusions from the results of this meta-analysis do support the use of GEM combination in patients with APCa, but toxicities from intensive chemotherapy may obliterate the survival benefit of GEM combination. In another meta-analysis, we had reported that the regimens GEM plus DDP were not superior to GEM alone in patients with APCa, which produced more side effects[32]. Furthermore, the subgroup analyses did not show any significant survival advantage in most of GEM combination groups, such as GEM plus 5-FU, GEM plus topoisomerase I inhibitor, and so on. It indicates that not all GEM combined chemotherapy have therapeutic advantage. We suggest that GEM combination, including GEM plus oxaliplatin, and GEM plus erlotinib, should be considered as optimal treatment for patients with APCa. In addition, we found that patients with good perforemance status gained great survival advantage in the sub-group analyses as reported by many other authors[28,29,12]. In our opinion, GEM combination should be applied to patients with good performance status, but carefully to the weak patients.

We found that patients receiving GEM-based combination therapy developed side effects more frequently, including neutropenia, thrombocytopenia and vomiting/nausea , which might lead to a deterioration in

**Table 3 Subgroup analyses on 6-mo survival rate**

| Subgroups | Trials | Patients | Mode | RD [95% CI] | *P* |
|---|---|---|---|---|---|
| GEM plus targeted drug *vs* GEM alone | [13, 14, 26] | 1496 | Fixed | 0.06 [0.01, 0.11] | 0.02 |
| GEM plus DDP *vs* GEM alone | [9, 10, 18, 20, 23, 24] | 560 | Fixed | 0.05 [-0.03, 0.13] | 0.24 |
| GEM plus 5-FU *vs* GEM alone | [12, 22, 28] | 881 | Random | 0.04 [-0.09, 0.17] | 0.57 |
| GEM plus topoisomerase I inhibitor *vs* GEM alone | [16, 21, 24, 27] | 928 | Fixed | 0.01 [-0.05, 0.08] | 0.72 |
| GEM plus capecitabine *vs* GEM alone | [8, 29] | 399 | Fixed | 0.00 [-0.08, 0.10] | 0.97 |

quality of life (QOL). However, the significant advantage of CBR and TTP/PFS in the GEM combination might be converted to the improvement of QOL**.** Because the primary role of chemotherapy in patients with APCa is palliative, the influence on the QOL of the patients is an important issue in determining the true value of the therapy. However, because the methods for QOL assessment from the included trials were quite different, there was no valid meta-analysis of QOL . We also noted that the CBR analysis was made in only six trials, so the result was still unreliable.

The mata-analysis was based on RCTs with high quality. We carried out a comprehensive search of the literature with barely all of cancer database. Publication bias is frequently cited as a reason for lack of validity in meta-analyses. It could occur if studies finding no association between exposure and disease were less likely to be submitted and accepted for publication than studies finding a positive association. In fact, the results of most of the studies in our meta-analyses were negative, as stated by the authors. The funnel plots also showed no evidence of publication bias. Therefore, our meta-analysis provided a valid assessment and creditable results.

Several technical issues have to be mentioned regarding this meta-analysis. One major limitation is the data source extracted from abstracted data and not individual patient data (IPD). In general, an IPD-based meta-analysis would give a more robust estimation for the association, therefore, we should interpret the results with care, especially for a positive result. Clearly, further investigations using IPD should be conducted to examine the main end points. Publication bias is a significant threat to the validity of meta-analysis. Although we detected no evidence of publication bias using the graphical method, it is difficult to completely rule out this possibility. Heterogeneity among trials can be another limitation of our meta-analysis. Although we applied a random-effect model that takes possible heterogeneity into consideration, there were still many factors causing heterogeneity, such as different drug combination, two infusion methods of gemcitabine and so on.

In conclusion, the meta-analysis indicates that GEM-based combination therapy may improve the overall survival and palliation in optimal patients with APCa as compared with GEM alone. Although the application of GEM combination is still controversial, it is a progressive method from the prospective view of point. At the same time, new regimens of drug administration should be explored in future studies.

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*CLINICAL RESEARCH*

# Relationship between antral distension and postprandial symptoms in functional dyspepsia

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## Abstract

**AIM:** To investigate in patients with functional dyspepsia (FD) after an every-day meal whether (1) gastrointestinal (GI) and extra-GI symptoms had any relation with the degree of antral volume, (2) the onset of postprandial symptoms was associated with, and may predict, delayed gastric emptying.

**METHODS:** In 94 symptomatic FD patients, antral volume variations and gastric emptying were assessed with ultrasonography after a 1050 kcal meal. Symptoms were evaluated with a standardized questionnaire. The association of GI and extra-GI symptoms with antral volumes and gastric emptying were estimated with logistic regression analysis.

**RESULTS:** Forty percent of patients did not report any symptoms after a meal. Compared to the healthy controls, the antrum was more distended in patients throughout the entire observation period and 37 (39.4%) patients had delayed gastric emptying. Only postprandial drowsiness was associated with antral volume variations (AOR = 1.42; $P < 0.001$) and with delayed gastric emptying (AOR = 3.59; $P < 0.03$).

**CONCLUSION:** In FD patients, GI symptoms are neither associated with antral distension nor with gastric emptying. Drowsiness is associated with antral distension and delayed gastric emptying. The onset of drowsiness is preceded by an increment of antral distension and the duration of the symptom appears to be related to the persistence of antral distension.



**Key words:** Antral distension; Functional dyspepsia; Gastric emptying, Ultrasonography

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## INTRODUCTION

Patients with functional dyspepsia (FD)[1] complain of several gastrointestinal (GI) and extra-gastrointestinal (extra-GI) symptoms[2-5] that are usually associated with food ingestion[6]. Several pathophysiological abnormalities have been implicated in the etiology of symptoms, but so far they failed to establish a clear-cut association between symptoms and specific function abnormalities and often similar studies provided contrasting findings[6-13]. Despite symptoms are often related to food ingestion, few studies have assessed the relationship between the occurrence of GI symptoms after meal ingestion and gastric functions[6,10,11,14-19] and reported non-univocal results. In addition, investigations performed while patients are symptom-free may miss their underlying pathophysiological mechanisms because as the symptoms, they may wax and wane over time[20]. Ricci *et al*[14] firstly and others subsequently[15,16,18,21,22] reported a distended fasting antrum and an increased post-prandial antral volume in patients with FD as compared with healthy controls, suggesting that an impaired motor function of the distal stomach is present in a subgroup of patients with FD. However, only two studies[14,15] evaluated the temporal association between the onset of postprandial GI symptoms and antral distension assessed with ultrasonography (US). The first study reported a close association between the onset of the postprandial GI complaints[14] and antral volume increase in the majority (71%) of patients. The second study reported an association between bloating and increased antral area[15]. We have recently shown that a subgroup of patients with FD reported postprandial drowsiness as a bothersome symptom, in addition to the GI dyspeptic ones[5]. Drowsiness is a subjective experience often reported after food ingestion[23]. It has also been shown that solid but not liquid meal results in a decreased sleep onset latency in healthy volunteers[24], suggesting that a post-ingestion mechanism rather than a cephalic stimulus is involved as an initial trigger in mediating the phenomenon.

We aimed to further evaluate the relationship between the onset of postprandial symptoms, both GI and extra-GI symptoms, such as headache and drowsiness, and the modality of the postprandial antral volume increase in FD patients.

The primary aim of the present study was to investigate with regression analysis models, in controlled condition, after an every-day balanced meal, whether upper GI and extra-GI symptoms had any relation with the degree of antral volume assessed with US in FD patients. The secondary aim was to evaluate whether the onset of postprandial symptoms was associated to, and may predict, delayed gastric emptying.

## MATERIALS AND METHODS

### Subjects

Two hundred and seventeen consecutive patients with chronic symptoms of dyspepsia (143 females, 74 males; age, 42.4 ± 12.2 years;) referred to the gastroenterology outpatient clinic were prospectively assessed. Functional dyspepsia (FD) was defined according to the Rome Ⅱ criteria[1]. Organic abnormalities, psychiatric illnesses, eating disorders, history of alcohol and caffeine abuse, use of NSAID, steroids or drugs affecting gastric function, previous GI surgery (except appendectomy and cholecystectomy) and systemic disorders were ruled out by history, clinical examination, biochemical investigations, upper GI endoscopy, and transabdominal US. Dyspeptic patients with Rome diagnostic criteria of irritable bowel syndrome (IBS) and/or referring heartburn and/or regurgitation as predominant or frequent symptoms were excluded from the study.

Severity of epigastric pain and upper abdominal discomfort was graded 0-4 according to its effect on patient's daily activities: 0 = absent; 1 = mild (present but easily bearable if distracted by usual activities); 2 = moderate (bearable but not influencing usual activities); 3 = relevant (influencing usual activities); and 4 = severe (interruption of usual activities)[5]. Patients were symptomatic at the time of, and in the 3 weeks preceding, the investigation. Dyspeptic symptoms had to be present more than 3 days a week, with pain and discomfort scored at least as moderate (≥ 2).

Overall 123 patients (80 females, 43 males; age, 44 ± 13.5 years) were excluded from the study because of the following diagnosis: 59 with gastroesophageal reflux disease; 15 with IBS; 14 with peptic ulcer disease; 16 with migraine; 3 with psychiatric disorders; 2 with celiac disease; and 14 with not properly reporting symptoms during the test.

Ninety-four consecutive patients (63 females, 31 males; age, 42 ± 12 years) fulfilling Rome Ⅱ diagnostic criteria of functional dyspepsia[1] and 21 healthy subjects (13 females, 8 males; age, 30 ± 8.5 years) without GI symptoms participated in the study.

Informed consent was obtained from each subject and the Local Ethics Committee approved the study protocol.

### Assessment of symptoms

An experienced gastroenterologist (EC) interviewed the patients before the study. GI symptoms were enquired by means of the validated Italian version of the Rome Ⅱ modular questionnaire[25]. The validation process and validity of the translated questionnaire have been formally assessed and approved by the Coordinating Committee of the Rome Foundation (on files of the Committee). The questionnaire also includes items inquiring on demography (5 items), daily habits (10 items), meal timing and composition, alcohol consumption, smoking and sleep patterns, past medical history (3 items), somatic extra-GI symptoms (5 items) as previously reported[2].

Frequency and time relationship with meal ingestion were assessed for each of the following dyspeptic symptoms, as defined by the Rome Ⅱ criteria[1]: pain or discomfort centered in the upper abdomen, nausea, vomiting, fullness, bloating, early satiety, epigastric burning and belching. Postprandial drowsiness was defined as a state of impaired awareness associated with a desire or inclination to sleep[26]. Furthermore, patients were requested to refer any other symptom they considered to be bothersome and related to meal ingestion.

### Study protocol

Gastric antral volume was evaluated at US as previously described[27,28] with 3.5-MHz convex probe (Tosbee, Toshiba, Japan). Gastric emptying was evaluated with US according to previously validated and standardized methods[27-32]. All drugs affecting the GI tract were discontinued at least 3 d before the study. Subjects refrained from smoking 12 h before and during the examination. Beverages, including water, coffee and tea were not allowed before and during the examination. The presence of sleep disturbances the night before the test and the quality of sleep in the last month were specifically enquired. After an overnight fast, subjects ate an ordinary standard solid meal of 1050 kcal containing 140 g bread, 70 g cheese, 80 g ham, (50 % carbohydrates, 25% lipids, 25% proteins) and 250 mL of water. The time of meal ingestion did not exceed 30 min (range, 15-30 min). The same proven-skilled operator (NP) performed gastric antral US measurements, with the subjects standing in the upright position. Subjects were studied in fasting condition, soon after the meal ingestion, at 30 and 60 min after the end of the meal ingestion, and at 60 min intervals thereafter for 300 min. In the intervals between measurements subjects could move freely.

Delayed gastric emptying was defined as the final antral volume exceeding the mean value plus 2SDs of the healthy controls[5].

Patients and healthy controls were requested to report every 30 min any symptom occurring after the meal ingestion. The ultrasonographer was blind for the referred historical symptoms.

### Statistical analysis

To compare patients and healthy controls as well as temporal meal-related antral volume variations, arithmetic mean, standard deviation, median values and interquartile ranges were calculated. Continuous variables (age, body mass) were compared using student's t-test. Box-plots[33] were used to provide an immediate graphical evaluation of

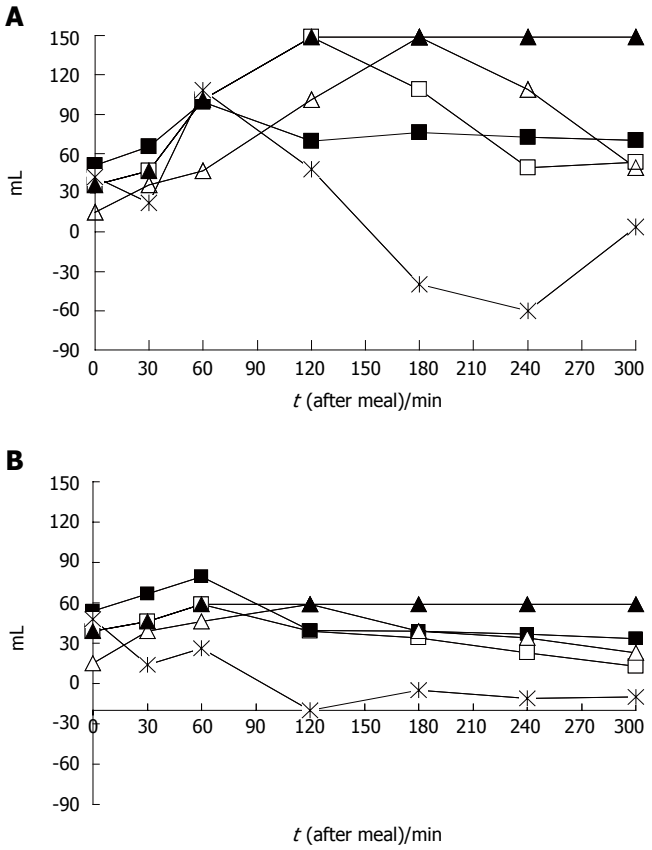


**Figure 1** Antral volume measurements and their transformations. **A**: Patients referring postprandial drowsiness during the study period; **B**: Patients not referring symptoms during the study period. Open quadrangle: current antral volume; asterisk: antral volume delta variation between two consecutive measurements; Closed quadrangle: mean weighted antral volume; Closed triangle: maximal antral volume; Open triangle: antral volume value at the preceding time intervals.

the postprandial gastric antral volume distribution during the study period. Linear regression analysis for repeated measurements was applied to evaluate during the study period whether there were differences in the antral volumes between patients and healthy controls. The cumulative probability of developing symptoms during the 5 h study period was evaluated using the Kaplan-Meier method. Logistic regression models[34] were applied to estimate crude (OR) and adjusted odds ratios (AOR) for age, body mass index (BMI) and gender, and 95% confidence intervals (95%CI) of having an association between antral volume variations and presence/absence of each GI and extra-GI symptom at each time interval evaluated. We also investigated specific transformations of the current gastric antral volume evaluated for a 10 mL unit change as follows (Figure 1 A and B): (1) the antral volume value at the preceding time interval; (2) the antral volume delta variation between two consecutive measurements; (3) the mean weighted antral volume value at each measurement (i.e., at each time when the volume was measured, the value obtained as the arithmetic mean of the current value together with all previous measurements); (4) the maximal antral volume value reached after a meal between the current and the previous measurement.

Logistic regression analysis was also applied to estimate crude and adjusted odds ratios of having delayed gastric emptying for each symptom, fasting antral volume, gender, age, and BMI. We reported results from multiple logistic regression analyses, obtained through a backward selection strategy having excluded factors with a $P$ value >0.20 obtained by a likelihood-ratio test[33]. Two-sided $P$ values were defined statistically significant when $P < 0.05$, and marginally significant when $0.05 < P < 0.2$. All the analyses were performed using STATA release 8.0[35].

**Table 1 Major demographic characteristics of investigated subjects**

| | Healthy controls ($n$ = 21) | FD patients ($n$ = 94) | $P$ |
|---|---|---|---|
| Age median (IQR) yr | 27 (26-29) | 42 (31-52) | < 0.001 |
| BMI median (IQR) kg/m$^2$ | 22.4 (20-23) | 22.0 (19.8-25.0) | 0.47 |
| Female (%) | 13 (61.9%) | 62 (66.0%) | 0.72 |

IQR: Interquartile range.

## RESULTS

### *Study population and GI and extra-GI symptoms profile*

At inclusion, all FD patients reported frequent pain or discomfort (60% of them almost daily) graded at least as moderate. Demographic characteristics of the subjects evaluated are summarized in Table 1. There was no significant difference between the healthy controls and the FD patients for BMI and gender, whereas the controls were younger ($P < 0.001$) than the FD patients. After a meal, none of the healthy subjects reported any symptoms. Of the FD patients, 39 (41.5%) did not refer any symptoms and 55 (58.5%) reported one or more GI and extra-GI symptoms. In this group of 55 symptomatic patients, postprandial drowsiness was reported as a predominantly bothersome symptom in 17 patients, presenting as the only symptom in 3, in association with pain in 3, with fullness in 6, with pain and fullness in 2, with belching in 2 and with bloating in 1. Reported symptoms' severity in the medical history did not significantly differ between the patients with and without postprandial symptoms during the investigation. The cumulative probability to develop at least one of the GI and extra-GI symptoms was 58.5% (Figure 2). None of the patients referred early satiety, vomiting and heartburn (not shown in Figure 2). Despite the patients with history of regurgitation were excluded from the study, 2 patients referred postprandial acid regurgitation (not shown in Figure 2 and Tables 2 and 3). The percentage of patients with symptoms at each evaluated time interval is reported in Table 2. Among the patients who referred at least one symptom, 55.5% had the first symptom within 60 min after meal ingestion. Postprandial drowsiness started later (71.5 ± 64.8 min) than dyspeptic symptoms (38.3 ± 55.4 min, $P < 0.001$).

### *Antral volume assessments and gastric emptying*

Antral volume before, immediately (time 0) and during the 300 min observation period after meal ingestion in

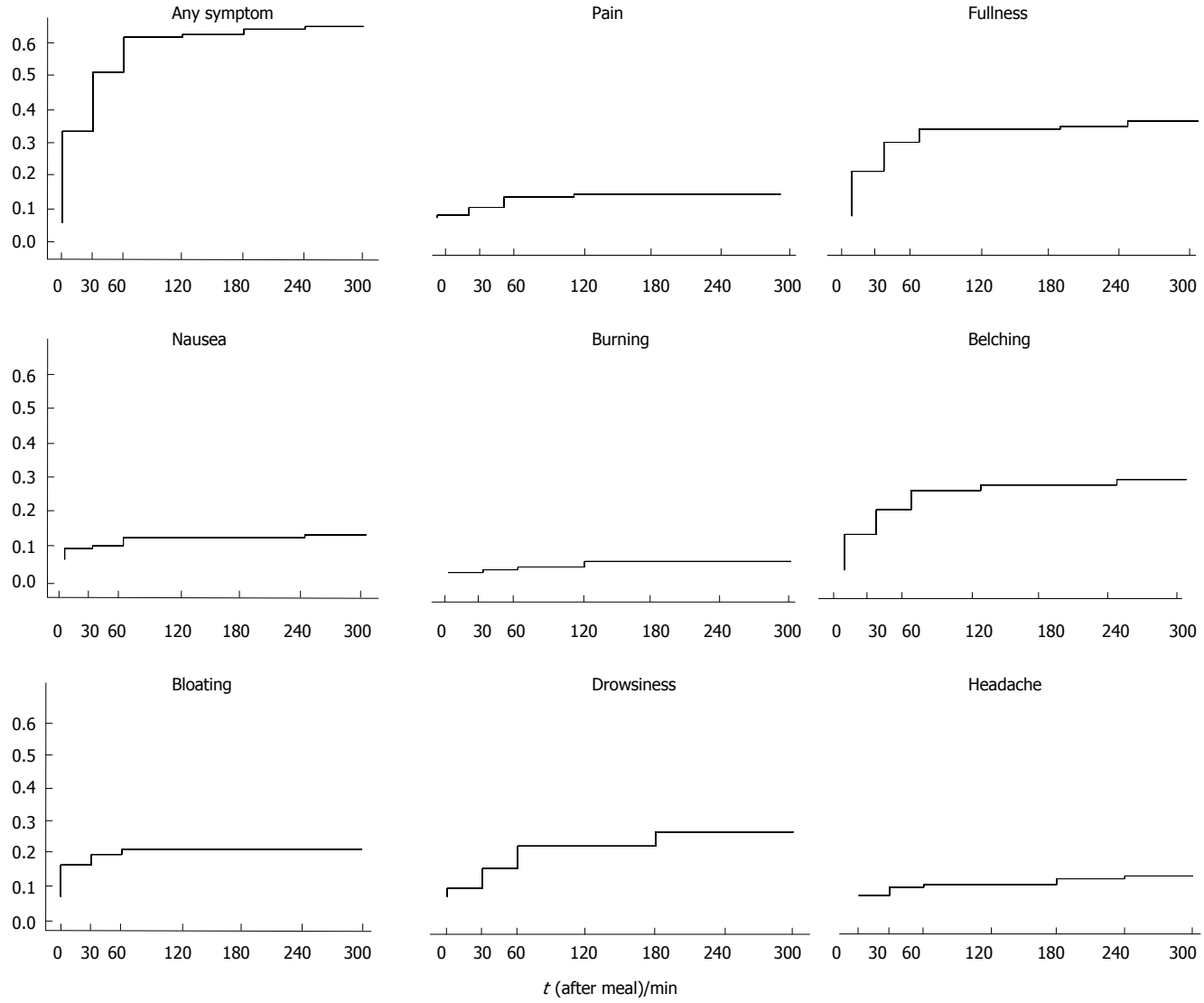


**Figure 2** Kaplan-Meier survival curves showing the cumulative probability to develop the evaluated symptoms.

**Table 2 Percentage of FD patients with symptoms at each time interval evaluated**

| Time (min) | Pain (%) | Fullness (%) | Bloating (%) | Nausea (%) | Belching (%) | Burning (%) | Headache (%) | Drowsiness (%) | Any (%) |
|---|---|---|---|---|---|---|---|---|---|
| 0 | 1 | 13.8 | 9.6 | 3.2 | 10.6 | 0 | 0 | 2.1 | 26.6 |
| 30 | 3.2 | 22.3 | 12.8 | 4.3 | 18 | 1 | 2.1 | 7.5 | 44.7 |
| 60 | 6.4 | 24.5 | 14.9 | 5.3 | 22.3 | 2.1 | 3.2 | 13.8 | 54.3 |
| 120 | 6.4 | 24.5 | 14.9 | 5.3 | 24.5 | 3.2 | 3.2 | 13.8 | 54.3 |
| 180 | 4.3 | 20.2 | 12.8 | 5.3 | 20.2 | 3.2 | 3.2 | 17 | 48.9 |
| 240 | 3.2 | 14.9 | 10.6 | 5.3 | 18 | 3.2 | 4.3 | 15.9 | 43.6 |
| 300 | 2 | 12.8 | 9.6 | 5.3 | 15.9 | 2.1 | 2.1 | 12.8 | 38.3 |
| At least once | 7.4 | 28.7 | 14.9 | 7.4 | 26.6 | 3.2 | 5.3 | 18 | 58.5 |

the controls and the FD patients is shown in Figure 3. In the FD patients, the postprandial antral volume was significantly larger compared to the controls ($P < 0.05$) from the end of meal ingestion up to the final observation. The multiple linear regression model showed that in the postprandial period antral volume, adjusted for gender and age, was higher in the dyspeptic patients compared to the controls with a difference in volume value of 11 mL during the entire period ($P < 0.01$). Gastric emptying was delayed in 37 (39.4%) of the FD patients.

### *Predictors of postprandial symptoms and gastric emptying in functional dyspepsia*

Odds ratios simultaneously adjusted for minutes after meal, BMI, age and gender in predicting the occurrence of GI and extra-GI symptoms evaluated for each of

**Table 3 AOR with 95% CI of having GI and extra-GI symptoms on the basis of antral volume variations**

| | Current AV (per 10 mL unit increase) | | | Previous AV (per 10 mL unit increase) | | | Delta AV (per 10 mL unit increase) | | | Mean weighted AV (per 10 mL unit increase) | | | Maximal AV (per 10 mL unit increase) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Symptom | AOR | 95% CI | *P* | AOR | 95% CI | *P* | AOR | 95% CI | *P* | AOR | 95% CI | *P* | AOR | 95%CI | *P* |
| Pain | 0.96 | 0.79-1.18 | 0.72 | 0.96 | 0.78-1.17 | 0.67 | 1.03 | 0.93-1.14 | 0.54 | 0.94 | 0.67-1.31 | 0.72 | 0.9 | 0.69-1.19 | 0.49 |
| Fullness | 0.99 | 0.84-1.17 | 0.97 | 0.98 | 0.82-1.18 | 0.88 | 1 | 0.95-1.06 | 0.82 | 0.95 | 0.73-1.25 | 0.74 | 0.97 | 0.83-1.13 | 0.68 |
| Bloating | 1.21 | 0.98-1.49 | 0.07 | 1.22 | 0.98-1.51 | 0.06 | 0.99 | 0.93-1.05 | 0.7 | 1.37 | 0.99-1.89 | 0.05 | 1.21 | 0.99-1.48 | 0.06 |
| Nausea | 0.99 | 0.76-1.3 | 0.98 | 1.06 | 0.8-1.4 | 0.67 | 0.84 | 0.7-1 | 0.05 | 1.18 | 0.82-1.69 | 0.37 | 1.17 | 0.94-1.44 | 0.15 |
| Burning | 0.87 | 0.55-1.38 | 0.57 | 0.81 | 0.39-1.67 | 0.58 | 1.1 | 0.87-1.39 | 0.39 | 0.68 | 0.2-2.25 | 0.53 | 0.64 | 0.17-2.33 | 0.5 |
| Belching | 1 | 0.84-1.19 | 0.95 | 0.96 | 0.78-1.17 | 0.67 | 1.07 | 1.01-1.12 | 0.008 | 0.94 | 0.69-1.27 | 0.69 | 0.98 | 0.82-1.17 | 0.81 |
| Headache | 1.04 | 0.77-1.4 | 0.79 | 1 | 0.77-1.31 | 0.96 | 1.03 | 0.88-1.2 | 0.69 | 0.9 | 0.58-1.43 | 0.68 | 0.93 | 0.7-1.23 | 0.64 |
| Drowsiness | 1.42 | 1.16-1.73 | 0.001 | 1.33 | 1.08-1.65 | 0.007 | 1.12 | 1.06-1.19 | 0.001 | 1.46 | 1.05-2.01 | 0.02 | 1.26 | 1.03-1.53 | 0.02 |
| Palpitation | 1 | 0.72-1.4 | 0.96 | 0.99 | 0.71-1.38 | 0.97 | 0.98 | 0.9-1.06 | 0.67 | 0.89 | 0.49-1.62 | 0.7 | 0.93 | 0.65-1.32 | 0.68 |
| Any symptom | 1.13 | 0.97 -1.31 | 0.12 | 1.1 | 0.94 -1.3 | 0.24 | 1.03 | 0.99 -1.08 | 0.11 | 1.15 | 0.9-1.46 | 0.25 | 1.11 | 0.95-1.29 | 0.18 |

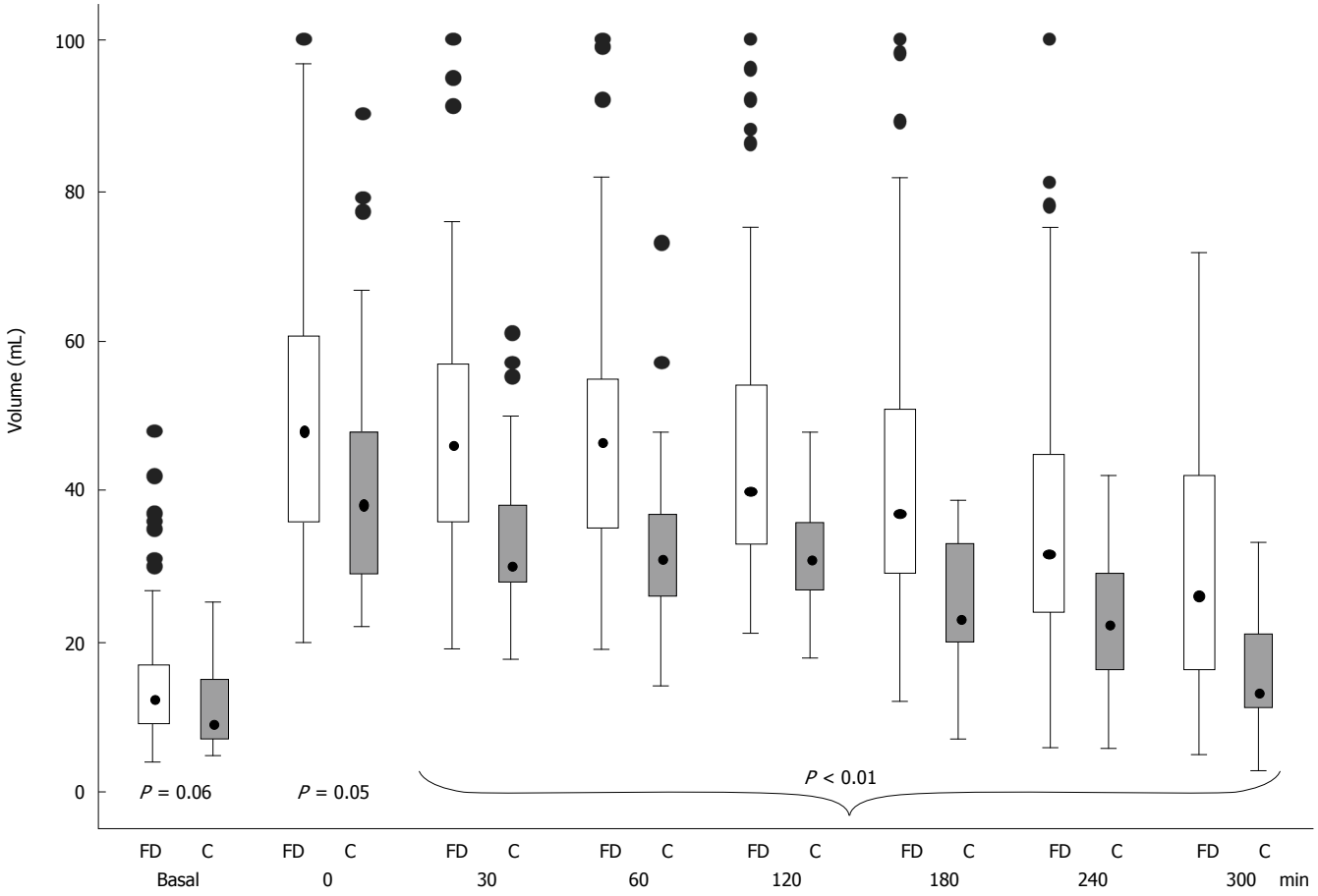


**Figure 3** Box-and-whiskers plots of gastric antral volume before (fasting), immediately (0), at 30 and at 60 min intervals, after the end of the ingestion of a standard meal in controls (gray box) and in dyspeptic (white box). The boxes at each time unit extend from the 25th percentile ($x_{[25]}$) to the 75$^{th}$ percentile ($x_{[75]}$) [i.e., the interquartile range (IQ)]; the lines inside the boxes represent the median values. The lines emerging from the boxes (i.e., the "whiskers") extend to the upper and lower adjacent values. The upper adjacent value is defined as the largest data point $\leqslant x_{[75]} + 1.5 \times IQ$, and the lower adjacent value is defined as the smallest data point $\geqslant x_{[25]} - 1.5 \times IQ$. Observed values more extreme than the adjacent values, if any, are individually plotted (circles).

the antral volume transformations described in Figure 1 are reported in Table 3. The occurrence of postprandial drowsiness was related to (1) the current antral volume value, (2) the antral volume value at the previous time interval, (3) the antral volume delta variation between two consecutive measurements, (4) the mean postprandial weighted antral volume and (5) the maximal antral volume value reached after a meal. The occurrence of nausea and belching was related to the antral volume delta variation between two consecutive measurements. The occurrence of bloating was significantly related to a mean postprandial weighted antral volume, and marginally associated with (1) the current antral volume value, (2) the antral volume value at the previous time interval, and (3) the maximal antral

**Table 4 AOR with 95% CI of having delayed gastric emptying on the basis of symptoms, fasting antral volume (FAV) and BMI**

| | AOR | 95% CI | *P* |
|---|---|---|---|
| Drowsiness | 3.59 | 1.12-11.54 | 0.03 |
| Fasting antral volume[1] | 1.09 | 1.02-1.17 | 0.007 |

AOR: Adjusted odds ratio; [1]AOR estimated per 5 mL increase (e.g. FAV of 20 mL *vs* 15 mL).

volume value reached after a meal.

Age was the only factor associated with the occurrence of some of the GI and extra-GI meal-related symptoms. Particularly, the occurrence of postprandial drowsiness was significantly associated with older age (AOR = 1.05 per 1 year increase, $P < 0.02$), while pain was marginally related to younger age (AOR = 0.96, $P < 0.04$). Epigastric burning was marginally related to older age (AOR = 1.11, $P < 0.03$).

The estimated AORs of having delayed gastric emptying are shown in Table 4. After a backward selection, only fasting antral volume and postprandial drowsiness were significantly associated with delayed gastric emptying, while no statistically significant relationship was found with age, gender and BMI. Fasting antral volume was significantly associated with delayed gastric emptying, increasing the OR of 93% for any additional volume increase of 5 mL.

## DISCUSSION

An unexpected finding of this study was that more than 40% of symptomatic patients at the time of, and in the 3 wk preceding, the investigation did not refer any symptoms when challenged with a normal meal in controlled condition. The lack of any difference in the demography and symptom presentation between symptomatic and symptom-free patients during the investigation excludes a patient selection bias and indicates that the well known long-term variability[20] of dyspeptic symptoms may occur even over a short period of time.

Ultrasonography is a reliable method to estimate in normal physiological conditions gastric antral volume during fasting and after a meal[27-32]. The serial US measurements of the antral volume enable to assess directly the time curve of antral distension, and indirectly the gastric emptying time. Ricci *et al*[14] first reported a close association between the onset of the usual postprandial complaints and an antral volume increase in the majority of FD patients. Hausken *et al*[15] found an association between a wide antral area and bloating. Both studies, however, did not comparatively investigate the antral volume of the FD patients who remained symptom-free after meal ingestion.

Several other studies reporting non-univocal results have attempted to correlate the occurrence of symptoms in FD patients with proximal and distal stomach distension[10,11,17,36,37], gastric volumes[16,19,38-40] or gastric food retention[6,21,22]. These studies were performed at the end[16], 30 min[19] and 1 h[11] after meal ingestion, with either invasive techniques or not physiological ingestion of meals that caused dyspeptic symptoms also in healthy controls[16,17,19, 39,40].

This study assessed, in physiological conditions after a normal meal, the relationship between gastric emptying, antral volume variation and postprandial symptoms in symptomatic compared to asymptomatic dyspeptic patients and healthy subjects. The meal was a normal every day meal. Differing from previous findings at multivariate analysis, gender did not have any relationship with postprandial antral distention or with the modality of gastric emptying[22]. The different results of this study may be explained by the larger number of patients studied.

The antral volume reached its maximal value soon after the end of meal ingestion and subsequently decreased throughout the observation period in both controls and FD patients (Figure 3). However, independently from the concomitant presence of GI dyspeptic symptoms, FD patients showed an antral volume greater than healthy controls, confirming the presence of an altered intragastric meal distribution in FD patients[14-16,21,22]. It has been suggested that impaired accommodation of the proximal stomach to a meal underlies the increased distribution of gastric contents in the distal stomach[21,38].

The antral volume variations and gastric emptying were evaluated for a long postprandial period, i.e. 5 h, thus enabling to detect a subgroup of patients in whom the over distension of the antrum occurred later than, and independently from, an early postprandial altered motor function of the proximal stomach.

The most relevant finding of this study was that postprandial drowsiness was the only of the GI and extra-GI symptoms to be highly significantly associated with antral volume and its transformations.

In patients with postprandial drowsiness, antral volume reached the maximal value 2 h after the meal and did not show a progressive decrement throughout the observation period (Figure 4). The occurrence of postprandial drowsiness was significantly associated with the current and mean weighted antral volume, supporting the time-relationship with antral distension. However, the association with delta antral volume change suggests that its onset is associated with the degree of time-related antral distension rather than the antral volume *per se*. Finally, the finding of an association between drowsiness and the antral volume assessed at the preceding observation period suggests that the antral distension precedes the onset of drowsiness. It could be argued that the fasting and the immediate postprandial antral volumes included in the analysis might have inappropriately contributed to the subsequent antral volume transformation, therefore affecting the results of the present study, but their exclusion from the analyses did not change the results. Furthermore, it has been shown that subtracting the fasting from the final postprandial antral volume, the latter is still greater in dyspeptic patients than in controls, thereby indicating a genuine greater postprandial antral distension in patients compared to healthy controls[5].

At present, whether increased antral volumes may reflect hypotonia of the antral muscular wall or intraluminal distension secondary to gastric retention or to an overload caused by an impaired accommodation of the proximal stomach[21,38], or an increased duodenogastric reflux could

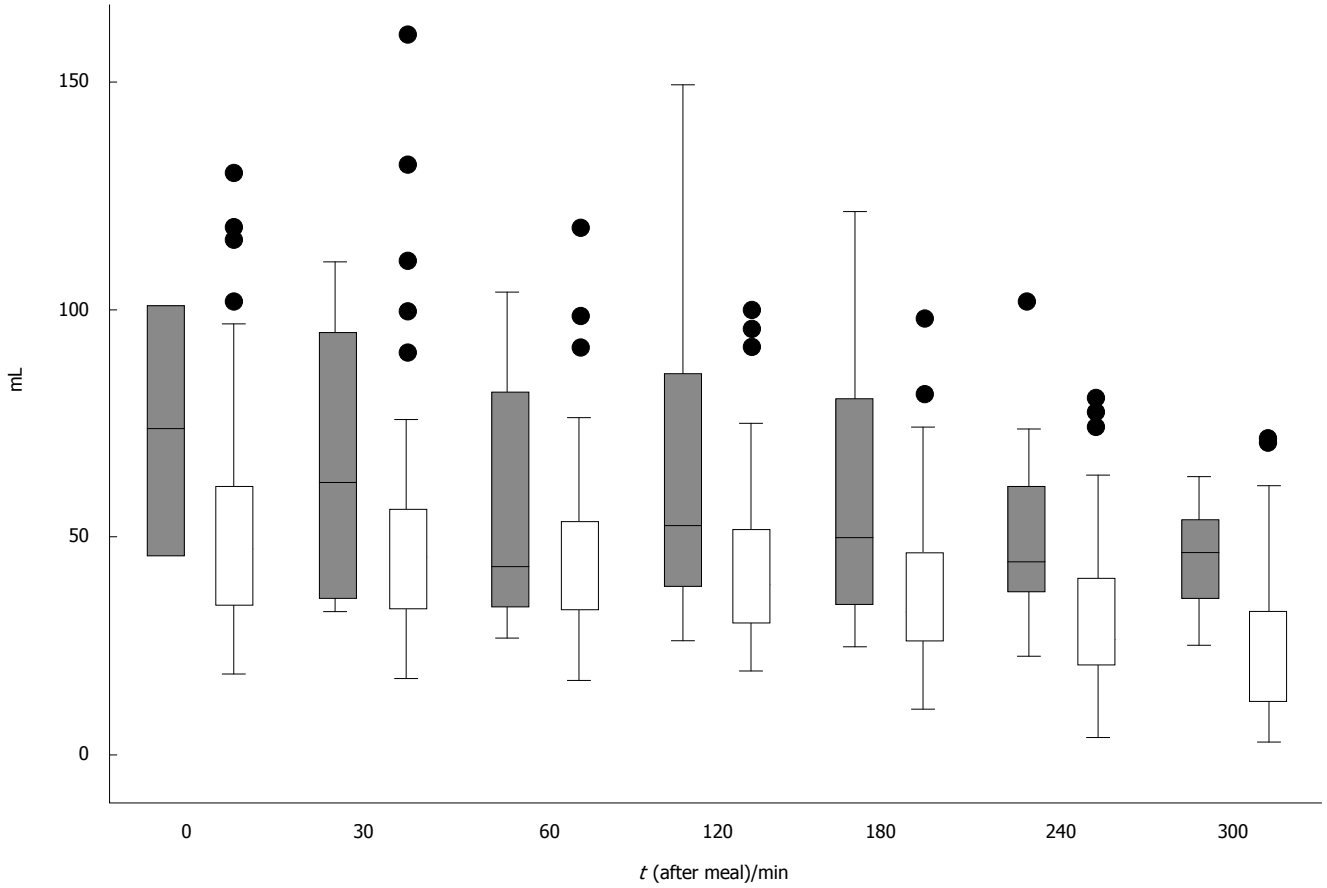


**Figure 4** Box-and-whiskers plots of gastric antral volume immediately (0), at 30 and at 60 min intervals, after the end of the ingestion of a standard meal in FD patients with (gray box) and without (white box) postprandial drowsiness. The boxes at each time unit extend from the 25th percentile ($x_{[25]}$) to the 75th percentile ($x_{[75]}$) [i.e., the interquartile range (IQ)]; the lines inside the boxes represent the median values. The lines emerging from the boxes (i.e., the "whiskers") extend to the upper and lower adjacent values. The upper adjacent value is defined as the largest data point $\leq x_{[75]} + 1.5 \times IQ$, and the lower adjacent value is defined as the smallest data point $\geq x_{[25]} - 1.5 \times IQ$. Observed values more extreme than the adjacent values, if any, are individually plotted (circles).

not be addressed in the present study.

Several studies indicate that a variable degree of sleepiness after a meal is a common sensation[23,24,41-43] in healthy subjects and may also influence food intake[44]. Studies assessing daytime sleepiness with standard tests, in healthy subjects showed that (1) sleep onset latency was significantly shorter after a caloric meal than after water[24] and sham feeding[45], and (2) the occurrence of postprandial sleepiness was neither related to the fat composition of the test meal nor to the circadian variation in sleepiness[23]. Finally, in healthy subjects, it has been shown that in comparison with an equal volume of water and equicaloric liquid meal, a solid meal results in decreased sleep onset latencies[24]. These results, taken together with the observation that drowsiness may occur after intravenous administration[46] of cholecystokinin (CCK), support the hypothesis of a peripherally circulating hormone of GI origin that mediates postprandial sleepiness. It is conceivable that meal ingestion may release CCK and other neuroendocrine substances, such as 5-$HT_3$, that affect the state of consciousness directly or activating vagal nerve afferences or releasing other sleep-promoting substances, such as insulin[47,48]. Several animal studies indicate that CCK sleep-promoting and food intake-reducing effects are closely associated, presumably expressing different, yet related, manifestations of satiety[47].

Postprandial drowsiness reported in this study refers to a sensation regarded to be bothersome enough to interfere with the daily activities. However, the sensation of drowsiness may vary from slight to severe and may be related to sleep disturbances. Sleep disturbances were enquired specifically and patients reporting postprandial drowsiness did not report any sleep disturbances. In the present study, older age in FD patients was an independent factor adjusted for antral volume variables for the occurrence of drowsiness. Control subjects and dyspeptic patients were not balanced for age, however, in the age-adjusted model, the presence of drowsiness in FD patients was associated only with antral volume variations. In addition, it has been shown that in healthy subjects, postprandial drowsiness is related to a younger age and to a greater food intake compared to older subjects[44]. In the present study, healthy controls, although significantly younger than FD patients, did not report any symptom. It would therefore appear that, in contrast with healthy controls, drowsiness reported by older dyspeptic patients was neither related to sleepiness nor to physiological change of postprandial sleep latency. However, we neither evaluated with objective measures postprandial sleep latency or the severity of postprandial drowsiness nor we specifically evaluated physiological adaptive variation or an altered state of the autonomic nervous system that could

justify the occurrence of postprandial drowsiness in our patients[41].

Of the GI symptoms conventionally considered to be a manifestation of dyspepsia, only bloating showed a statistically significant association with the postprandial weighted antral volume and, to a lesser degree, with all the other antral volume variables, confirming previous observations[15,16,37]. Bloating has been frequently reported in healthy subjects in barostat studies, in caloric and even in water drink tests, without any relationship with altered gastric motor function[11,17,44]. In our study, nausea and belching were associated only with antral volume delta change, whereas all the other GI dyspeptic symptoms were not related to any of the assessed antral volume variables. Postprandial fullness is a common sensation even in healthy subjects and it has been reported to be associated with an increase of the antral area[49,50]. In the present study, none of the healthy subjects and about 50% of the study patient population referred fullness that was not associated with any of the antral volume variables evaluated. The frequent occurrence of bloating and postprandial fullness in healthy subjects together with the absence of a constant and univocal association with gastric functions limits the interpretation of the pathophysiological mechanisms of these symptoms in FD patients. Nausea is a non-specific GI symptom that could be elicited by the direct instillation of acid in the duodenum[51], i.e. a condition likely mimicking a physiological condition. Several studies highlighted the potential key role of the duodenum in the symptom generation in functional dyspepsia[51-53]. Hypersensitivity of the duodenum to acid infusion as well as duodenal distension have been reported to elicit upper GI symptoms[51-53]. However, the present study does not allow one to draw any conclusion about the role of the duodenum in the occurrence of symptoms and further investigations are needed.

Early satiety, which is one of the cardinal dyspeptic symptoms and was referred in the medical history by 45% of our patients population, did not occur after a meal in controlled condition in any of the investigated patients. It is conceivable that the sensation referred by the patients was more likely fullness or bloating that had induced the patients to terminate prematurely food ingestion to prevent further discomfort. It has been indeed shown that perception of fullness is a useful predictor of food intake[44,50]. If so, the definition of early satiety as an unpleasant sensation that forces one to stop eating, and the numerous studies in which this symptom has been related to specific function disorder of the stomach, should be reevaluated.

Nevertheless, a relevant limitation of this study is that except for pain and discomfort, severity of postprandial symptoms was not assessed, thus precluding any evaluation of its possible relationship with antral distension.

A significant proportion of FD patients complain of psychological symptoms and the presence of anxiety has been demonstrated in about 70% of these patients[22]. We excluded the patients with eating disorders and clinically evident psychological disorders like major depression from our study; however, we did not fully evaluate the psychological status of the patients.

Despite that it has been widely debated whether a delayed gastric emptying is a relevant factor in causing dyspeptic symptoms, to our knowledge, none of the previous studies evaluated concurrently and in physiological conditions the modality of the gastric emptying and the occurrence of symptoms in FD patients after an ordinary every day meal. In the present study, 39% of FD patients had delayed gastric emptying, but it did not have any relationship with the occurrence of dyspeptic symptoms during the test. A previous study reported a delayed gastric emptying in 41% of the patients and a rapid initial gastric emptying in 43% of them that were associated with higher symptoms score after a challenge meal[19]. Thus, it would appear that a delayed gastric emptying *per se* does not play any role in the origin of the usual dyspeptic symptoms.

The present study confirms our previous finding[5] that patients referring postprandial drowsiness have a greater probability to have delayed gastric emptying than controls and dyspeptic patients without post-prandial drowsiness. Differently from the usual dyspeptic symptoms, drowsiness is a late postprandial complaint occurring significantly later than GI symptoms and is related with antral distension and delayed gastric emptying. It would therefore appear that the two conditions are associated with the onset, and the persistence of postprandial drowsiness in this subgroup of FD patients. We cannot draw any definitive conclusion about the separate role, if any, played by delayed gastric emptying and antral distension, either alone or in combination, in the occurrence of postprandial drowsiness in FD patients.

In conclusion, our study assessed in controlled conditions the relationship between gastrointestinal and extra-gastrointestinal symptoms arising after a normal balanced meal, and antral distension and gastric emptying. Postprandial gastrointestinal symptoms do not have any constant or predictable relationship with antral distension and gastric emptying in functional dyspepsia patients. Of the extra-gastrointestinal symptoms, postprandial drowsiness is associated with antral distension. On the average, drowsiness occurs late after meal ingestion and after the onset of gastrointestinal dyspeptic symptoms. The onset of postprandial drowsiness is usually preceded by an increment of antral distension and the duration of the symptom appears to be related to the persistence of antral distension. Finally, a delayed gastric emptying is significantly associated with postprandial drowsiness and the degree of the fasting antral volume.

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*CLINICAL RESEARCH*

# Milan criteria are useful predictors for favorable outcomes in hepatocellular carcinoma patients undergoing liver transplantation after transarterial chemoembolization

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## Abstract

**AIM:** To evaluate whether the Milan criteria are useful in patients with hepatocellular carcinoma (HCC) who received transarterial chemoembolization (TACE) before liver transplantation (LT).

**METHODS:** Thirty-six HCC patients who fulfilled the Milan criteria after having received TACE and subsequently underwent LT were included (TACE + LT group) in the study. As controls, 21 patients who also met the Milan criteria and underwent LT without prior treatment were selected (LT group). Post-LT clinical outcomes, such as HCC recurrence, survival rate, and histologic features of explanted livers, were compared between the two groups.

**RESULTS:** Baseline characteristics were not different between the two groups. Pre-LT maximal tumor diameter in TACE + LT group was similar to that of LT group (2.0 ± 0.6 cm *vs* 2.3 ± 0.9 cm; $P = 0.10$). Post-LT histologic findings also revealed similar maximal tumor diameter in the two groups (2.4 ± 1.4 cm *vs* 2.3 ± 0.9 cm; $P = 0.70$). Explanted livers showed similar incidence of unfavorable pathologic features. The morality within 60 d after transplantation was not different between the two groups (8.3% *vs* 9.5%; $P = 0.99$). Post-LT 5-year survival rate (57% *vs* 74%; $P = 0.70$) and cumulative recurrence rate (8.3% *vs* 4.8%; $P = 0.90$) were not significantly different between the two groups.

**CONCLUSION:** The Milan criteria are still a useful selection criteria showing favorable outcomes in HCC patients receiving TACE before LT.



**Key words:** Milan criteria; Hepatocellular carcinoma; Transarterial chemoembolization; Liver transplantation

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## INTRODUCTION

Hepatocellular carcinoma (HCC) is a major global health problem involving more than 500 000 new cases a year. Several treatment modalities, such as liver transplantation (LT), surgical resection, radiofrequency ablation (RFA), and percutaneous ethanol injection (PEI), are accepted for curative therapies for HCC. Theoretically, LT remains the only ideal treatment option because LT has been claimed to simultaneously cure the malignant disease and replace the premalignant cirrhotic liver. Early series of LT for HCC yielded poor outcomes[1-4]. In those series, 3- and 5-year survival after LT ranged 15%-67% and 15%-48%, respectively. These inferior results come from inclusion of patients with far advanced HCC. In spite of initial dismal experiences with LT for patients with HCC, patients with confined HCC (solitary lesion $\leqslant$ 5 cm or $\leqslant$ 3 lesions with diameter $\leqslant$ 3 cm, no major vessel invasion, and no extrahepatic involvement; Milan criteria) were reported to show an excellent long-term outcome with a 5-year survival rate of 70% and a recurrence rate below 15%[5]. With pathologic review, modestly expanded selection criteria (solitary lesion $\leqslant$ 6.5 cm or $\leqslant$ 3 lesions with the largest one $\leqslant$ 4.5 cm and total tumor diameter $\leqslant$ 8 cm; UCSF criteria) were suggested to offer an excellent outcomes with a 1- and 5-year survival rate of 90% and 75.2%, respectively[6]. In clinical practice, however, the Milan criteria based on pre-LT radiologic findings could be more useful and a widely accepted selection criteria than

the UCSF criteria based on post-LT pathologic findings.

Because of donor organ shortage or other limits including economic problem in HCC patients waiting for LT, various treatment modalities including resection, RFA, PEI, and transarterial chemoembolization (TACE) were tried to prevent the progression of HCC. Among these, TACE is the most commonly used procedure in patients with unresectable HCC in our country. Up to now, it is not known whether a favorable outcome after LT can also be achieved in HCC patients who have been treated by TACE and meet the Milan criteria, as in treatment-naïve HCC patients.

Hence, we conducted a study to assess the usefulness of the Milan criteria in HCC patients who had been treated with TACE prior to LT.

## MATERIALS AND METHODS

### Subjects

Between September 1996 and April 2004, a total of 105 patients with HCC underwent LT at our institute. Among them, 63 (59.4%) patients met the Milan criteria based on pre-LT imaging. Excluding 27 patients within Milan criteria treated by RFA or surgical resection before LT, 36 patients with one or more sessions of TACE only prior to LT were selected (TACE + LT group). Twenty-one HCC patients who had not been given any treatment before LT were selected as control (LT group) (Figure 1). The Milan criteria was defined as the presence of a tumor 5 cm or less in diameter in patients with single HCC or no more than 3 tumor nodules, each 3 cm or less in diameter, in patients with multiple tumors, and no extrahepatic metastasis, and no major hepatic vessel invasion[5].

### TACE

One to eight sessions of TACE were performed *via* the transfemoral arterial approach under local anesthesia at intervals of 4-12 wk in the TACE + LT group. Selective celiac and superior mesenteric angiography was performed to define the hepatic artery anatomy and to evaluate the portal venous system. The feeding artery to the lesion was catheterized as selectively as possible using a highly flexible coaxial catheter, and the chemotherapeutic agent lipiodol mixture was injected under fluoroscopic guidance. The mixture contained 20-50 mg of doxorubicin, 3-20 mL of lipiodol (Lipiodol Ultrafluide; Guerbet, Aulnay-sous-Bis, France), and 3 mL of water-soluble contrast agent. Embolization was performed with gelatin pellets (Gelfoam; Upjohn, Kalamazoo, Michigan) thereafter in patients with liver function of Child-Pugh class A and tumor confined to single lobe of the liver. This Gelfoam embolization was also performed for patients with Child-Pugh B, if superselection of the feeding vessel was feasible. Abdominal computerized tomography (CT) scan was performed 4 wk after TACE to evaluate the anti-tumor effect of TACE, including lipiodol uptake by the tumor tissue. If viable tumor was still observed in CT, repeated TACE was performed. If lipiodol was compactly uptaken by all tumor nodules and any new lesion was not seen, follow-up CT scan was repeated every 3 mo.

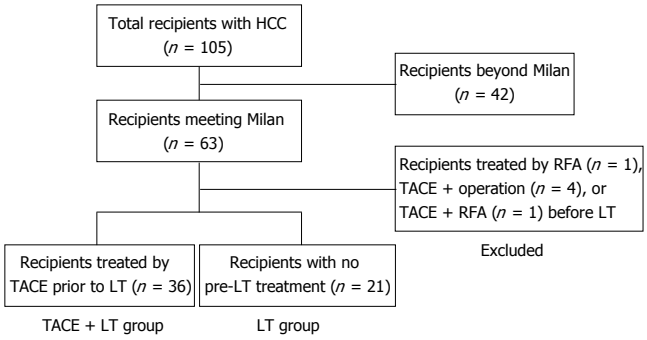


**Figure 1** Selection of patients according to the treatments given prior to LT. Among a total of 105 HCC patients, 63 patients met the Milan criteria at the time of LT. After excluding 6 patients who received treatments other than TACE, there were 36 patients in TACE + LT group and 21 in LT group.

### Pre-LT radiologic measurement of tumor size

As for tumor size in the LT group, any nodular lesion showing arterial enhancement and delayed washout in 3-phase helical CT was regarded as a viable HCC and its largest diameter was considered as a tumor size. In the TACE + LT group, a tumor nodule showing compact lipiodol uptake without arterial enhancement or delayed washout in CT was considered as non-viable tumor and was excluded from the measurement of tumor size or number. For a nodule showing arterial enhancement and delayed washout at the margin, tumor size was defined as the difference from diameter of the entire nodule to diameter of lipiodol-uptaken portion. Representative cases showing how to measure the HCC lesions treated by TACE are illustrated in Figure 2.

### Histopathology

All total hepatectomy specimens were processed by a hepatopathologist using a routine protocol consisting of 1 cm or thinner sections throughout the entire liver. With standard histological staining, the specimens were examined to evaluate tumor characteristics, such as number of nodules, size, pathologic tumor grade (Edmonson grade), percentage of necrosis, and presence of tumor capsule invasion, satellite nodule, or microvascular invasion. By pathologic review of explanted liver, it was evaluated whether the enrolled patients also met the UCSF criteria.

### Post-LT management and follow-up

Post-transplant immunosuppression consisted of corticosteroid plus either tacrolimus or cyclosporin. Corticosteroid was gradually tapered and was discontinued within 1 year. Tacrolimus and cyclosporin were continued after LT unless contraindicated. Acute rejection was treated with steroid pulse therapy. Antithymocyte globulin (ATG) or muromonab (OKT3) antibody infusions were reserved for the patients with acute rejection resistant to intravenous corticosteroids. The interval of the outpatient clinic visits after discharge from the hospital was adjusted according to the patient's condition.

Tumor recurrence was screened by measurement of alpha-fetoprotein and abdominal CT or ultrasonography

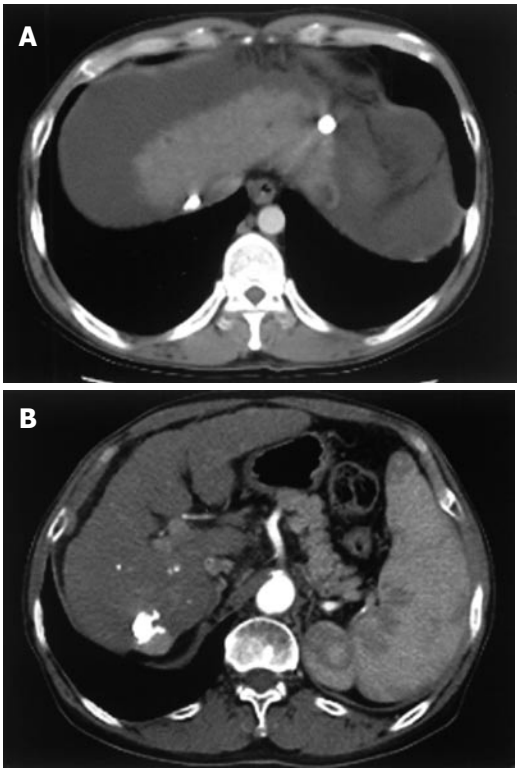


**Figure 2** Representative cases for measurement of viable tumor size treated by TACE prior to LT. **A:** A tumor nodule with compact lipiodol uptake, no enhancement at arterial phase, and no washout at portal or delayed phase was considered as a non-viable tumor and was excluded from the measurement of tumor size or number; **B:** For a nodule showing arterial enhancement and delayed washout at the margin, tumor size was defined as the difference from diameter of the entire nodule to diameter of lipiodol-uptaken portion.

every 3 mo. Additional imaging modalities, such as chest CT and bone scan, were performed if HCC recurrence at lung or bone was suspected. No adjuvant chemotherapy was administered to any patients after LT.

### Comparison of clinical outcomes

In this study, we retrospectively analyzed the medical records, radiologic and pathologic findings of the 36 patients in TACE + LT group and 21 in the LT group. Baseline clinical characteristics of the patients, tumor characteristics, clinical outcomes including recurrence of HCC, and post-LT survival rate were compared between the two groups.

### Statistical analysis

Baseline characteristics of the patients were expressed as mean ± SD. Comparison between the two groups was done by using the independent $t$ test for continuous variables and by chi-square test or Mann-Whitney $U$ test for categorical variables. The overall survival rate and cumulative recurrence rate were calculated by the Kaplan-Meier method. The survival curves were compared by means of the log-rank test. All statistical tests were two-tailed and $P < 0.05$ was considered statistically significant. All statistical analyses were performed with SPSS 11.0 (SPSS Inc., Chicago, IL).

**Table 1 Baseline clinical, demographic, and imaging-based tumor characteristics of the enrolled patients (mean ± SD)**

| Characteristics | TACE + LT group ($n$ = 36) | LT group ($n$ = 21) | $P$ |
|---|---|---|---|
| Age (yr) | 49 ± 8.2 | 52 ± 8.0 | 0.13 |
| Sex (M/F) | 31/5 | 18/3 | 0.99 |
| Etiology of liver disease | | | 0.12 |
| HBV | 34 (94.4%) | 18 (85.7%) | |
| HCV | 0 (0%) | 2 (9.5%) | |
| HBV + HCV | 1 (2.8%) | 0 (0%) | |
| Alcoholic | 0 (0%) | 1 (4.8%) | |
| α-fetoprotein (μg/L) | 193 ± 472 | 1012 ± 4110 | 0.24 |
| TACE | | | |
| 1 | 12 (33.3%) | - | |
| 2 | 16 (44.4%) | - | |
| ⩾ 3 | 8 (22.2%) | - | |
| MELD score | 19 ± 9 | 22 ± 9 | 0.10 |
| Type of graft | | | |
| Cadaveric graft | 8 (22.2%) | 2 (9.5%) | 0.30 |
| Living graft | 28 (77.8%) | 19 (90.5%) | |
| Number of nodules | | | |
| 1/2/3 | 24/6/6 | 18/2/1 | 0.26 |
| Diameter of the largest tumor (cm) | | | 0.10 |
| Mean ± SD | 2.0 ± 0.6 | 2.3 ± 0.9 | |
| Range | 0.9-4.0 | 1.0-4.0 | |
| Sum of the tumor diameters (cm) | | | 0.38 |
| Mean ± SD | 2.5 ± 1.1 | 2.8 ± 1.3 | |
| Range (cm) | 0.9-6.1 | 1.0-5.9 | |

TACE: Transarterial chemoembolization; LT: Liver transplantation; HBV: Hepatitis B virus; HCV: Hepatitis C virus; MELD: Model for end stage liver disease.

## RESULTS

### Patient characteristics

A total of 57 patients (49 men and 8 women; median age 51 years, range 30-68 years) were included in this study. Fifty-three patients (93.0%) had HCC associated with hepatitis B virus (HBV) infection. Other causes consisted of hepatitis C virus (HCV) infection in 2 patients (3.5%), co-infection of HBV/HCV in one (1.8%), and alcohol-related liver disease in one (1.8%). The mean alpha-fetoprotein levels were 494.9 μg/L and model for end stage liver disease (MELD) score was 19.4. One to eight sessions of TACE were performed in TACE + LT group; 1 session in 12 (33.3%) patients, 2 sessions in 16 (44.4%) patients, and 3 or more sessions in 8 (22.2%) patients. Cadaveric grafts were used in 10 (17.5%) and living grafts in 47 (82.5%) cases. As for baseline clinical and demographic characteristics, there was no significant difference between TACE + LT group and LT group (Table 1).

### Tumor characteristics

According to pre-LT radiologic evaluation, 42 (73.7%) patients had single tumor, and 8 (14.0%) and 7 (12.3%) patients had 2 and 3 tumors, respectively. The mean diameter of the largest nodule in all patients was 2.1 cm. In TACE + LT group, 24 (66.7%) patients had single tumor, 6 (16.7%) patients had 2 tumors, and 6 (16.7%)

**Table 2 Histologic finding-based tumor profiles of patients who met the Milan criteria**

| Variable | TACE + LT group ($n$ = 36) | LT group ($n$ = 21) | $P$ |
|---|---|---|---|
| Number of nodules | | | |
| 1/2/3/≥4 | 18/8/3/7 | 13/3/3/2 | 0.79 |
| Diameter of the largest tumor (cm) | | | |
| Mean ± SD | 2.5 ± 1.4 | 2.3 ± 0.9 | 0.70 |
| Range | 0.6-7.5 | 0.5-4.2 | |
| Sum of the tumor diameters (cm) | | | |
| Mean ± SD | 3.8 ± 2.6 | 3.3 ± 1.3 | 0.36 |
| Range | 0.6-14.0 | 0.5-7.0 | |
| Tumor differentiation[1] | | | 0.59 |
| Edmonson grade Ⅰ | 5 (16.7%) | 6 (28.6%) | |
| Ⅱ | 22 (73.3%) | 13 (61.9%) | |
| Ⅲ | 3 (10%) | 2 (9.5%) | |
| Presence of satellite nodule | 5 (13.9%) | 2 (9.5%) | 0.99 |
| Tumor capsule invasion | 11 (30.6%) | 3 (14.3%) | 0.21 |
| Microvascular invasion | 11 (30.6%) | 9 (42.9%) | 0.40 |

[1]Assessment of tumor differentiation was possible in only 30 patients in TACE + LT group due to complete necrosis in 6 patients. TACE: Transarterial chemoembolization; LT: liver transplantation.

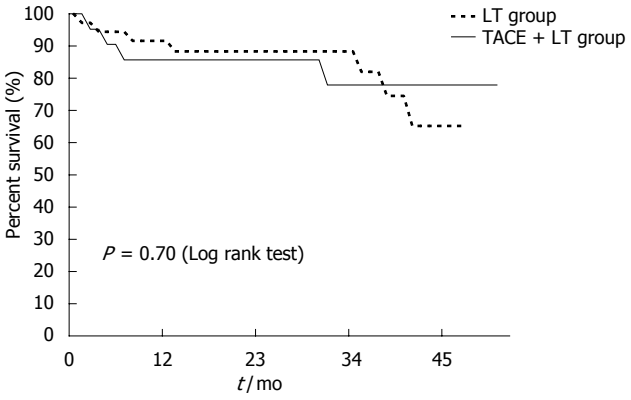


**Figure 3** Comparison of overall survival rate between TACE + LT and LT groups. There was no obvious difference in 5-year survival rate after LT between the two groups (57% *vs* 74%; $P$ = 0.70).

patients had 3 tumors. The distribution of tumor number was similar in the LT group; 1 in 18 (85.7%) patients, 2 in 2 (9.5%) patients, and 3 in 1 (9.5%) patient. No statistical difference was found in the mean diameter of the largest tumor between TACE + LT and LT groups (2.0 cm *vs* 2.3 cm; $P$ = 0.10; Table 1).

According to histopathologic findings of explanted livers, a total of 114 nodules were found from all the patients; 77 in the TACE + LT group and 37 in the LT group. The number of tumor nodule was 1 in 31 (54.4%) patients, 2 in 8 (14.0%) patients, 3 in 3 (5.3%) patients, and 4 or more in 15 (26.3%) patients. The mean diameter of the largest tumor was 2.4 cm and the sum of the tumor diameters was 3.6 cm. There was no significant difference in distribution of tumor number between the two groups. The mean diameter of the largest tumor and the sum of the tumor diameters were also similar between both groups (2.4 cm *vs* 2.3 cm, $P$ = 0.70; 3.8 cm *vs* 3.3 cm, $P$ = 0.36, respectively, Table 2). Twenty-eight (77.8%) patients

**Table 3 Detailed clinical data of the patients in whom HCC recurred after LT**

| Patient | Group | Time to recur (mo) | Site of recur | Treatment after recurrence | Survival (mo) |
|---|---|---|---|---|---|
| 1 | TACE + LT | 9.6 | Liver | None | 10.0 |
| 2 | TACE + LT | 3.3 | Liver, bone | TACE | 7.1 |
| 3 | LT | 15.4 | Lymph node | Excision, TACE | 21.0 |
| 4 | TACE + LT | 0.9 | Lung, liver | TACE | 5.7 |

TACE: Transarterial chemoembolization; LT: Liver transplantation.

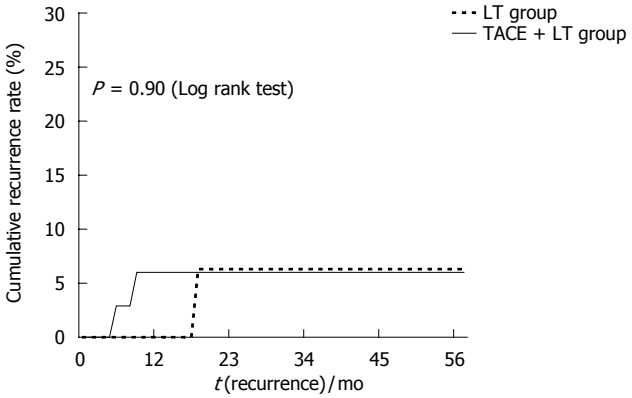


**Figure 4** Comparison of cumulative HCC recurrence rate between TACE + LT and LT groups. Five-year recurrence rate was similar between the two groups (8.3% *vs* 4.8%; $P$ = 0.90).

in the TACE + LT group and 19 (90.5%) in the LT group met the UCSF criteria.

As to the tumor differentiation, Edmonson grade 1 was found in 11 (21.6%), grade 2 in 35 (68.6%), and grade 3 in 5 (9.8%) patients. Of 57 patients, 7 (12.3%) and 14 (28.8%) patients had satellite nodules and tumor capsule invasion, respectively. In addition, 20 (35.1%) patients had microvascular invasion. The incidence of unfavorable pathologic features was similar in the two groups (Table 2). The explanted liver showed TACE-induced complete tumor necrosis without histologic evidence of viable carcinoma in 23 of 77 (29.9%) lesions.

### *Clinical outcomes after LT*

During median follow-up of 24.3 (range: 0.1-99) mo, 14 of 57 (24.6%) patients died. Post-LT early mortality, defined as death within 60 d after transplantation, was not different between TACE + LT and LT groups (8.3% *vs* 9.5%; $P$ = 0.99). The overall survival rates of the patients at 1-, 3-, and 5-year were 86%, 72%, and 67%, respectively. The 1-, 3-, 5-year survival rates between the TACE + LT group and the LT group were not significantly different (89% *vs* 81%, 68% *vs* 74%, and 57% *vs* 74%, respectively; $P$ = 0.70) (Figure 3). The causes of death consisted of graft failure in 3 patients, graft *versus* host disease (GVHD) in 2 cases, hepatic vein problem with hepatic congestion in 1 case, HBV recurrence in 3 cases, HCC recurrence in 4 cases, and intracerebral hemorrhage in 1 case.

During the follow-up period, HCC recurrence was found in 4 (7.0%) patients (Table 3). Five-year cumulative HCC recurrence rate in the TACE + LT group was similar to that in the LT group (8.3% *vs* 4.8%; $P$ = 0.90) (Figure 4).

## DISCUSSION

HCC is one of the serious complications of chronic liver disease associated with high mortality. Although surgical resection is traditionally regarded as treatment of choice for HCC, it is a major problem that resection is feasible in only limited cases due to poor liver function and/or advanced stage of HCC at diagnosis. In addition, many patients suffer from recurrent HCC and aggravation of cirrhosis after operation. In this regard, LT gives patients with HCC an advantage over resection, because it addresses the multifocal potential of HCC in many patients that limits the success and applicability of resection and also treats the underlying liver disease[6].

The criteria developed by Mazzaferro and associates, known as the Milan criteria, have been widely applied around the world in the selection of patients with HCC for LT. However, the Milan criteria were originally made for patients with treatment-naïve HCC. In the clinical setting, a significant number of HCC patients are treated with TACE or RFA prior to LT because of a long waiting list for LT. It remains uncertain whether excellent outcomes can be obtained in HCC patients who previously underwent locoregional treatments and still meet the Milan criteria at the time of LT. In the present study, we retrospectively selected the patients who had received only TACE before they underwent LT since TACE is the most widely used procedure for HCC in our country. Patients undergoing other therapies, such as resection or RFA, were excluded to eliminate confounding effect of those treatments. For validation of usefulness of the Milan criteria in HCC patients treated with TACE before LT, their survival rates following LT were compared with those of HCC patients undergoing LT only.

The current study demonstrates that HCC patients who had undergone one or more sessions of prior TACE showed as good post-LT survival as treatment-naïve HCC patients, if they met the Milan criteria at the time of transplantation. One- and 5-year survival rates of patients in the TACE + LT group and LT group were comparable (89% *vs* 81% and 57% *vs* 74%, respectively; $P = 0.70$). Taniguchi *et al*[7] showed a long-term survival and marked TACE-induced tumor necrosis in patients with unresectable HCC. A recent report on randomized controlled trial showed that TACE with doxorubicin and gelatin sponge, compared with conservative management, provided survival benefits to patients with unresectable HCC[8]. However, as a bridge to transplantation for patients on waiting lists, TACE showed varying results[9,10]. Among these, an European study showed a 5-year survival of 93% in 48 patients receiving TACE, with no dropout over a mean waiting period of 6 mo[10]. Moreover, prospective studies have shown that the probability of preventing tumor progression is significantly higher in patients treated with TACE than those untreated controls[11,12]. In a recent series, pre-LT TACE in 54 predominantly early-stage cases yielded a reasonable 5-year post-LT survival of 74%[13]. However, the benefit of TACE could not be inferred given that waiting list dropout and post-LT recurrence rates were not markedly lower than historical controls. In contrast to previous studies regarding the role of TACE before LT, we elucidated the usefulness of the Milan criteria at the time of LT in patients receiving TACE prior to LT.

Up to now, there is no data on whether pre-LT TACE increases early mortality after transplantation compared to LT without prior therapy. In a previous study, some patients who underwent LT within 30 d of the last TACE developed unexplained severe pneumonia, leading to death very early after transplantation[9]. On the other hand, our results showed that mortality within 60 d after transplantation was not different between TACE + LT and the LT groups. Thus, the issue concerning pre-LT TACE and early mortality after operation still remains controversial.

In our study, 1-year survival rates of LT group were lower than those of TACE + LT group, albeit not statistically significant (89% *vs* 81%). Although the reason for this observation is not clearly understood, it might be due to the poorer liver function of patients in LT group as compared with the TACE + LT group. Urgent transplantations might have been undertaken in patients of the LT group whose liver functions were too poor to perform locoregional therapy. The tendency of higher MELD score (22 *vs* 19; $P = 0.10$) and more frequent living donor transplantation in the LT group compared to the TACE + LT group (90.5% *vs* 77.8%; $P = 0.30$) support the more aggravated liver function and urgent condition of the LT group.

Our data demonstrated that 5-year cumulative recurrence rates of patients in the TACE + LT group were similar to those in the LT group (8.3% *vs* 4.8%; $P = 0.90$). HCC recurred in 3 of 36 patients in the TACE + LT group with a recurrence site of liver in one and distant organs in two patients. In the LT group, the recurrence occurred in one patient at the perihepatic lymph node. Increased incidence of hepatic or extrahepatic recurrence after TACE followed by resection has been an important concern on the grounds that partial necrosis of the tumor favors the shedding of neoplastic cells in a few previous studies[14-17]. However, such a concern was not substantiated in our patients.

There was no significant difference between the TACE + LT group and the LT group in terms of baseline clinical and demographic characteristics, radiologic finding-based or histologic finding-based tumor profiles, and histologic parameters indicating unfavorable prognosis. At this point, we have to comment on the radiologic measurement of HCC lesions previously treated with TACE. In the TACE + LT group, albeit statistically not significant, the radiologically measured diameter of the largest tumor was shorter than the histologically measured one ($2.0 \pm 0.6$ cm *vs* $2.5 \pm 1.4$ cm; $P = 0.06$), and 4 of 36 patients in the TACE + LT group were found to have 4 or more nodules on explanted specimens. In the LT group, the radiologically measured diameter of the largest tumor was similar with the histologically measured one ($2.3 \pm 0.9$ cm for both), and 2 of 21 patients in the LT group had 4 or more nodules on explanted livers. These results imply that pre-LT staging of HCC by the current imaging modalities may be underestimated in terms of number in both groups and in terms of size in the TACE + LT group.

Although the extents of HCCs were estimated by

expert radiologists using a pre-defined method, it was difficult to precisely measure the maximal diameter of lesions in a portion of the TACE + LT group, especially in cases with incomplete or scattered lipiodol uptake in nodules. In a previous study measuring the tumor volume (TV) to improve the selection criteria based on number and diameter of HCC, TV > 28 $cm^3$ was reported to be a predictive factor for HCC recurrence after LT[18]. Other investigators measuring the TV with a region-of-interest CT technique also evaluated the prognostic value of volumetric CT in patients treated with repeated TACE[19]. These alternative methods might better represent the tumor size, but are much more complex and time-consuming.

In conclusion, our data demonstrated that the Milan criteria would be useful selection criteria in HCC patients who underwent the TACE procedure before LT, showing favorable prognosis if they fulfill the criteria at the time of transplantation.

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*CLINICAL RESEARCH*

# mRNA expression, functional profiling and multivariate classification of colon biopsy specimen by cDNA overall glass microarray

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## Abstract

**AIM:** To understand the local pathophysiological alterations and gene ontology-based functional classification of colonic biopsies into inflammatory and neoplastic diseases.

**METHODS:** Total RNA was extracted from frozen biopsies and amplified by T7-method. Expression profile was evaluated by Atlas Glass 1K microarrays. After microarray quality control, applicable data were available from 10 adenomas, 6 colorectal adenocarcinomas (CRCs), and 6 inflammatory bowel diseases (IBDs). Multivariate statistical and cell functional analyses were performed. Real-time RT-PCR and immunohistochemistry were used for validation.

**RESULTS:** Discriminant analysis of selected genes, could correctly reclassify all 22 samples using 4 parameters (heat shock transcription factor-1, bystin-like, calgranulin-A, TRAIL receptor 3). IBD samples were characterized by overregulated chemokine (C-X-C motif) ligand 13, replication protein A1, E74-like factor 2 and downregulated TNF receptor-associated factor 6, BCL2-interacting killer genes. In adenomas upregulation of TNF receptor-associated factor 6, replication protein A1, E74-like factor 2 and underexpression of BCL2-associated X protein, calgranulin-A genes were found. CRC cases had significantly increased epidermal growth factor receptor, topoisomerase-1, v-jun, TNF receptor-associated factor 6 and TRAIL receptor 3, and decreased RAD51 and RAD52 DNA repair gene, protein phosphatase-2A and BCL2-interacting killer mRNA levels. Epidermal growth factor receptor RT-PCR and immunohistochemistry, topoisomerase-1 RT-PCR confirmed the chip results.

**CONCLUSION:** Different histological alterations can be reclassified by functional, multivariate analysis using cDNA microarrays. Further studies with expanded sample number are needed for subclassification of pathological alterations.



**Key words:** Adenoma; Biopsy samples; Colorectal cancer; Gene expression; Inflammatory bowel diseases; Microarray technology

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## INTRODUCTION

mRNA expression array analysis is usually performed on high volume surgery or blood samples. However, evaluation of routine biopsy specimens could yield information, as to how the local pathological processes differ from healthy counterparts. In the gastrointestinal tract, biopsy samples are routinely taken. The mRNA expression study of these samples could allow further insight into the development of inflammatory, preneoplastic and neoplastic diseases.

These specimens could not be applied previously for expression array studies, because array technology even today needs significantly more RNA than can be isolated from the tiny biopsy specimen. However, new techniques and commercial kits have recently become available for the reliable mRNA amplification without an effect on the original gene expression pattern[1].

Previous microarray analyses reported in the literature were performed predominantly from surgically resected colon adenocarcinoma samples[2], with gene expression analysis of colonic biopsies done in only two cases[3,4].

In a few studies, local pathological alterations were examined using cDNA microarrays in cells and tissue structures laser-microdissected from surgical material[4,5]. The mRNA expression patterns of tumorous and normal tis-

sues were usually compared by cDNA microarrays[5,7-11] or oligonucleotide microarrays[12,13] containing some hundreds to several thousand fixed target sequences.

Microarray gene expression profiling of adenomas as a precancerous stage of colon adenocarcinoma is less represented in the scientific literature. Oligonucleotide or cDNA microarray-based molecular diagnostics of malignancy in colon adenoma and colorectal cancer samples was described using 10[14], 9[6] and 4[15] adenoma samples compared to adenocarcinoma and normal colonic tissues.

Inflammatory bowel diseases (IBDs) were rarely analyzed by microarrays. Lawrance *et al*[16] published an oligonucleotide microarray comparison between surgically resected human ulcerative colitis (UC), Crohn's disease (CD) and normal colonic tissues. Recently, Langmann *et al*[3] used mucosal biopsy specimens for global gene expression profiling in patients with UC and CD.

cDNA-based mid-sized (1K) commercial arrays have recently appeared, which do not need any special hybridization, washing or scanner apparatus. These open-platform arrays, if of good quality and reproducibility, can contribute to a widespread use of the array technology. Mid-sized so called "overall" microarrays with fixed target cDNAs from the most important cell processes (adhesion, apoptosis, cell cycle, DNA replication and repair, extracellular matrix remodeling, cytoskeleton, immune regulation, metabolism, stress response, oncogenesis and tumor suppression, growth factor-related cell proliferation, neuroendocrine regulation, signal transduction, transcription and transport) give us opportunity for analysis of different cell functions that may be associated with diseases.

Bioinformatical analysis of these types of arrays can then be used to detect the expression pattern differences between limited numbers of diagnostic groups. Multivariate statistical analysis methods can be applied for the development of automated classification methods like image analysis based cervical cancer screening[17,18].

In the present study we aimed to prove that normal, inflammatory, premalignant and malignant colon biopsies that can be used for mRNA expression analysis and the expression differences can be utilized in a multivariate classification system. As colorectal adenocarcinoma (CRC) frequently arises in the setting of various high-risk conditions such as adenomatous polyps and IBD, we looked for gene expression pattern-based connections between these types of diseases.

## MATERIALS AND METHODS

### Patients and samples

Routine biopsy specimens were collected from the pathological and normal part of the colon, placed in RNALater Stabilization Reagent. Total RNA was extracted from frozen biopsy specimens from 11 patients with adenomatous polyps, 12 with CRC and 11 with IBDs (5 UC, 6 CD). After quality control of the microarrays, applicable data were available from 10 patients with adenomatous polyps, 6 with CRC and 6 with IBD (3 UC, 3 CD).

Four male and two female patients of 53-86 years (median, 72.17 years) with CRC were involved in the study. Five patients had left side (2 rectal, 33%; 3 sigmoid , 50%) and 1 had right side (coecal, 17%) involvement. Two patients had localized disease without nodal or distant organic involvement (Dukes B stage, 33%), 1 patient had nodal involvement (17%, Dukes C stage) and 3 had liver metastases (50%, Dukes D stage).

Nine male and one female patient of 17-77 years (median, 61.2 years) with colorectal adenoma were involved in the study. Five of them had left side (1 rectal, 10%; 3 sigmoid, 30%; 1 descendent colonic, 10%), and 5 had right side (2 ascendant colonic, 20%; 1 coecal, 10%; 2 total colonic, 20%) involvement. Six patients had tubular (60%), 2 had tubulovillous (20%) and 2 had villous adenoma (20%). Only one of the villous adenoma patients' biopsy sample contained severe dysplastic alteration.

One male and five female IBD patients of 23-72 years. (42 years. median) were involved in the study. Three of them [50%; 1 UC (33%) and 2 CD (66%)] had total and three [50%; 2 UC (66%) and 1 CD (33%)] had left side colonic disease. The grade of inflammation was severe in 3 cases (50%) and moderate in 3 cases (50%).

### Methods

**Total RNA isolation using Qiagen RNeasy Mini Kit:** Frozen biopsy samples were lysed and homogenized in a mixture of 300 μL GITC-containing lysis buffer and 3 μL β-mercaptoethanol by Polytron homogenizator for 30-40 s. The lysed samples were digested in proteinase K solution at 55℃ for 10 min. After silica membrane cleaning, according to the manufacturer's description and DNase I treatment (in order to absolutely remove genomic DNA), the total RNA was eluted in 50 μL RNase-free water. Quantity and quality of the isolated RNA was tested by measuring the UV absorbance, by using real-time RT-PCR (Light Cycler G6PDH Housekeeping Gene Set, Roche) and by agarose gel electrophoresis. The high quality, intact total RNA samples, which showed regular 18S and 28S ribosomal RNA bend pattern during the agarose gel analysis, and showed positive real-time RT-PCR reaction were used for microarray analysis.

**T7 RNA amplification and labeling:** Because the total RNA content of the biopsy samples was lower than 10 μg, which was not enough for one hybridization reaction-the mRNA fraction was amplified by the T7-method (MessageAmp Ⅰ aRNA Kit, Ambion Inc., US), according to the manufacturer's instructions, fluorescently labeled probes were synthesized using amino allyl UTPs and Cy-3 and Cy-5 monoreactive dyes (Amersham Biosciences Ltd., England).

**Atlas Glass 1.0K microarray analysis:** The mRNA expression profile was evaluated by Atlas Glass microarrays (BD Clontech Inc. US, 1081 genes). Two hundred μL fluorescently labeled probes (mixture of the appropriate Cy3- and Cy5-labelled cDNA) were mixed with the prewarmed hybridization solution and hybridized to the microarray for 16 h at 50℃. Washing steps were done at room temperature in washing solutions (containing 0.75 mmol/L DTT) according to the manufacturer's instructions. The slides were dried by blowing with carbone-dioxyde and were scanned by Axon GenePix4000B reader on 532 nm (Cy3) and 635 nm (Cy5) wavelengths.

**Data analysis:** Scanned arrays were evaluated by the GenePix Pro 4.1 software. Automated spot detection using local background determination was done and feature extraction (ratio of medians, ratio of means, Cy3/Cy5) was performed. Microarrrays with nonhomogeneous background and/or incomplete housekeeping gene spot set were removed from further analysis. Ratios of medians of the detected features were applied in the normalization. Means of the ratio of medians were normalised to be 1. Results were exported into Acquity 3.1 software (Axon Inc.) and datasets were established. Dataset selection into classification categories was performed. Mean, median, SD of each parameter was calculated. Underexpression was defined as ratio of means ± SD < 0.5, overexpression as ratio of means ± SD > 2.0. One-way (group) ANOVA and multivariate exploratory techniques (discriminant analysis, factor analysis and hierarchical cluster analysis) were performed by SAS 6.12 version statistical software. Hierarchical cluster analysis was done using Ward's method (Euclidean distances). The colonic cases were clustered according to their expression pattern based on the filtered ANOVA results. Gene annotation and functional classification were done using Atlas Gene List Version 4.0. Functional analysis and visualization of biological association network were done using Pathway assist 2.53 software.

**Validation:** Real-time RT-PCR was used for validation of expression microarray results (Roche LightCycler). One-step RT-PCR was carried out using the LightCycler h-β2M Housekeeping Gene Set and RNA Master Hybridization Probes kit. For the relative quantification, commercially available β-2-microglobulin (142 bp length fragment) was used as a reference, and newly designed and synthesized epidermal growth factor receptor (EGFR) (227 bp length fragment) or DNA topoisomerase I (Top1) (184 bp length fragment) were used as a target gene. The following primer and hybridization probe sequences were used: EGFR9 S primer: 5'-atcctgccggtggcatt-3', EGFR12 A primer: 5' -gttcaggctgacgactgca-3', EGFR-FL probe: 5'-caggacggac ctccatgcctttga-3', EGFR-LC probe: 5'-LC Red 640-cctag aaatcatacgcggcaggacc-3' in case of EGFR, and Top1-fw primer: 5'-acatcatgcttaaccctagttcac-3', Top1-as primer: 5' -cagagcaagcttgtcgatg-3', TOP1-FL probe: 5'-cggatcttgtcc acacatttttttcagc-3', TOP1-LC probe: 5'-LC Red 640-ccgag cagtctcgtatttctgccag-3' in case of DNA-topoisomerase-I. Evaluation of relative ratios (diseased/normal/same patient) was prepared using RelQuant software.

**EGFR immunohistochemistry:** Formalin fixed paraffin embedded 4 μm thick colonic biopsy tissue sections were dewaxed and rehydrated. Antigen unmasking was carried out by nuclease free Proteinase K digestion for 20 min at room temperature. After washing twice in PBS, endogenous peroxidase activity was blocked by incubation in 3% hydrogen peroxide for 30 min at room temperature. After washing 3 times in PBS for 3 min, a specific blocking was done with 1% BSA-PBS solution for 10 min at room temperature. Then the slides were incubated with diluted EGFR culture supernatant antibody (1 μL EGFR antibody and 40 μL PBS) (Clone: H-11, DAKO) at 37℃ for 60 min in a humidified chamber. After washing 3 times in PBS, signal conversion was carried out with the LSAB2 system (DAKO) according to the manufacturer's instructions. Haematoxylin co-staining was done.

**Ethical consideration:** All routine colonic biopsy specimens from the patients were taken after informed consent and ethical permission was obtained for participation in the study.

## RESULTS

### *Identification of commonly over-and underexpressed genes in colonic diseases*

Genes that were up- and downregulated in at least 2/3 of cases per sample group in colorectal cancer (Supplemental Table 1), in adenoma (Supplemental Table 2) and in IBD (Supplemental Table 3) were considered as a commonly over- and underexpressed genes. CRC cases are characterized by upregulated genes in the DNA replication (such as replication protein A1, DNA topoisomerase Ⅱα, DNA topoisomerase Ⅰ), cell cycle (including cyclin A1, cyclin-dependent kinase 10, protein NIMA-interacting 1), extracellular matrix remodeling (like keratin 5, perlecan, enactin), transcription regulation (such as IRF5, 6, and E74-like factor 2) , oncogenesis (including v-jun, BRCA2) and growth factor related cell proliferation (EGFR, VEGFB, hepatocyte growth factor, transforming growth factor β2) cell function groups; and downregulated genes in the DNA repair (RAD51 and 52 homolog), tumor suppression and apoptosis (like BCL2-interacting killer) cell function groups. Adenoma cases in comparison showed altered gene expression data in apoptosis (such as TNF receptor-associated factor 6, BAX), growth factors, receptors and their signal transduction (like calgranulin A, KIT ligand, Ran GTPase activating protein 1), oncogenesis and tumor suppression (including growth factor receptor-bound protein 10, p53-induced protein and betaglycan) functional groups. IBD cases are featured by the gene expression changes of immune regulation (including GM-CSF2, CXCL13, MMP-3, MMP-12 and interleukin 1 receptor antagonist), transport [like transferring and solute carrier family 25 (mitochondrial carrier; adenine nucleotide translocator), member 5], and growth factor-related cell proliferation (such as B cell growth factor 1, GM-CSF).

### *The most differentially expressed genes between the 3 sample groups in colon-filtered results of ANOVA*

We analyzed the expression differences between all 1081 genes using ANOVA. The results of ANOVA were filtered according to the number of cases. The genes without enough data from all colonic samples were removed from the analysis. 19 genes were found to be significantly differently expressed ($P < 0.05$) between the colonic sample groups using filtered ANOVA method (Table 1).

### *Factor analysis and discriminant analysis*

Factor analysis was prepared on the basis of the results of variance analysis. Factor analysis resulted in two different factor groups. Factor 1 had the most considerable explorative variance value (6.331196), but the factor 2 also showed significant explorative variance values (4.710563). The factor analysis gives information about the functional gene groups which can differentiate the observed diseases

**Table 1 The most differentially expressed genes between the 3 sample groups in the colon**

| Name | GenBank ID | Differences between sample groups | *P* | Number of valid cases/ sample groups | Ratio of means[1] in adenoma | Ratio of means[1] in CRC | Ratio of means[1] in IBD | Cell function |
|---|---|---|---|---|---|---|---|---|
| Adhesion | | | | | | | | |
| Bystin-like | L36720 | 1-3<br>2-3 | 0.0005<br>0.0008 | 5, 2, 2 | 0.612 ± 0.62676 | 1.47 ± 0.789131 | 7.3375 ± 1.134906 | Other cell adhesion proteins |
| Apoptosis | | | | | | | | |
| Tumor necrosis factor receptor superfamily, member 10c, decoy without an intracellular domain | AF016267 | 1-2<br>2-3 | 0.0044<br>0.0046 | 4, 4, 5 | 0.49075 ± 0.315247 | 2.7695 ± 1.308503 | 0.5032 ± 0.270649 | Death domain receptors |
| Extracellular matrix, cytoskeleton | | | | | | | | |
| Matrix metalloproteinase 13 (collagenase 3) | X75308 | 2-3 | 0.0395 | 10, 6, 6 | 0.8075 ± 0.406413 | 0.557 ± 0.324015 | 1.222333 ± 0.558757 | Metallo proteinases |
| Metabolism, blood coagulation | | | | | | | | |
| Ubiquitin C | M26880 | 1-3<br>2-3 | 0.0325<br>0.0227 | 10, 5, 5 | 0.9329 ± 0.687387 | 0.8456 ± 0.51739 | 2.3002 ± 1.110219 | Protein turnover |
| Plasminogen activator, urokinase | M15476 | 1-3 | 0.0089 | 6, 5, 4 | 0.482167 ± 0.248489 | 1.0554 ± 0.524046 | 1.513 ± 0.417319 | Serine proteases |
| Oncogenes and tumor suppression | | | | | | | | |
| AXL receptor Tyrosine kinase | M76125 | 1-2 | 0.0209 | 7, 5, 4 | 0.258571 ± 0.237985 | 2.0162 ± 1.429626 | 1.3225 ± 0.780286 | Oncogenes and umor suppressors |
| Ras homolog gene family, member H | Z35227 | 1-2 | 0.0076 | 7, 5, 3 | 0.806429 ± 0.732179 | 2.736 ± 1.083434 | 1.115 ± 0.249638 | Oncogenes and tumor suppressors |
| RAB5A, member RAS oncogene family | M28215 | 1-3 | 0.0151 | 8, 5, 5 | 0.5745 ± 0.438766 | 0.9958 ± 1.224974 | 2.263 ± 0.863959 | General trafficking |
| Receptors and ligands | | | | | | | | |
| Small inducible cytokine A5 (RANTES) | M21121 | 1-2 | 0.0087 | 8, 4, 6 | 0.706125 ± 0.402215 | 1.94025 ± 0.799844 | 1.0615 ± 0.371365 | Growth factors, cytokines, and chemokines |
| Pleiotrophin (heparin binding growth factor 8) | M57399 | 1-2 | 0.0373 | 5, 5, 2 | 0.3474 ± 0.242632 | 2.7236 ± 1.872401 | 0.477 ± 0.166877 | Growth factors, cytokines, and chemokines |
| Patched (Drosophila) homolog | U43148 | 1-3<br>2-3 | 0.0043<br>0.0058 | 9, 5, 5 | 0.653667 ± 0.463297 | 0.7752 ± 0.448416 | 3.8946 ± 2.578342 | Growth factor and chemokine receptors |
| Stem cell growth factor; lymphocyte secreted C-type lectin | D86586 | 1-2 | 0.01755 | 5, 5, 4 | 0.3264 ± 0.168316 | 1.6386 ± 0.957384 | 0.5585 ± 0.420226 | Ggrowth factors, cytokines, and chemokines |
| Interleukin 7 receptor | M29696 | 1-2<br>2-3 | 0.0096<br>0.0066 | 8, 4, 4 | 0.40775 ± 0.12511 | 1.774 ± 1.113227 | 0.32975 ± 0.133809 | Interleukin and interferon receptors |
| Signal transduction | | | | | | | | |
| Myotonic dystrophy protein kinase like protein | Y12337 | 1-2<br>1-3 | 0.0253<br>0.0131 | 10, 6, 6 | 0.7611 ± 0.296331 | 1.375167 ± 0.301345 | 1.4415 ± 0.521753 | Intracellular kinase network members |
| Transcription | | | | | | | | |
| Heat shock transcription factor 1 | M64673 | 1-2 | 0.0136 | 9, 6, 3 | 0.732444 ± 0.421368 | 1.7565 ± 0.747317 | 0.385333 ± 0.320644 | Transcription activators and repressors |
| Immediate early protein | M62831 | 2-3 | 0.0209 | 8, 5, 5 | 0.552875 ± 0.271426 | 0.4708 ± 0.276956 | 0.989 ± 0.255144 | Basic transcription factors |
| High-mobility group (nonhistone chromosomal) protein isoforms I and Y | M23619 | 1-3<br>2-3 | 0.0003<br>0.0011 | 9, 6, 5 | 0.734556 ± 0.324623 | 0.897667 ± 0.371581 | 1.7982 ± 0.238334 | Chromatin proteins |

[1]Cy3/Cy5 (disease/normal).

according to their different expression levels (Table 2).

According to the expression changes of the following genes the three colonic disease groups can be significanty distinguished: HSF1 ($P$ = 0.012537), bystin-like ($P$ = 0.001027), calgranulin A ($P$ = 0.043831), and TNFR superfamily member 10c ($P$ = 0.037888) (Figure 1).

### Cluster analysis

Four different clusters can be identified: two IBD-related clusters containing one CRC case, a carcinoma group containing one adenoma and one ulcerative colitis case, and an adenoma cluster with nine adenoma cases (Figure 2). Tree diagram of 22 colonic cases showed considerable accordance with the conventional histopathological diagnoses. Excluding one case (AD1), all adenoma cases belong to a significantly distinct cluster. The cluster of severely inflammed IBD cases contained one colorectal adenocarcinoma case, as it showed a similar gene expression pattern. This

**Table 2 Results of the factor analysis**

| STAT. factor analysis | Factor loadings (Varimax raw) Extraction: Principal components (Marked loadings > 0.700000) | | Cell function |
|---|---|---|---|
| | Factor 1 | Factor 2 | |
| Stem cell growth factor | 0.896313 | -0.053631 | Growth factors, cytokines,and chemokines |
| Pleiotrophin (heparin binding growth factor 8) | 0.882657 | 0.006966 | Growth factors, cytokines, and chemokines |
| Small inducible cytokine A5 (RANTES) | 0.802949 | 0.169587 | Growth factors, cytokines, and chemokines |
| Interleukin 7 receptor | 0.835349 | -0.151447 | Interleukin and interferon receptors |
| Tumor necrosis factor receptor superfamily, member 10c | 0.742897 | -0.145755 | Death domain receptors |
| Signal transducer and activator of transcription 2113 kDa | 0.820315 | 0.066954 | Transcription activators and repressors |
| Heat shock transcription factor 1 | 0.745691 | 0.032620 | Transcription activators and repressors |
| Patched (Drosophila) homolog | -0.113082 | 0.768421 | Growth factor and chemokine receptors |
| RAB5A, member RAS oncogene family | -0.044311 | 0.834146 | General trafficking |
| Ubiquitin C | -0.045668 | 0.719247 | Protein turnover |
| High-mobility group protein isoforms I and Y | -0.029882 | 0.943945 | Chromatin proteins |
| Explorative variance | 6.331196 | 4.710563 | |

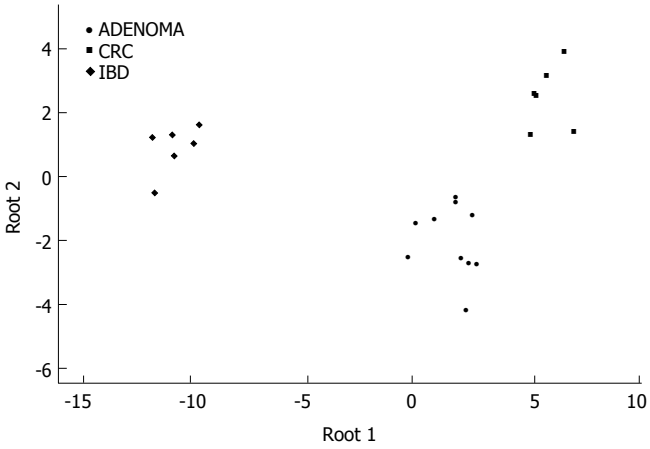


**Figure 1** Discriminant analysis of colonic biopsy specimens. Note the clear separation of the single classification groups based on the discriminatory genes detailed in the results section.

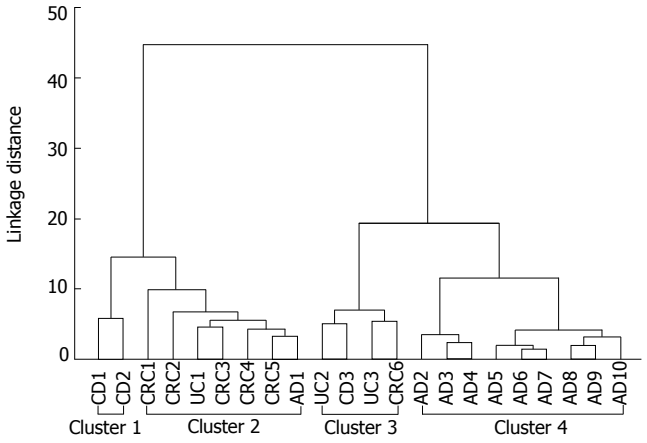


**Figure 2** Tree diagram for 22 colonic diseases (Ward's method euclideandistances). Four different clusters can be identified: 1 and 3 are IBD-related clusters containing one CRC case; 2 is a carcinoma group containing one adenoma and one ulcerative colitis cases; 4 is an adenoma cluster with nine adenoma cases. AD: Adenoma; CD: Crohn's disease; CRC: Colorectal cancer; UC: Ulcerative colitis.

observation could reflect that colorectal cancer can develop from chronic inflammation and some types of adenomas.

### *Validation*

All samples with remaining total RNA (9 CRCs, 2 adenomas and 3 CD samples), were tested using one-step EGFR and TOP1 real-time RT-PCR. Using β-2-microglobulin as a standard control, EGFR- and TOP1 mRNA levels were measured, and ratios of these concentrations to the standard were determined. Ratios for each disease group along with normal samples were compared, and the resulting relative ratios determined (diseased/normal in the same patient). The CRC samples showed higher EGFR mRNA level than the normal paired sample (relative ratio: 3.008, SD: 4.591), while there was no differential expression found in the adenoma samples (relative ratio: 0.772, SD: 0.060). Lower EGFR expression was measured in CD samples (relative ratio: 0.451, SD: 0.173) compared to the normal adjacent mucosa. Increased, 2.802-fold TOP1 mRNA level was evaluated in the CRC samples (SD: 3.884), but TOP1 level of the other sample groups was within the normal range (realtive ratio-adenoma: 0.774, SD: 0.0368; realtive ratio-CD: 0.942, SD: 0.337). The expression differences of EGFR and TOP1, found by microarray analysis, could be confirmed in most, but not in all cases. First, because of the limited total RNA amount in several of the small biopsy samples, the starting isolated total RNA was used in its entirety for probe synthesis. Secondly, differences between the two gene expression analysing method could be due to different gene sequences being amplified and detected during the real-time RT-PCR and the microarray analysis.

### *EGFR-immunohistochemistry*

In colorectal carcinoma samples, EGFR was found to be mildly and moderately expressed in all carcinoma cells. Diffuse cytoplasmatic EGFR staining was found in all carcinoma samples. In normal samples, moderate and high EGFR expression was found in some epithelial cells, but in total amount, EGFR expression was decreased compared to carcinoma samples (Figure 3). EGFR protein expression data were in correlation with EGFR mRNA expression data from both microarray and real-time RT-PCR analysis.

## DISCUSSION

We compared the gene expression pattern of biopsy samples from patients with adenoma, CRC and IBD, on

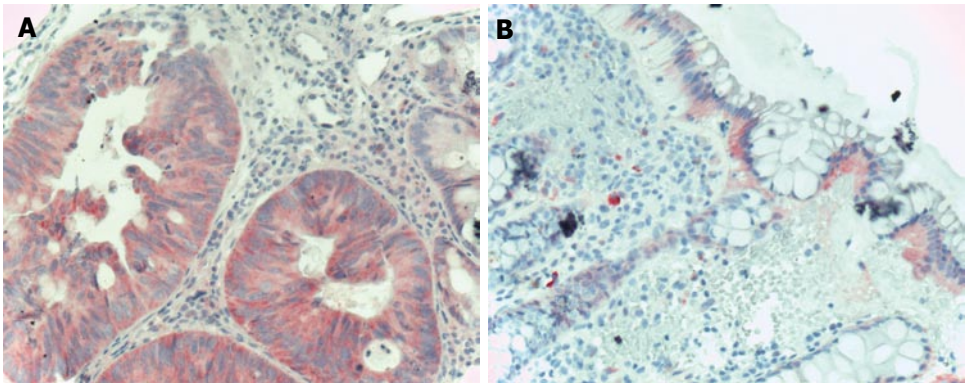


**Figure 3** EGFR immunohistochemistry in CRC. **A**: Carcinomatous glands of the colon showing diffuse cytoplasmatic, moderately intensive EGFR staining (Hematoxylin co-staining × 200); **B**: Normal colonic epithelia showing mildly, moderately intensive basolateral intracytoplasmatic EGFR staining. The lower 2/3 of the crypts do not show EGFR positivity (Hematoxylin co-staining × 100).

Human Atlas Glass 1.0 microarrays containing 1081 target sequences. Biopsies were taken from disease-involved areas of the colon and surrounding disease-free colonic mucosa from the same patient. Multiple filtering methods for reduction of array-array variability origins from the spotted microarray hybridization procedure, and multiple statistical analyses were applied for finding colorectal carcinogenesis-associated genes, which can enhance the conventional histological diagnosis.

The Atlas Glass Human 1.0 microarray platform is a spotted microarray with each probe consisting of a single "long oligo" (80 mer) spotted on a glass slide. On the Atlas Glass microarray, all of the weight of detection rests on a single oligonucleotide. If cross-hybridization with an inappropriate target occurs, then the readout for that gene is incorrect. This could result in an erroneous quantification of mRNA levels[19,20] and relatively high standard deviation values. This main disadvantage of spotted opened system microarrays was apparent in our analyses: several hybridized microarrays (12 from 34) were removed from the further analysis after the quality testing, because of the problems mentioned above. Hence, comparison results from analysis of different microarray types, besides the facts mentioned above, requires due foresight, because of the different signal detection techniques, various sample amounts, sample types, sample processing and storage, and different experimental controls. The results are strongly affected by choice of biopsy samples or surgically resected tissue as a starting material, heterogeneous tissue samples or homogeneous cells such as laser microdissected cells. Our concept was that biopsies taken from patients during endoscopical examination with minimal intervention, are the most suitable samples for identifying early diagnostic target molecules.

CRC cases are characterized by upregulated genes in the DNA replication, cell cycle, extracellular matrix remodeling, transcription regulation, oncogenesis and growth factor related cell proliferation cell function groups; and downregulated genes in the DNA repair, tumor suppression and apoptosis cell function groups, while adenoma cases showed altered gene expression data in apoptosis, growth factors, receptors and their signal transduction, oncogenesis and tumor suppression functional groups. Despite the differences in samples and microarray types, there are genes, which were found differently expressed in our study that were also found commonly with other research groups. This fact emphasizes the importance of these CRC-related genes. Similarly to our findings, overexpression of several oncogenes (v-jun[20], BRCA2[8], growth factor genes (VEGF[22], HGF[23], TGFβ[5,9,24,25]), DNA replication and repair genes (TOP2A[8,26], tankyrase[9]), signal transduction gene (ephrin B2[27]), cell cycle genes (cell growth regulatory with zinc finger domain[8]) and transcription factors (several interferon regulatory factors[8]) was previously found by others using microarray analysis in gastrointestinal cancers.

Over- and underexpression of several apoptosis-, proliferation- and cell survival-related genes were observed in our microarray analysis (Table 1). These cell processes and their balance play a critical role in carcinogenesis and tumor progression. Functional analysis and visualization of biological association networks, especially pathways involved in apoptosis and cell survival were done using Pathway Assist 2.53 software. Figure 4 shows a graphical overview illustrating the role of the most important upregulated apoptosis-related genes. We found differently expressed genes in our study that are involved in or affected by not only one, but four different apoptotic pathways which are known in mammalian cells. These genes include TNFRSF10c (tumor necrosis factor receptor superfamily, member 10c) so called TRAILR3, TRAF6 (TNF receptor-associated factor 6), TNFRSF6 (Fas) and caspase 4 (CASP4) genes, downregulated apoptotic BIK (Bcl-2 interacting killer) and PP2A (protein phosphatase 2A). Two other genes that showed increased mRNA level in CRC, and were verified by real-time RT-PCR and/or immunohistochemistry, DNA topoisomerase I (TOP1) and EGFR (epidermal growth factor receptor), have a possible role in cell processes leading to CRC. Overexpression of EGFR correlates with CRC progression and metastatic potential[28]. EGFR activation can promote proliferation and maintain survival via MAPK (Ras/JNK), Ras/ERK and JAK/STAT signalling pathways. Amplification of receptor signaling by means of overexpression may promote tumor growth and resistance to apoptosis. Human TOP1 associated with intensive cell division and DNA replication occurring in the malignant cells.

We also aimed to find genes that can enhance the molecular classification of CRC and its precancerous stages. Several genes from different functional groups were found to be overexpressed in both adenoma and CRC samples compared to adjacent normal mucosa, and the degree of upregulation of these genes follows the normal-adenoma-

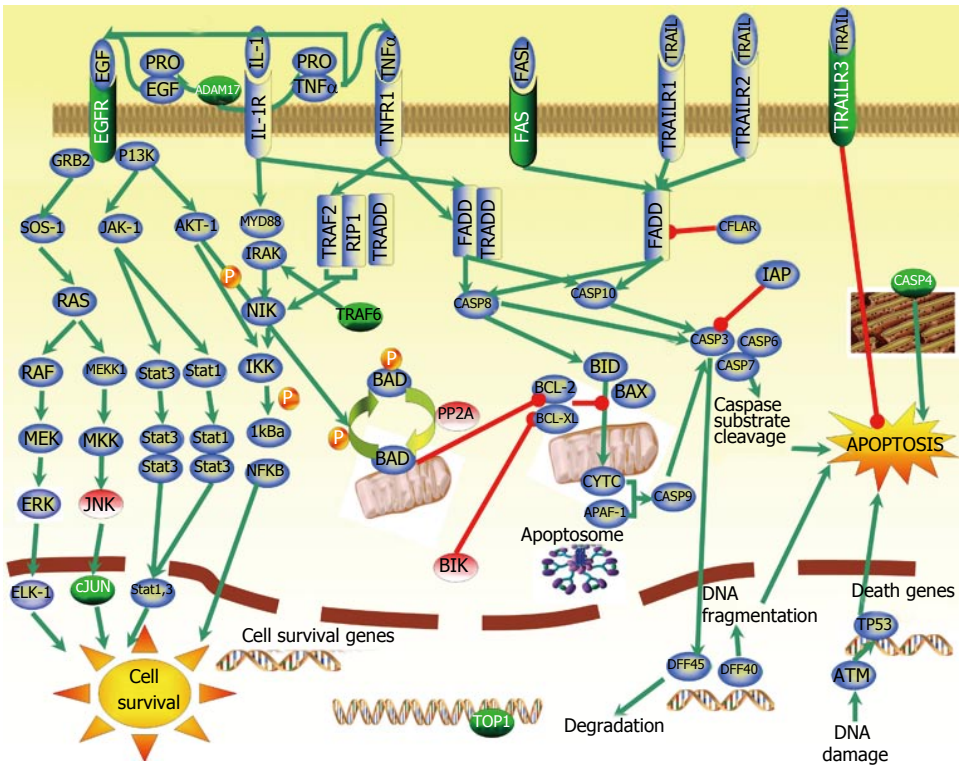


**Figure 4** The main apoptotic and cell survival pathways identified in CRC gene expression study. Genes marked with green were found to be overexpressed, while genes marked with red were found to be underexpressed in our microarray analysis. Genes in blue are previously described as apoptotic and cell survival pathways-related genes. Green arrows refer to the positive regulation, while reds mean negative regulation (inhibition).

CRC sequence. Genes found to be important for this classification include cell survival promoting molecules (TRAF6, Grb10, ELF2, TACE), DNA synthesis and cell cycle involved molecules (RanGAP1, replication protein A1) and CFTR chloride channel. CFTR (cystic fibrosis transmembrane conductance regulator) is the most important chloride channel in the luminal membrane of the colon. A central role has been suggested for CFTR in coordinating electrolyte transport by changing absorption into secretion in colon carcinogenesis[29]. Recent studies demonstrate an enhanced cAMP-activated $Cl^-$ secretion in the hyperproliferative colonic mucosa that is caused by elevated CFTR expression[30].

IBD cases are featured by the gene expression changes of immune regulation, transport and growth factor-related cell proliferation in our study. In correlation with our findings, elevated chemokine (small inducible cytokine A4, interleukin-1 receptor antagonist) and matrix metalloprotease (MMP-3, MMP-12) mRNA levels were detected in IBD compared to normal mucosa by microarray analysis[16]. Small inducible protein A4 is highly expressed in IBD according to the degree of inflammation[31]. IL-1 receptor antagonists inhibit the activity of IL1 and modulate a variety of IL1-related immune and inflammatory responses. Programmed expression of MMPs is involved in tissue remodeling during inflammation, moreover MMP-12 may play a role in macrophage movement and epithelial cell shedding[32]. Several other growth factors and chemo attractants were found overexpressed in our microarray analyses. Secretion of GM-CSF2 (macrophage-granulocyte colony stimulating factor 2) is increased in mucosal lesions in IBD. B cell growth factor 1 released by T cells after antigen stimulation, and supports the clonal proliferation of B cells. CXCL13 B cell chemoattractant is mostly produced by the monocyte/macrophage lineage in UC[33].

### *Discrimination of colonic diseases according to the gene expression markers*

Discriminant analysis shows the genes which can help us to classify an unknown sample into one of the groups of observed diseases, considering their expression levels. According to the expression changes of the following four genes the three colonic disease groups can be significantly distinguished: HSF1, calgranulin A, TNFRSF10c (TRAILR3) and bystin-like.

HSF1 is a heat-shock transcription factor, that was found to be upregulated in CRC compared to normal mucosa in both our and other research groups' gene expression studies[34]. In adenoma and IBD cases it showed lower expression level compared to the CRC samples. Induction of HSF1 gene expression could activate the HSF1 heat shock stress signal pathway in sporadic CRC. The heat shock stress signaling pathway is highly involved in carcinogenesis since heat shock proteins are responsible for maintaining the conformation, stability and function of key oncogenic client proteins involved in signal transduction pathways leading to proliferation, cell cycle progression and apoptosis, as well as other features of the malignant phenotype such as invasion, angiogenesis and metastasis[35-37].

Calgranulin A (S100A8) is a calcium-binding protein that showed higher expression in the IBD sample group, than in CRC and adenoma cases. S100 proteins are involved in the regulation of a number of cellular processes such as cell cycle progression, differentiation and immune response[38].

Significantly increased TRAILR3 level was found in

CRC samples, but not in IBD and adenoma cases. This receptor inhibits the TRAIL-induced apoptosis via binding TRAIL ligand which in this case cannot interact with the pro-apoptotic, death domain containing other TRAIL receptors[39,40].

Interestingly, significant bystin-like 7-fold mRNA overexpression was found in IBD samples in our microarray analysis. Suzuki *et al*[41] identified a cytoplasmic protein, named bystin, that directly binds trophinin and tastin cell adhesion molecules that are involved in the process of the embryo implantation. A role for bystin-like protein in inflammation has not been described.

The factor analysis gives information about the functional gene groups which can differentiate the observed diseases according to their different expression levels. Five of the seven genes (HSF1, TRAILR3, stem cell growth factor, interleukin-7 receptor and pleiotrophin) with the most considerable explorative variance value (belong to Factor 1) were mentioned previously in the scientific literature as cell proliferation and cancer related genes (HSF1[34-37], TRAILR3[39,40], stem cell growth factor[42], interleukin-7 receptor[43] and pleiotrophin[44,45]).

Twenty two colonic biopsy samples were clustered in correlation with the conventional histopathological diagnoses. Excluding one case, all adenoma cases belong to a significantly distinct cluster. The cluster of severely inflammed IBD cases contained one colorectal adenocarcinoma case, as it showed a similar gene expression pattern. This fact can refer that colorectal cancer can develop on the basis of chronic inflammation and some types of adenomas.

In summary, we can say that the overall mid-size glass arrays are suitable for identification of disease-specific genes which are considered as gene expression markers. Detection of the mRNA expression levels of marker gene panels gives an opportunity for classification of colonic samples, even in the case of small biopsy specimens. The limited starting RNA amount arising from the small sample size makes microarray analysis difficult. The standardization of the opened manual microarray systems is more difficult; however, automatization of this technique would further improve the efficacy, reproducibility and quality of microarray analysis.

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*RAPID COMMUNICATION*

# Colorectal cancer screening by non-invasive metabolic biomarker fecal tumor M2-PK

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## Abstract

**AIM:** To evaluate the utility of the innovative fecal tumor M2-Pyruvate kinase (M2-PK) test in our daily clinical routine, as a marker for the pre-selection of patients who should subsequently undergo colonoscopy for the diagnosis or exclusion of colorectal cancer.

**METHODS:** Fecal tumor M2-PK was measured in stool samples of 96 study participants (33 patients with colorectal cancer, 21 patients with rectal carcinoma and 42 controls) who all underwent total colonoscopy.

**RESULTS:** In 39 of 42 individuals in the control group, fecal tumor M2-PK was below 4.0 kU/L (93% specificity). Colorectal tumors were accompanied by a highly significant increase ($P < 0.001$) in fecal tumor M2-PK levels (median: colon carcinoma, 23.1 kU/L; rectal carcinoma, 6.9 kU/L; colorectal carcinoma, 14.7 kU/L), which correlated with Duke's staging and T-classification. The overall sensitivity was 78% for colorectal cancer, increasing from 60% for stage T1 to 100% for stage T4 and from 60% for Duke's A to 90% for Duke's D tumors.

**CONCLUSION:** Fecal tumor M2-PK is an appropriately sensitive tool to pre-select those patients requiring colonoscopy for the further diagnostic confirmation or exclusion of colorectal cancer.



**Key words:** Tumor M2-Pyruvate kinase; Pyruvate kinase type M2; Colon cancer; Rectal cancer; Adenoma; Feces; Cancer screening

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## INTRODUCTION

In Germany, about 70 000 people are diagnosed with colorectal cancer each year[1]. This figure is about 1 million worldwide, with approximately 528 000 deaths from colorectal cancer each year[2]. The gold standard for the early detection of colorectal cancer is colonoscopy. However, the acceptance of this costly and invasive method is low. Only 1.7% of people entitled to colonoscopy under the German national colorectal cancer screening program actually undergo the procedure[3].

In order to increase the participation in colorectal cancer screening programs, an easy, fast and economical initial screening method, with good patient compliance, is absolutely necessary. This allows identification of those patients most likely to have colorectal cancer, who require further investigation by colonoscopy.

The guaiac-based fecal occult blood test (FOBT), which is based on the premise that polyps and cancer bleed more than normal mucosa[4], is currently the most commonly used test for colorectal cancer screening. Guaiac-based FOBTs have been investigated in a number of large studies and shown to reduce mortality by about 15%-33% in screened populations[5,6]. However, they have limited sensitivity. For example, Lieberman *et al*[7] and Koss *et al*[8] found their sensitivity was less than 30% for colorectal cancer and less than 15% for advanced adenomas. Newer immunological FOBTs showed higher sensitivities[9,10] with the advantage of no dietary restrictions. In most studies with immunological FOBTs to date, however, colonoscopy has been performed only in FOBT-positive cases. Non-bleeding colorectal tumors and those not consistently discharging sufficient blood into the gut lumen are not detected by either guaiac or immunological FOBTs.

Recently a new screening test for the early detection of adenomas and colorectal tumors has been described. The tumor M2-Pyruvate kinase (M2-PK) stool test is based on the measurement of a key enzyme involved in tumor metabolism[8,11-14].

Tumor M2-PK is the dimeric form of the glycolytic pyruvate kinase isoenzyme type M2[15]. The enzyme catalyzes the last reaction step within the glycolytic

sequence from phosphoenolpyruvate (PEP) to lactate and is responsible for net ATP production within this pathway. Enzymatic characterization of a wide range of different tumors revealed that tumorigenesis is accompanied by an increase in total pyruvate kinase v-max activities. There is also a shift towards the expression of the pyruvate kinase isoenzyme type M2 (M2-PK) and away from the tissue-specific isoenzymes (L-PK in liver and kidney, M1-PK in muscle and brain and R-PK in erythrocytes)[16-18]. The increased expression of M2-PK is under the control of ras, and the transcription factors SP1 and HIF-1. Ras and HIF-1 are both consistently altered in gastrointestinal tumors[19-22]. M2-PK can occur in a tetrameric form which is characterized by a high affinity to its substrate PEP and in a dimeric form with a low PEP affinity. The tetramer: dimer ratio of M2-PK determines the proportion of glucose carbons used for glycolytic energy production (tetrameric form) or channeled into synthetic processes (dimeric form). In tumor cells, M2-PK is mainly found in the dimeric form (tumor M2-PK) due to direct interaction with various oncoproteins, i.e. $pp60^{v\text{-}src}$ kinase and HPV-16 E7[15,21,23]. Tumor M2-PK is released into the blood, and in the case of adenomas and tumors in the lower gastrointestinal tract also into the stool of patients. An increase in tumor M2-PK in EDTA plasma samples is found in gastrointestinal cancers, as well as a wide range of other tumors such as lung, renal, breast and cervical cancer. The EDTA plasma test is highly suitable for patient monitoring[24-30].

The fecal tumor M2-PK test has been described as a promising new screening tool for adenomas and colorectal cancer[8,11-14]. Therefore, the aim of our study was to evaluate the utility of the tumor M2-PK test in our own daily clinical routine as a marker for the pre-selection of patients requiring subsequent diagnostic colonoscopy.

## MATERIALS AND METHODS

### Patients

Our study consisted of 96 participants who underwent complete colonoscopy. The control group consisted of 42 healthy individuals (15 male and 27 female; median age: 58 years; range: 25-79 years) without any findings at colonoscopy, who were participating in the national screening colonoscopy program provided by the German health insurance system. All screening colonoscopies were conducted between September 2005 and April 2006 in a primary care gastroenterology and hepatology medical center. Healthy individuals were included in the control group.

The 54 participants with colorectal cancer underwent diagnostic colonoscopy at the Offenbach Municipal Hospital between January 2003 and April 2006. Rectal carcinomas were diagnosed in 21 patients (15 male and 6 female; median age: 70 years; range: 52-84 years). Colonic adenocarcinomas were diagnosed in 33 patients (24 male and 9 female; median age: 70 years; range: 43-84 years).

All participants received a stool sample collection pot and were instructed to collect a single stool sample (naturally produced, walnut sized) one day prior to the laxative administration in preparation for colonoscopy. No special diet was recommended. Paper collecting devices were used to avoid stool contact with water in the toilet bowl. Stool samples were initially stored at room temperature by the participants until the day of colonoscopy. Thereafter, these pre-colonoscopy stool samples were stored at -20℃ at the medical center or hospital until analyzed for tumor M2-PK.

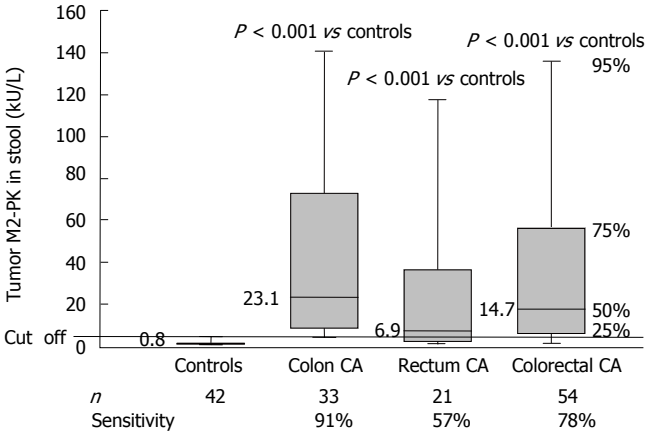


**Figure 1** Tumor M2-PK levels in stool samples of healthy control individuals and patients with colon or rectal cancer.

### Measurement of fecal tumor M2-PK concentrations

Fecal tumor M2-PK concentrations were determined using a commercially available sandwich ELISA based on two different monoclonal antibodies which specifically recognize the dimeric form of M2-PK (ScheBo® · Biotech AG, Giessen, Germany). A positive test result was defined as > 4.0 kU/L, as indicated by the manufacturer.

### Statistical analysis

Since the data were skewed to the right, the statistical analysis was conducted using the Kruskall-Wallis ANOVA test (Statistica, StatSoft® Inc., Tulsa, USA).

## RESULTS

This study evaluated 54 patients with colorectal cancer and 42 healthy controls with no indication of gastrointestinal diseases at colonoscopy. In the control group, fecal tumor M2-PK levels were below 4.0 kU/L in 39 of the 42 subjects (median: 0.8 kU/L), resulting in 93% specificity (Figure 1). In two of the three control samples which were above the cut-off value only a slight increase of tumor M2-PK (4.4 kU/L and 5.3 kU/L) was measured.

Colorectal tumors were accompanied by a highly significant increase in fecal tumor M2-PK levels. The median value was 23.1 kU/L for colon carcinoma ($P < 0.001$), 6.9 kU/L for rectal carcinoma ($P < 0.001$) and 14.7 kU/L ($P < 0.001$) for colorectal carcinoma when both groups were combined.

At a cut-off level of 4.0 kU/L, the sensitivity was 91% for colon carcinoma, 57% for rectal carcinoma and 78% when both groups were combined. Both T classification and Duke's staging of the colorectal tumors revealed a

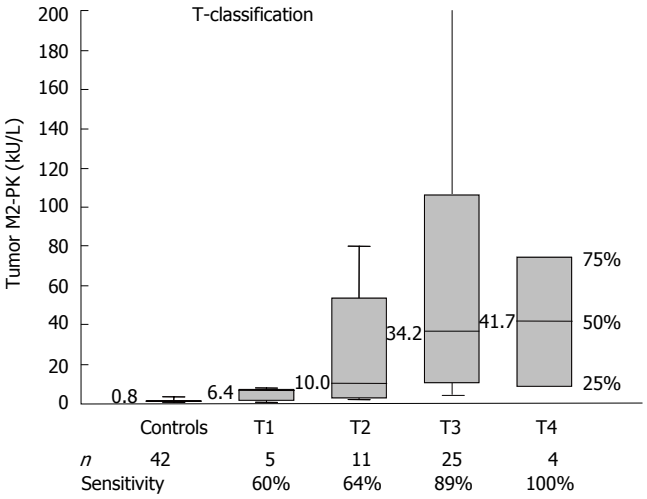


**Figure 2** Correlation between fecal tumor M2-PK levels and TNM staging.

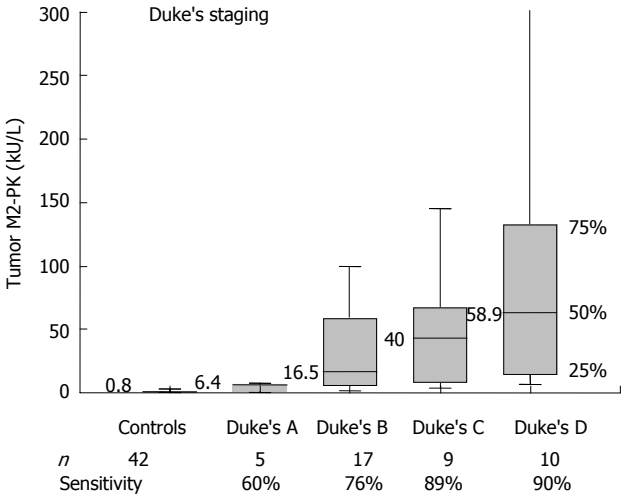


**Figure 3** Correlation between fecal tumor M2-PK and Duke's staging.

**Table 1 Correlation between fecal tumor M2-PK levels and TNM classification or duke's staging**

| Classification | *n* | Median (kU/L) | Mean (kU/L) | SE (kU/L) | Range (kU/L) |
|---|---|---|---|---|---|
| Controls | 42 | 0.8 | 1.5 | 0.4 | 0.1-17.3 |
| T1 | 5 | 6.4 | 4.5 | 1.6 | 0.3-7.7 |
| T2 | 11 | 10.0 | 30.5 | 10.1 | 1.5-100 |
| T3 | 25 | 34.2 | 106.3 | 35.2 | 1.7-620 |
| T4 | 4 | 41.7 | 41.4 | 19.2 | 6.6-76 |
| Duke's A | 5 | 6.4 | 4.5 | 1.6 | 0.3-7.7 |
| Duke's B | 17 | 16.5 | 63.5 | 34.7 | 0.2-604 |
| Duke's C | 9 | 40.0 | 50.0 | 17.8 | 1.9-176 |
| Duke's D | 10 | 58.9 | 138.5 | 65.3 | 4.5-620 |

strong correlation between fecal tumor M2-PK levels and staging. The sensitivities increased from 60% for stage T1 to 100 % for stage T4 and from 60% for Duke´s A to 90% for Duke's D (Figures 2 and 3; Table 1).

## DISCUSSION

Tumor M2-PK is the synonym for the dimeric form of the glycolytic pyruvate kinase isoenzyme type M2[15]. M2-PK is the pyruvate kinase isoenzyme which is characteristic of all proliferating cells and can occur in a tetrameric form as well as a dimeric form. Previous studies describe that tumor M2-PK is released into the stool of patients with adenomas and colorectal tumors and can easily be quantified with a commercially available sandwich ELISA[8,11-14].

In order to evaluate whether tumor M2-PK is a practical tool for the pre-selection of patients with colorectal cancer in our daily routine, we measured fecal tumor M2-PK in a cohort of 96 individuals. All 42 healthy control individuals, 33 patients with colon carcinoma and 21 patients with rectal carcinoma underwent total colonoscopy in order to confirm or exclude colorectal cancer. Our study revealed a highly significant increase ($P < 0.001$) in tumor M2-PK in the stool samples of those patients with colorectal cancer, whereby fecal tumor M2-PK values correlated well with Duke's staging and T-classification (Figures 1-3; Table 1). Even stage T1 or Duke's A showed 60% sensitivity, increasing to 100% in stage T4 and to 90% in Duke's D tumors.

At a cut-off value of 4.0 kU/L, our overall sensitivity for colorectal carcinoma was 78 %. These data correspond well with the results of Hardt *et al*[13] who reported a sensitivity of 78% in 60 colorectal cancer patients, and those of Naumann *et al*[31] who found a sensitivity of 85.2% in a cohort of 27 colorectal cancer patients. A higher sensitivity was reported by McLoughlin *et al*[14] (92% in 25 colorectal cancer patients and 67% in 30 patients with adenomas) and by Koss *et al*[8] (92.3 % in 26 colorectal cancer patients and 60% for adenomas > 1 cm in ten patients).

The most commonly used fecal test in current screening programs is the guaiac-based FOBT[32,33]. Liebermann *et al*[7] and Koss *et al*[8] have reported an overall sensitivity for guaiac-based FOBTs of less than 30% for colorectal cancer and less than 15% for advanced adenomas. Results with newer, immunological FOBTs showed higher sensitivities than guaiac-based FOBTs for colorectal cancers[9,10] but in most studies colonoscopy was performed only in FOBT-positive cases.

The overall sensitivity of tumor M2-PK is increased if a higher proportion of late stage patients are included, but this is also true for FOBTs[10]. Nevertheless, McLoughlin *et al*[14] reported a sensitivity of 67% for adenomas with the fecal tumor M2-PK test. Similarly, Koss *et al*[8] found a sensitivity of 60% for adenomas > 1 cm.

In a head-to-head comparison of fecal tumor M2-PK and the commonly used guaiac-based FOBT, Koss *et al*[8] demonstrated a sensitivity for colorectal cancer of 92.3% for fecal tumor M2-PK and 20% for FOBT. No comparative study of fecal tumor M2-PK and immunochemical FOBTs is currently available.

The high sensitivity of the tumor M2-PK test is due to its ability to detect bleeding and non-bleeding tumors. From a practical viewpoint, the use of a single random formed stool sample for tumor M2-PK analysis, without

requiring dietary restrictions, might be of greater patient convenience compared with the need to collect stool on three consecutive days for the guaiac FOBT.

In our study, the control group consisted of individuals without any signs of gastrointestinal diseases at colonoscopy. The median tumor M2-PK value in this group was 0.8 kU/L. In 39 of 42 subjects, tumor M2-PK levels were below the cut-off value; in two further control samples tumor M2-PK levels were only slightly increased (4.4, 5.3 kU/L). The resulting specificity at a cut off value of 4.0 kU/L is 93%, which is in general accordance with the studies of Hardt *et al*[13], Koss *et al*[8] and McLoughlin *et al*[14] who report specificities between 78% and 98%. Naumann *et al*[31] found increased fecal tumor M2-PK levels in cases of active Crohn's disease and ulcerative colitis in which increased cell proliferation is expected. In addition, patients with inflammatory bowel disease have an increased risk of developing colorectal cancer, probably linked to frequent cycles of damage and regeneration of the colonic mucosa associated with flares of active disease.

Another new approach for pre-selective colorectal cancer screening is the determination of mutated oncogenes and anti-oncogenes[22,34-38]. These tests have the advantage of very high specificities. However, due to high genetic heterogeneity within colorectal cancers, a panel of different targets (k-ras, p53, APC genes, as well as microsatellite instability marker) must be measured in order to reach acceptable sensitivity which makes the test extensive and expensive. Using a 21-target multipanel, sensitivities between 44% and 91% and specificities between 93% and 100% are described[36-38]. Furthermore, to assure the stability of DNA within the stool, samples have to be frozen at -80℃ within 12 h after defecation. Fecal tumor M2-PK is stable for 48 h at room temperature and for up to one year when frozen at -20℃ (manufacturer's data sheet), which makes it practical for routine use. In addition, the tumor M2-PK test could be conducted in virtually all hospital and private diagnostic laboratories because it can either be run manually combined with an ELISA plate reader or automated using existing commercially available equipment.

Overall, our results are in general agreement with previous studies which have demonstrated that fecal tumor M2-PK is an appropriate tool to achieve a sensitive pre-selection by identifying those patients with the greatest need to undergo diagnostic colonoscopy to confirm or exclude colorectal cancer.

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*RAPID COMMUNICATION*

# Steroids reduce local inflammatory mediator secretion and mucosal permeability in collagenous colitis patients

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## Abstract

**AIM:** To study the effect of oral steroids upon clinical response and rectal mucosa secretion of eosinophil cationic protein (ECP), myeloperoxidase (MPO), basic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF) and albumin in patients with collagenous colitis (CC).

**METHODS:** A segmental perfusion technique was used to collect perfusates from rectum of CC patients once before and twice (one and four weeks) after the start of steroid treatment. Clinical data was monitored and ECP, MPO, bFGF, VEGF and albumin concentrations were analyzed by immunochemical methods in perfusates and in serum.

**RESULTS:** Steroids reduced the number of bowel movements by more than five times within one week and all patients reported improved subjective well-being at wk 1 and 4. At the same time, the median concentrations of ECP, bFGF, VEGF and albumin in rectal perfusates decreased significantly. MPO values were above the detection limit in only 3 patients before treatment and in none during treatment. VEGF, bFGF, ECP and albumin concentrations correlated with each other with the exception of ECP and albumin. A decrease of serum ECP and VEGF concentrations was also seen even if the overtime reduction was not significant.

**CONCLUSION:** Oral steroid treatment in CC patients induced a simultaneous reduction of bowel movements and rectal release of ECP, bFGF, VEGF and albumin, suggesting that these polypeptides and increased mucosal permeability are important components of the pathophysiology in collagenous colitis.



**Key words:** Collagenous colitis; Inflammatory mediator; Steroid treatment; Eosinophil cationic protein; Basic fibroblast growth factor; Vascular endothelial growth factor

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## INTRODUCTION

Collagenous colitis (CC) is characterized by watery diarrhea and an increased subepithelial collagen layer with infiltration of inflammatory cells in the surface epithelium and lamina propria of the colon[1]. The thickened collagen in CC is an amorphous eosinophilic band located beneath the intercryptal epithelial surface and is best visualized using special collagen staining, such as Masson´s trichrome[1]. The collagen composition of the basal membrane of colonic mucosa in CC patients is identical to that of normal individuals[1]. In contrast, the subepithelial collagen layer differs in CC patients and consists of collagen type I and Ⅲ or Ⅵ[2-4].

Intestinal inflammatory cells present in CC are lymphocytes, plasma cells, often eosinophil and sometimes neutrophil granulocytes. In the epithelium, lymphocytes are most frequent and dominated by $CD8^+$ T-cells[5]. In the lamina propria, the inflammatory cell infiltration includes plasma cells, eosinophils, macrophages and mast cells, but rarely neutrophils. Unlike epithelial lymphocytes, the lymphocytes in the lamina propria are mainly composed of $CD4^+$ T-cells[5].

Eosinophils are one of the main components of the inflammatory cell population in colonic mucosa of CC patients[1]. Eosinophils produce not only cytotoxic granule proteins, such as eosinophil cationic protein (ECP), major

basic protein (MBP) or eosinophil peroxidase (EPO), but also a wide range of polypeptides, such as transforming growth factor-β1 (TGF-β1), vascular endothelial growth factor (VEGF), *etc*[6-10]. Eosinophilopoietic cytokines, such as granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin-5 (IL-5), activate eosinophils by inhibiting apoptosis and prolonging the life span of eosinophils[7]. Eosinophils interact with fibroblasts and contribute to fibrosis in inflammatory diseases like asthma and inflammatory bowel disease (IBD). Activated fibroblasts/myofibroblasts are regarded as the major contributors of collagen accumulation in the intestinal wall of IBD[11,12] including CC patients[13].

In asthma, basic fibroblast growth factor (bFGF), separately or in combination with other growth factors, perpetuate chronic inflammation and triggers fibrosis leading to airway tissue remodeling[14] including excessive extra cellular matrix deposition in the subepithelial region. The role of bFGF, as one contributing factor in tissue remodeling, is supported by perfusion studies in ulcerative colitis (UC)[15] and CC[16] patients, where the secretion of bFGF was increased.

VEGF is a pleiotropic polypeptide, which has physiological and pathophysiological actions on the extra cellular matrix and may enhance fibrosis through induction of connective tissue growth factor (CTGF)[17]. A recent study on the pathophysiological role of VEGF in CC showed that VEGF, by changing the local matrix metalloproteinase -1 and tissue inhibitor metalloproteinase-1 (MMP-1/TIMP-1) balance, causes the accumulation of immature subepithelial ECM in CC[18].

Glucocorticosteroids have several anti-inflammatory effects, including the ability to block the recruitment of proinflammatory cells like eosinophils, downregulation of the secretion of proinflammatory cytokines, enhancement of apoptosis in eosinophils[19-21] and engulfment of apoptotic eosinophils by phagocytic cells[7]. In asthma, prolonged treatment with inhaled corticosteroids reverses tissue remodeling by downregulating fibroblast-functions[14,20,22]. In our previous perfusion studies, we found increased colorectal concentrations of ECP[23], bFGF[16], VEGF[24] and the permeability marker albumin in CC patients compared with control patients. We put forward the hypothesis that these mediators enhanced chronic inflammation and fibrosis, leading to pathological accumulation of subepithelial collagen. Oral steroid treatment is effective for inducing remission in CC patients[25,26] but the mechanisms leading to relief of symptoms are not fully known. The aim of this study was to elucidate the relationship between steroid treatment and rectal mucosa secretion of ECP, MPO, bFGF, VEGF and albumin in CC patients.

## MATERIALS AND METHODS

### Patients

Twelve patients (10 women, 2 men; mean age 52 years; range 34-66 years) who fulfilled the diagnostic criteria of CC[1] were recruited between November 2000 and November 2002 at the Department of Medicine, University Hospital, Uppsala, Sweden.

### Ethics

Committee of the Medical faculty at Uppsala University approved the study and informed consent was obtained from all participants. No patient had anti- or pro-inflammatory drug treatment during the investigation period, but one patient (number eight) was taking indomethacin (Indomee$^{R}$, Merck Sharp and Dohme, Stockholm, Sweden) up until one week before the first perfusion. Patient number seven also had celiac disease and lactose intolerance, while patients five and eight suffered from hypothyroidism. Patients eleven and twelve were smokers.

### Design of study

Steroid treatment was initiated after the first perfusion with an oral dose of 40 mg prednisolone daily, which was reduced successively by 5 mg/wk. Three rectal perfusions were fulfilled per patient, one before and two after the start of steroid treatment, at completed weeks one and four respectively. Serum samples were also collected on those occasions. Clinical data were recorded at each perfusion as the patients estimated their average daily number of loose stools and degree of general well-being during the week preceding each perfusion.

### Perfusion technique

A colonoscope-based segmental perfusion technique[23] was used to obtain perfusion fluid from the rectum. Sodium phosphate (Phosphoral$^{R}$, Ferring, Limhamn, Sweden) was used for bowel preparation. The rectal perfusions were carried out with the patients lying in a supine position and started with an endoscopic examination of the rectum and sigmoid colon. The segment, positioned at the end of the flexible endoscope, was established by inflating air into two balloons delimiting the segment in the rectum. The perfusion procedures were carried out for one hour[23] and the perfusates were collected in three portions at 20 min intervals in tubes immersed in ice. Intravenous injections of the sedative diazepam (Stesolid$^{R}$, Alpharma, Stockholm, Sweden) were given at a median dose of 7.5 mg (range: 5-15) and the analgesic meperidine (Petidin$^{R}$, Ipex, Stockholm, Sweden) at a median dose of 25 mg (range: 25-50).

### Perfusion buffer, perfusates and serum samples

A buffer was used, consisting of 120 mmol/L NaCl, 5.4 mmol/L KCl, 2 mmol/L $Na_2HPO_4$, 10 mmol/L glucose, 35 mmol/L mannitol, and 1 g/L polyethylene glycol (PEG; MW 4 kDa) at pH of 8.20-8.48 and osmolarity of 290 mosm/L. The perfusion fluid was kept at 37℃ and then infused at a speed of 3 mL/min for 60 min. By adding ten milliliters of aprotinin (Trasylol$^{R}$ Bayer 1.0 × $10^7$ kIU/L) to every liter of perfusion buffer, proteolytic activity was inhibited. Phenol red solution (50 mg/L in physiological saline) was intermittently infused (5 mL every 20 min) above the proximal balloon to check that the perfusion segment was closed and that there was no contamination of the perfusion fluid by intestinal contents located proximally. All perfusate samples were collected on ice and frozen in aliquots of 2 mL at -70℃. The protease inhibitor, phenylmethylsulphonyl fluoride (PMSF; Sigma

**Table 1 The clinical data and the individual concentrations of ECP, MPO, bFGF, VEGF and albumin in rectal perfusion fluid of CC patients before and after one and four weeks of treatment with steroids**

| Demography | | | Bowel movements per day | | | Mediators analyzed in Perfusion fluid | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | ECP (μg/L) | | | MPO (μg/L) | | | bFGF (pg/mL) | | | VEGF (pg/mL) | | | Albumin (mg/L) | | |
| Patients | Age (yr) | Gender | | | | wk | | | | | | | | | | | | | | |
| | | | 0 | 1 | 4 | 0 | 1 | 4 | 0 | 1 | 4 | 0 | 1 | 4 | 0 | 1 | 4 | 0 | 1 | 4 |
| 1 | 34 | Female | 3-4 | 0-1 | 0-1 | 3.7 | < 2 | < 2 | 29.1 | 14.9 | < 8 | 14.03 | 18.26 | 0.65 | 299.7 | 92.8 | 41 | 20.3 | 44.6 | < 2.1 |
| 2 | 57 | Female | 4 | 0-1 | 0-1 | 2.6 | < 2 | < 2 | 12.5 | < 8 | < 8 | 7.85 | 7.11 | 0.37 | 54.7 | 97 | 40.2 | 95.8 | 57.7 | < 2.1 |
| 3 | 59 | Female | 10-12 | 0-1 | 0-1 | 3.6 | 7 | < 2 | < 8 | < 8 | < 8 | 1.21 | 4.1 | < 0.25 | 113.1 | 146.7 | 81.1 | 20.3 | 10.7 | 3.4 |
| 4 | 43 | Female | 4-6 | 3-4 | 0-1 | < 2 | < 2 | < 2 | < 8 | < 8 | < 8 | 0.72 | 0.41 | < 0.25 | 28.4 | 22.9 | 40.2 | 2.8 | 10.9 | 17.5 |
| 5 | 62 | Female | 3-4 | 0-1 | 0-1 | < 2 | < 2 | < 2 | < 8 | < 8 | 11.3 | 0.35 | < 0.25 | 0.29 | 66.2 | 46.6 | 53.1 | 2.7 | 10.7 | 5.5 |
| 7 | 66 | Female | 4-5 | 0-1 | 0-1 | < 2 | < 2 | < 2 | < 8 | < 8 | < 8 | < 0.25 | < 0.25 | < 0.25 | 58.8 | 19.1 | 22.2 | 3.9 | 6.5 | 3.2 |
| 8 | 59 | Female | 5-10 | 2 | 0-1 | 6.5 | < 2 | < 2 | < 8 | < 8 | < 8 | 5.28 | < 0.25 | 0.35 | 86.1 | 37.8 | 58.8 | 17.1 | 3.1 | 2.3 |
| 9 | 64 | Female | 3 | 0-1 | 0-1 | < 2 | < 2 | < 2 | < 8 | < 8 | < 8 | < 0.25 | < 0.25 | < 0.25 | 17.6 | 9.6 | 12.3 | 5 | < 2.1 | 7 |
| 10 | 39 | Male | 7-10 | 2-3 | 0-1 | < 2 | < 2 | < 2 | < 8 | < 8 | < 8 | 2.7 | 1.21 | < 0.25 | 100.4 | 94.5 | 90.3 | 43 | 13.7 | 11.7 |
| 11 | 34 | Female | 5-6 | 2 | 0-1 | 3.1 | < 2 | < 2 | 18.3 | < 8 | < 8 | 11.99 | 2.35 | < 0.25 | 153.6 | 84.4 | 80.2 | 116 | 22.1 | 10.7 |
| 12 | 53 | Male | 6-7 | 2-3 | 0-1 | 2.3 | < 2 | < 2 | < 8 | < 8 | < 8 | 12.29 | 0.91 | 0.61 | 95.3 | 48.2 | 29.1 | 21.5 | 5.2 | < 2.1 |

Chemical Co., St Louis, MO, USA), was added to a final concentration of 2 mmol/L immediately before analysis to counteract the effect of even small amounts of proteases in the perfusion fluid. Serum samples were drawn simultaneously with the perfusions being performed and frozen at -70℃ for later analysis.

### Quantification of ECP, MPO and albumin

The concentrations of ECP and MPO in perfusion fluids and serum samples were analyzed by means of a specific RIA (Pharmacia & Upjohn, Diagnostic AB, Uppsala, Sweden). The inter- and intra-assay coefficients of variations were < 10% for both tests. The lower detection limits of ECP and MPO in perfusion fluid were 2 and 8 μg/L, respectively. The reference intervals for ECP and MPO in serum were 2.3-16 μg/L and 170-478 μg/L, respectively. Albumin in perfusion fluid was analyzed by rate nephelometry on a Beckman Array protein system (Beckman Instruments, Brea CA), according to the recommendations of the manufacturer. The lower detection limit in perfusion fluids of albumin was 2.14 mg/L. Analysis of albumin in serum was performed by a photometric assay (Boehringer Manheim, Mannheim, Germany) and the reference intervals were 40-51 g/L (age < 50 years) and 37-48 g/L (age > 50 years). The concentration of phenol red was assessed, after alkalinization of the perfusate samples to pH 11, by a spectrophotometric method at 520 nm.

### Quantification of bFGF and VEGF

ELISA technique was used to measure bFGF (Human bFGF, Quantikine High Sensitivity, R&D Systems, Minneapolis, MN, USA) and VEGF (DVE00, R&D Systems, Minneapolis, MN, USA) as recommended by the manufacturer. The bFGF and VEGF concentrations in the samples were determined by comparing the optical density of the samples with the standard curve. The lowest detectable value for bFGF was 0.25 pg/mL and for VEGF 9 pg/mL in perfusion fluid. Reference intervals of S-bFGF were < 4.0 ng/L (males) and < 10.8 ng/L (females). The reference interval of S-VEGF was < 500 ng/L.

### Statistical analysis

The Wilcoxon signed rank test for two variables was used to compare the differences between wk 0, 1 and 4, while the statistical analysis of changes over time in the perfusate and serum concentrations of ECP, MPO, bFGF, VEGF and albumin, was done by Friedman's ANOVA. Spearman rank correlation test was applied to study co-variation between each mediator and the frequency of bowel movements per day. All statistical calculations were performed on a Macintosh computer by means of a statistical package, Stat View 4.51 (Abacus, Concepta Inc.). If concentrations of the mediator proteins and albumin were below the detection limit of the assay, the value of the detection limit was used in the calculations. $P < 0.05$ was considered significant.

## RESULTS

### Perfusions and clinical outcome

Out of 12 CC patients studied, 11 successfully completed steroid treatment and all three perfusions according to the protocol. The excluded patients stopped taking steroids because of side effects. The number of bowel movements per day declined from median 5 before start of treatment, to 0-1 after one and 0-1 after four weeks, respectively. These differences were significant ($P < 0.0001$) (Table 1). All patients reported that senses of general well-being were better after the start of steroid treatment, compared to the period before it.

### ECP, MPO and albumin

The concentrations of ECP, MPO and albumin in perfusates are shown in Table 1. The median values of ECP concentrations in perfusates were 2.3 μg/L (range: 1.9-6.5) at wk 0, 1.9 μg/L (range: 1.9-7.0) at wk 1 and 1.9 μg/L (range: 1.9-1.9) at wk 4, respectively. A significant

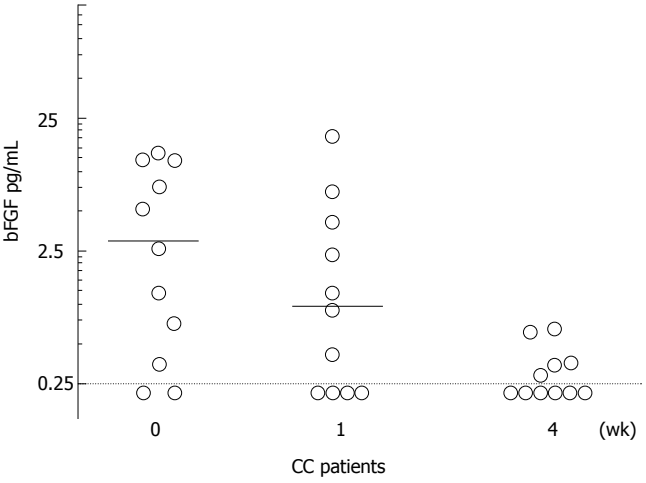


**Figure 1** The individual values of basic fibroblast growth factor (bFGF) concentrations in perfusion fluids from rectum of collagenous colitis (CC) patients before and after one and four weeks of steroid treatment. The bars indicate median values. The dotted line indicates the detection limit for the assay (bFGF: 0.25 pg/mL).

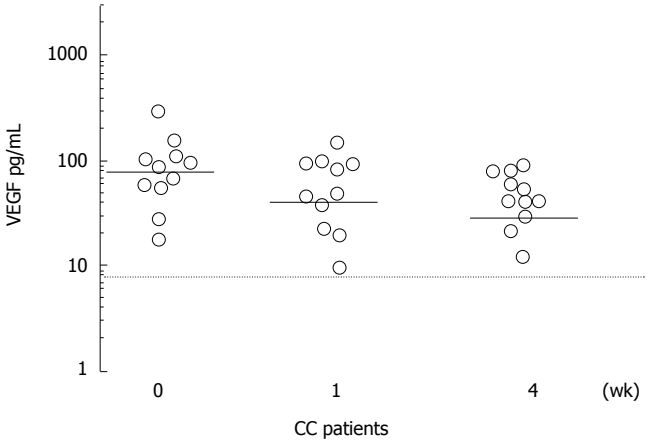


**Figure 2** The individual values of vascular endothelial growth factor (VEGF) concentrations in perfusion fluids from rectum of collagenous colitis (CC) patients before and after one and four weeks of steroid treatment. The bars indicate median values. The dotted line indicates the detection limit for the assay (VEGF: 9 pg/mL).

difference was only found between wk 0 and 4 ($P$ = 0.0277). The corresponding values for ECP in serum at wks 0, 1 and 4 were 11.3 μg/L (range: 1.9-29), 6.6 μg/L (range: 1.9-18.8) and 6.3 μg/L (range: 1.9-18.9), respectively. The decrease between wk 0 and 1 and wk 0 and 4 were significant ($P$ = 0.0076, $P$ = 0.045 respectively), while no difference was found when the decline of ECP concentrations in perfusate and serum over time was analyzed by the Friedman's ANOVA.

Perfusate concentrations of MPO were above the detection level in three out of 11 patients at wk 0, one at wk 1 and none at wk 4 (Table 1). Serum MPO concentrations were 432 μg/L (range: 197-649), 325 μg/L (range: 106-1805), 490 μg/L (range: 144-1190) at wk 0, 1 and 4, respectively. No difference was found between the concentrations during the study period.

The median values of albumin concentrations in perfusates were 20.3 mg/L (range: 2.7-116), 10.7 mg/L (range: 2.13-57.7) and 3.4 mg/L (range: 2.13-17.5) at wk 0, 1 and 4, respectively. The difference was significant only between wk 0 and 4 ($P$ = 0.0329). The decline in albumin concentrations over time was not significant. The corresponding median values of serum albumin at wk 0, 1 and 4 were 42 g/L (range: 38-45), 43 g/L (range: 34-48) and 42 g/L (range: 36-46), respectively ($P$ > 0.05). No correlation was found between the concentrations of ECP and albumin in perfusion fluids.

### *Analysis of bFGF*

The concentrations of bFGF in perfusion fluid are presented in Table 1. The median values of bFGF concentrations in perfusion fluid were at wk 0, 1 and 4, were 2.7 pg/mL (range: 0.2-14.0), 0.9 pg/mL (range: 0.2-18.3) and 0.2 pg/mL (range: 0.2-0.7), respectively. The decline of bFGF perfusate concentrations over time was significant when analyzed by the Friedman's ANOVA ($P$ < 0.01) (Figure 1). A correlation ($P$ < 0.05, $r$ = 0.702) that existed between bFGF and ECP perfusate concentrations at wk 0 disappeared at wk 1 and 4. A correlation between bFGF and albumin in perfusates remained significant throughout the study period ($P$ < 0.05 and $r$ = 0.709, $r$ = 0.805, $r$ = - 0.670 at wk 0, 1 and 4 respectively). The median values of bFGF concentrations in serum were 9.1 ng/L (range: 1.7-44), 2.9 ng/L (range: 0.9-17.6) and 3.6 ng/L (range: 0.3-20.9) at wk 0, 1 and 4, respectively, but the decline over time was not significant.

### *Analysis of VEGF*

Table 1 shows all the individual concentrations of VEGF in perfusion fluid. The median values of VEGF concentrations in perfusates of these individuals at wk 0, 1 and 4 were 86.1 pg/mL (range: 17.6-299.7), 48.2 pg/mL (range: 9.6-146.7), and 41.0 pg/mL (range: 12.3-90.3), respectively. When the Friedman's ANOVA was used to analyze the decline of VEGF concentrations, a significant difference ($P$ < 0.01) was seen (Figure 2). Furthermore, there was a significant correlation between VEGF and ECP at wk 0 and 1 ($P$ < 0.05; $r$ = 0.627 and 0.625 at wk 0 and 1 respectively) but not at wk 4. The correlation between VEGF and albumin in perfusate was significant only at wk 1 ($P$ < 0.05; $r$ = 0.666). Similar significant correlations were present between VEGF and bFGF concentrations at wk 0 and 1 ($P$ < 0.01; $r$ = 0.680 and $P$ < 0.05; $r$ = 0.850) but not at wk 4. The median values of VEGF serum concentrations at wk 0, 1 and 4 were 842 ng/L (range: 622-1517), 775 ng/L (range: 385-1215) and 811 ng/L (range: 574-1114), respectively. The decline in serum concentration of VEGF between wk 0 and 4 was significant ($P$ < 0.05) but the over time change was not significant.

## DISCUSSION

The immediate decrease in frequency of bowel movements and the improvement of the general well-being of these patients under steroid medication were similar to previous reports in IBD, including CC[25,26]. After four

weeks of treatment with the oral steroid prednisolone, the concentrations of ECP, bFGF, and VEGF in rectal perfusion fluids were reduced significantly. There were correlations between ECP, bFGF, VEGF and albumin concentrations initially, but these correlations disappeared at wk 4.

Local infiltration of the colonic mucosa by inflammatory cells is one of the histopathological characteristics of CC, and its composition includes eosinophils. The activation of eosinophils is supported by perfusion studies on UC and CC patients, where significantly increased ECP concentrations were found in these patients compared to controls[23,27,28]. Other studies have confirmed that eosinophils are activated and participate in the inflammatory process of CC[29,30]. The pro-inflammatory and profibrotic actions of eosinophils can be down-regulated by different medications including glucocorticoids[19-21]. In the present study, six, one and none out of 11 patients had ECP concentrations above the detections level at wk 0, 1 and 4, respectively. This decrease of ECP perfusate concentrations, according to Wilcoxon signed rank test between wk 1 and 4, suggests that glucocorticoids significantly reduce the secretion of ECP in the colonic mucosa of CC patients. The absence of significant difference when the over time changes in ECP concentrations according to Friedman's ANOVA did not contradict the finding because ECP median values were equal at wk 1 and 4. This happened because all the perfusate ECP values at wk 1 and 4 were below the detection level, with the exception of one case at wk 1. The anti-inflammatory actions of corticosteroids are numerous, including enhancement of eosinophilic apoptosis, increase of tissue eosinophilic clearance, deactivation of eosinophils and diminished recruitment of eosinophils as mentioned above[19-21]. The reduced perfusate concentrations of ECP found in this study, suggests that corticosteroids diminish the mucosal availability of ECP in CC. Corticosteroid treatment reduces the number of eosinophils in the colonic mucosa of CC, as shown in a small previous study[31]. Furthermore, our observations in CC resemble airway eosinophilia in asthma, which is quickly reduced by steroid treatment[32,33]. In a similarly designed therapeutic intervention study on ulcerative proctitis, the local release of ECP, EPO and MPO dropped significantly under steroid medication[34]. Contrary to CC, the numbers of neutrophils in the inflammatory infiltrate of UC is very high and this is well reflected by the high MPO concentrations found in the perfusion fluids of UC patients[35,36]. The similarity in design and the difference in results between these studies suggest that the role of neutrophils in the pathogenesis of CC is probably of little importance, except in cases where acute exacerbation of the disease cannot be excluded[37].

The bFGF concentrations in the rectal perfusates decreased 3 times from wk 0 to 1 and 11 times from wk 0 to 4. The significant downfall of intraluminal bFGF concentrations and the disappearance of the correlation between bFGF and ECP, imply that steroids markedly decrease mucosal bFGF secretion. Based on our previous findings[16] that local bFGF secretion is increased in CC patients, we suggested that bFGF enhances collagen synthesis in CC by stimulating pericryptal myofibroblasts. Furthermore, *in vitro* experiments showed that bFGF stimulated fibroblasts/myofibroblasts, collected from IBD patients, to produce more collagen, compared to corresponding cells from controls[12]. These observations and the findings in the present study suggest pericryptal myofibroblasts are synthetically active in CC[38,39], are de-activated by corticosteroids and this may be the reason behind the diminished collagen layer observed in corticosteroid-treated CC[30]and asthma patients[31]. The presence of correlations between bFGF and ECP perfusate concentrations in this study, suggests that bFGF, separately or in relation with other proteins, perpetuates chronic inflammation and triggers tissue remodeling in CC.

VEGF concentrations in the perfusion fluid were halved at wk 4 after the start of steroid treatment in CC patients. The amount of VEGF concentrations decreased significantly, as was the case for ECP, bFGF and albumin. Correlations existed between VEGF and ECP, bFGF and albumin. The correlation between VEGF and ECP disappeared at the end of this study, indicating a down-regulating effect of corticosteroids on eosinophils. VEGF and ECP are produced by eosinophils[7,29] and less activated eosinophils entail reduction of released cytokines. There is evidence that the synergetic relationship between VEGF and bFGF depends on the bFGF property to upregulate the expression of VEGF receptors (flt-1 and KDR)[40]. The correlations found between these mediators in this study may have reflected this synergism. The reduction of VEGF secretion may not only depend on steroids and de-activation of eosinophils, but also on deactivation of other VEGF producing cells, such as fibroblasts and other mesenchymal cells. This is in line with previous studies[24], which concluded that VEGF participates not only in the inflammatory process of UC and Crohn´s disease[15,41,42], but also in CC.

The clinical improvement during corticosteroid medication occurred simultaneously with a reduction in mediator concentrations, but no statistically significant correlation was found in relation to the decline in frequency of bowel movements (data not shown). In the present study, the patients themselves were the controls because the first perfusion (control) took place before starting steroid treatment.

The luminal release of albumin decreased, as in the case of ECP, significantly from wk 0 to 4 while the corresponding changes over time analyzed by the Friedman's ANOVA, did not show any significant difference. This may be due to the small number of patients included in this study. This finding, together with the correlation of VEGF and albumin, supports the hypothesis that VEGF enhances mucosal permeability in CC as previously suggested[24]. The disappearance of this correlation at wk 4 suggests that corticosteroids normalize permeability in CC. The correlation between bFGF and albumin indicates that bFGF might play a role in the increased colonic permeability. The appearance of a negative correlation at wk 4 is most probably caused by the numbers of bFGF values under detection level which have the same value, increased between wk 0 and

4 from two to six (Figure 1). Because the bFGF values under the detection levels are in the majority, the relevance of this correlation is questionable. The VEGF and ECP serum concentrations declined after introduction of corticosteroid treatment according to the Wilcoxon signed rank test but not according to Friedman's ANOVA. This may reflect a corticosteroid effect on blood eosinophils, as we did not find any difference in previous studies between the serum concentration of VEGF and ECP in CC patients, compared with controls[23,24]. This finding once again stresses the relevance of methods such as perfusion technique in studying predominantly locally restricted mucosal inflammatory processes, especially in CC.

In summary, our study confirms that the secretion of ECP, bFGF and VEGF is increased in CC and we suggest that corticosteroids relieve symptoms by decreasing the availability, release and functions of these inflammatory mediators in colonic mucosa of CC patients.

## ACKNOWLEDGMENTS

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*RAPID COMMUNICATION*

# Hepatitis B genotypes: Relation to clinical outcome in patients with chronic hepatitis B in Saudi Arabia

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## Abstract

**AIM:** To identify the most common hepatitis B virus (HBV) genotype in Saudi Arabia, and correlate the prevailing genotypes with the clinical outcome of patients.

**METHODS:** Patients were consecutively recruited from the hepatology clinics of two tertiary care referral centers. Patients were categorized into 4 different groups: group 1, patients with hepatitis B and normal liver enzymes; group 2, patients with hepatitis B and abnormal liver enzymes but without cirrhosis; group 3, patients with hepatitis B and liver cirrhosis; group 4, patients with hepatitis B and hepatocellular carcinoma. All patients had a positive hepatitis B surface antigen (HBsAg). Genotyping of HBV was performed by nested PCR-mediated amplification of the target sequence and hybridization with sequence-specific oligonucleotides.

**RESULTS:** Seventy patients were enrolled in this study. They were predominantly male (72.9%) in their mid-forty's (mean age 47 years). Forty-nine (70%) patients were hepatitis B envelope antigen (HBeAg) negative. The majority of patients (64%) acquired HBV through unknown risk factors. Hepatitis B genotyping revealed that 57 patients (81.4%) were genotype D, 1 patient (1.4%) had genotype A, 1 patient (1.4%) had genotype C, and 4 patients (5.7%) had genotype E, while 7 patients (10%) had mixed genotype (4 patients ADG, 1 patient DE, 1 patient DF, and 1 patient ADFG). Based on univariate analysis of genotype D patients, significant predictors of advanced liver disease were age, gender, aspartate transaminase, alanine transaminase, albumin, bilirubin, and alkaline phosphatase (all $P < 0.001$). In multivariate analysis decreased hemoglobin ($r = -0.05$; 95% CI: -0.08 to -0.03; $P = 0.001$) and albumin levels ($r = -0.004$; 95% CI: -0.007 to -0.001; $P = 0.002$) were highly significant predictors of advanced liver disease. In patients with HBV genotype D, HBeAg negativity was found to increase across advancing stages of liver disease ($P = 0.024$).

**CONCLUSION:** This study highlights that the vast majority of Saudi patients with chronic hepatitis B have genotype D. No correlation could be observed between the different genotypes and epidemiological or clinical factors. The relationship between genotype D and HBeAg status in terms of disease severity needs to be further elucidated in larger longitudinal studies.



**Key words:** Hepatitis B virus; Genotype D; Cirrhosis; Hepatocellular carcinoma; Saudi Arabia

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## INTRODUCTION

Hepatitis B virus (HBV) infection is a global health problem with over 350 million chronic carriers of the virus with the risk of developing chronic hepatitis, cirrhosis or hepatocellular carcinoma (HCC). HBV is a circular, partially double-stranded DNA virus of approximately 3200 nucleotides. This highly compact genome contains four open reading frames encoding the envelope (PreS1, PreS2, S), core (core, precore), polymerase, and X proteins[1]. HBV genotypes represent naturally occurring strains of HBV that have evolved over the years and reflect the geographical distribution of HBV throughout the world. Up to now, eight different HBV genotypes have been identified and shown to cluster in different areas of the world[2,3]. They display an 8% inter-group divergence in the complete nucleotide sequence of HBV and differences

in the nucleotide homology of the surface gene, which result in different hepatitis B surface antigen (HBsAg) serotypes[4-6].

Genotype A is mainly found in Northwestern Europe, North America and Africa[7], whereas genotypes B and C have been described in South-Eastern Asian populations[8,9]. Genotype E and F are seen in East Africa and the New World, respectively. Genotype D is most often found in southern Europe, parts of Central Asia, India, Africa and the Middle East. Genotype G is a recently determined genotype in France, America, and Germany while genotype H has been reported in patients from Central America[10,11].

Recently, a number of publications have examined the impact of HBV genotype on disease pathogenesis and the clinical outcomes in patients with chronic hepatitis B. Most of these natural history studies, in view of the bimodal distribution of HBV genotypes in Asia and Western countries, have compared either genotypes B and C[11,12], or genotypes A and D[13]. The clinical impact of HBV genotypes when studied in Indian patients with a mixed population of both genotypes A and D, has shown that genotype D has a higher likelihood of developing advanced cirrhosis compared to genotype A[13].

Chronic hepatitis B is an important medical problem in Saudi Arabia although, with the implementation of HBV vaccination of children, the prevalence has dramatically reduced from 6.7% in 1989 to 0.3% in 1997[14,15]. However, little is known about the prevalence and distribution of HBV genotypes in Saudi Arabia. Furthermore, the association between the distinct genotypes and the severity of liver disease in the country remains unreported. The epidemiological studies of HBV genotypes arising from the Middle-Eastern region suggest that genotype D is perhaps the most common[16-20]. Accordingly, the objectives of our study were to identify the most common HBV genotype in Saudi Arabia, and to elucidate the relationship between the prevailing genotypes with the clinical outcome of patients.

## MATERIALS AND METHODS

### Enrollment of study cohort

Patients were recruited prospectively from two tertiary-care hepatology clinics at King Khalid University Hospital and the Riyadh Military Hospital in Riyadh, Saudi Arabia. These medical centers serve as referral centers for population groups resident in different geographical regions of the country. The Medical Ethics Committee in both centers approved the study protocol and all patients signed an informed medical consent indicating agreement to participate in this study. Results of all abnormal tests were provided to patients or their immediate relatives, as was deemed appropriate.

Between May 2004 to December 2005, 70 consecutive adult HBsAg positive patients were recruited at the two centers. Patients were interviewed in person by participating investigators (AAA, FMS, NA, KS) at recruitment by using a structured questionnaire. Information on socio-demographic characteristics, alcohol consumption, personal medical and surgical history, time of disease diagnosis, area of birth and upbringing, and family history of liver disease or cancers was collected. Patients who were HBsAg positive for a period exceeding 6 mo and who had not received any antiviral therapy for HBV in the preceding 6 mo were included in the study.

### Serological evaluation

HBV markers [HBsAg and antibody to HBsAg (HBsAb)] were measured using standard commercial assays. In addition, further serological testing for hepatitis B envelope antigen (HBeAg) and anti-HBe antibody was also performed using the same commercial kits. HBV DNA was determined by a sensitive PCR based assay (COBAS Amplicor; Roche Diagnostics) with a lower limit of detection of approximately 200 copies/mL.

### Virologic testing

Genotypic testing was performed in only those with a detectable HBV DNA (qualitative) in serum. HBV genotyping was determined from serum samples by performing nested PCR-mediated amplification of the target sequence and hybridization with sequence-specific oligonucleotides at Bioscientia Laboratory in Germany.

### Study design

The patients were recruited into four groups: group 1, patients with hepatitis B and normal liver enzymes; group 2, patients with hepatitis B and abnormal liver enzymes and no laboratory or radiological features of cirrhosis; group 3, patients with liver cirrhosis secondary to hepatitis B; and group 4, patients with hepatitis B and HCC. General exclusion criteria included: (1) anti-HCV antibody positive; (2) identifiable other causes of chronic liver disease defined as (high serum iron and ferritin, abnormal serum ceruloplasmin, history of significant alcohol consumption, antinuclear antibody > 1:320, antismooth muscle antibody > 1:320, antimitochondrial antibody > 1:40); (3) history of hepatotoxic medications in the preceding three months of presentation; (4) history of antiviral therapy in the last 6 mo.

Group specific inclusion criteria for group 1 (HBV with normal liver enzymes) were: (1) persistently normal alanine transaminase (ALT) and aspartate transaminase (AST) (normal ALT and AST on at least two occasions separated by at least 3 mo), (2) normal serum bilirubin, albumin and International Normalized Ratio (INR), (3) normal complete blood count (CBC), (4) normal abdominal ultrasound (US) without features of liver cirrhosis or portal hypertension. Inclusion criteria for group 2 (HBV with abnormal liver enzymes) included: (1) persistently elevated ALT and AST (more than two times the upper limit of normal on at least two occasions separated by at least 3 mo), (2) normal CBC, (3) normal US without features of cirrhosis or portal hypertension. Patients were excluded from groups A and B if there was any abnormality in the CBC or any signs of cirrhosis or portal hypertension on abdominal US. Inclusion criteria for group 3 (HBV with liver cirrhosis) included: Any four of the following features of cirrhosis (1) platelet count < $100 \times 10^9$/L, (2) evidence of esophageal varices on endoscopy, (3) ultrasonographic features consistent with cirrhosis, (4)

albumin level less than 30 g/L, (5) INR more than 1.4 and (6) bilirubin level more than 30 μmol/L. Patients were also included in this group if there was histological evidence of liver cirrhosis regardless of the above criteria. Inclusion criteria in group 4 (HBV with HCC) included: Evidence of HCC defined as two of the followings: (1) α-fetoprotein > 400 ng/L, (2) liver mass detected by triphasic computed tomography (CT) or magnetic resonance imaging (MRI) of the abdomen, (3) fine needle aspiration (FNA) or liver biopsy showing HCC.

### Ascertainment of cirrhosis and hepatocellular carcinoma

All participants had screening abdominal ultrasonography (US) at the time of recruitment into the study. The US was performed and interpreted by trained radiographers according to a standardized protocol, and the records reviewed by the investigators. Cirrhosis was diagnosed ultrasonographically based on the appearance of the liver surface, liver parenchymal texture, portal vein size, splenic size, presence of ascites and varicose veins in the portal and perisplenic area.

All patients with an α-fetoprotein > 400 ng/L (or a persistently rising α-fetoprotein) or with high clinical suspicion of HCC underwent a CT and/or MRI of the liver. The diagnosis of HCC was based upon the two published European guidelines towards the diagnosis and management of HCC[21,22]. Enhancement of a liver lesion during the arterial phase and contrast washout during the portal phase, in patients with background cirrhosis secondary to HBV was considered diagnostic of HCC. FNA or liver biopsy was obtained only where considerable doubt existed towards HCC diagnosis.

### Statistical analysis

Descriptive statistics are summarized as mean ± SD. For continuous variables, ANOVA was used for comparison of the four groups. Fisher's exact or chi-square test was used for categorical variables. Univariate analysis was performed to identify important baseline characteristics associated with genotype D. Variables examined included age, gender, White blood cells, hemoglobin, platelet count, HBeAg status, albumin, ALT, AST and bilirubin. A multivariate logistic regression model was developed using forward model to assess the effect of baseline variables on disease advancement. All tests were two sided with a 5% level of significance. All analyses were performed using STATS 9.1.

## RESULTS

Seventy patients were enrolled in the period from May 2004 to Dec 2005. Mean age of the patients was 47 years, and 51 patients (72.9%) were male while 19 (27.1%) were female. All geographical regions of the Kingdom were represented, with 51.5% from the central region where the vast majority of the Saudi population reside and also where the study was conducted; 21.4% from the southern region, 8.5% from the eastern region, 13% from the western region and 5.6% from the northern region, vastly representing the population distribution across the country. There were 17 patients included in group 1, 22 patients in group 2, 19 patients in group 3, and 12 patients in group 4. The majority (45 of 70 patients, 64%) had acquired HBV through unknown risk factors, while 11 patients (15.7%) reported blood transfusion, 7 (10%) reported a prior history of surgery or dental procedures and 4 (5.7%) reported a family history of HBV infection.

**Table 1 Biochemical, hematological and virological parameters of all enrolled patients distributed across the four groups**

| Parameter (mean value) | Group 1 (*n* = 17) | Group 2 (*n* = 22) | Group 3 (*n* = 19) | Group 4 (*n* = 12) | *P* |
|---|---|---|---|---|---|
| ALT (U/L) | 41.88 | 177.7 | 52 | 96 | 0.001 |
| AST (U/L) | 22 | 80 | 55.3 | 150 | 0.001 |
| ALP (U/L) | 100 | 111 | 116.4 | 238.8 | 0.001 |
| INR | 1.3 | 1.1 | 1.4 | 1.2 | 0.003 |
| Bilirubin (μmol/L) | 10 | 13.8 | 40.4 | 30 | 0.001 |
| Albumin (g/L) | 40 | 37.6 | 28.9 | 25.4 | 0.005 |
| WBC ($10^9$/L) | 7.1 | 6.1 | 4.9 | 7.2 | 0.160 |
| Hemoglobin (g/L) | 125 | 129.6 | 118.4 | 62 | 0.740 |
| Platelets ($10^9$/L) | 253 | 202 | 121.3 | 213 | 0.230 |
| HBV DNA level | | | | | |
| < 200 copies/mL | 5 | 2 | 3 | 1 | NS |
| 200-$10^5$ copies/mL | 10 | 8 | 8 | 8 | NS |
| > $10^5$ copies/mL | 2 | 12 | 8 | 3 | NS |

ALT: Alanine transaminase; AST: Aspartate transaminase; ALP: Alkaline phosphatase; GGT: Gamma glutamyl transpeptidase; WBC: White blood cells.

Most patients (52 of 70 patients, 74%) did not express HBeAg including 65% (11 of 17 patients) in group 1, 55% (12 of 22 patients) in group 2, 95% (18 of 19 patients) in group 3, and 92% (11 of 12 patients) in group 4. Amongst the patients who were HBeAg positive, only two patients (11.1%) had cirrhosis or HCC. The vast majority of HBeAg positive patients were either carriers with normal ALT (6 of 18 patients, 33.3%), or raised ALT (10 of 18 patients, 55.6%). One patient expressed HBeAg as well as anti-HBe. Two patients were negative for both markers. In group 1, 15 patients (88.2%) had HBV DNA < $10^5$ copies/mL and were also HBeAg negative. Two patients (11.8%) had HBV DNA > $10^5$ copies/mL and were also HBeAg positive. In group 2, 9 patients (41%) had an HBV DNA < $10^5$ copies/mL and were all HBeAg negative. In group 3, 11 patients (58%) had an HBV DNA of < $10^5$ copies/mL and all were also HBeAg negative. In group 4, 9 patients (75%) had an HBV DNA of < $10^5$ copies/mL and all were also HBeAg negative. Although the proportion of patients with a high viral load (> $10^5$ copies/mL) was greater in groups 2 and 3, this did not reach statistical significance (Table 1).

Table 2 shows the genotyping results according to the clinical status of the studied patients. The majority of the patients were genotype D (57 of 70 patients; 81.4%), with 58.8% (10 of 17 patients) in group 1, 95.5% (21 of 22 patients) in group 2, 84.2% (16 of 19 patients) in group 3, and 83.3% (10 of 12 patients) in group 4. One patient (1.4%) had genotype A, and one (1.4%) had genotype C. Four patients (5.7%) were genotype E, and 7 patients (10%) were mixed genotypes (4 patients ADG, 1 patient DE, 1 patient DF, 1 patient ADFG). There were no significant differences between groups in terms of

**Table 2 HBV genotype distribution across different stages of liver disease and in relation to HBeAg status in 70 patients**

| Study cohort (*n*) | HBV genotype % (*n*) | | | | |
|---|---|---|---|---|---|
| | D | E | A | C | Mixed |
| Group 1 (17) | 58.8 (10) | - | 5.9 (1) | - | 35.3 (6) |
| Group 2 (22) | 95.5 (21) | - | - | 4.5 (1) | - |
| Group 3 (19) | 84.2 (16) | 15.8 (3) | - | - | - |
| Group 4 (12) | 83.3 (10) | 8.3 (1) | - | - | 8.3 (1) |
| HBeAg + (18) | 88.9 (16) | - | 5.6 (1) | 5.6 (1) | - |
| HBeAg - (52) | 78.8 (41) | 7.7 (4) | - | - | 13.5 (7) |

genotypes. In addition, we found no difference between different genotypes in terms of patient age, gender, area of upbringing, liver enzyme level, or serum albumin level. HBeAg negative genotype D patients comprised 50%, 57%, 93.8% and 75% across groups 1, 2, 3, and 4 respectively ($P = 0.024$) (Table 3).

The baseline characteristics according to disease severity (groups 1 to 4) are listed in Table 1. Patients in group 4 (HCC) were more likely to have a higher level of alkaline phosphatase (ALP) than those in groups 1, 2 and 3 respectively, (238 *vs* 100, $P = 0.004$; 95% CI 45-230), (238 *vs* 111, $P = 0.003$; 95% CI 45-208), (238 *vs* 116, $P = 0.036$; 95% CI 6.8-201), and also a higher level of AST than those in groups 1, 2 and 3 respectively (150 *vs* 22, $P = 0.001$; 95% CI 52-203), (150 *vs* 80, $P = 0.06$; 95% CI 3-145), (150 *vs* 60, $P = 0.02$; 95% CI 13-65). Furthermore, group 4 (HCC) patients were more likely to have a significantly lower level of albumin. In addition, 83.3% (10 of 12) of the patients in group 4 were genotype D compared to 81.4% (57 of 70) of the recruited patients who expressed the same genotype ($P = 0.30$). However, there was no significant difference in WBC, hemoglobin, and platelet count between the four groups.

Table 3 depicts the patients with genotype D according to disease severity. Patients in group 4 were older (mean age 61.5 years), tended to have higher levels of ALT, AST, and bilirubin, and lower levels of albumin. These differences were significant between the groups (all $P = 0.001$). However, there was no significant difference between the four groups with respect to hemoglobin ($P = 0.70$), platelet count ($P = 0.11$) and WBC ($P = 0.10$). In view of the small sample size in each group we did not perform a head-to-head comparison between the groups.

In univariate analysis of genotype D patients, age, gender, AST, ALT, albumin, bilirubin, and ALP were significant predictors of advanced liver disease (all $P < 0.001$). However, in multivariate analysis decreased hemoglobin ($P = 0.001$) and albumin levels ($P = 0.002$) were highly significant predictors of advanced liver disease (Table 4).

**Table 3 Hepatitis B virus genotype D patients' biochemical, hematological and virological parameters distributed across the four groups (mean ± SD)**

| Parameter | Group 1 (*n* = 10) | Group 2 (*n* = 21) | Group 3 (*n* = 16) | Group 4 (*n* = 10) | *P* |
|---|---|---|---|---|---|
| Age (yr) | 45 ± 18 | 35 ± 9.9 | 52.1 ± 7.7 | 61 ± 12 | 0.015 |
| ALT (U/L) | 40.3 ± 8 | 176.9 ± 100.7 | 54.1 ± 30.4 | 99.3 ± 79.9 | 0.001 |
| AST (U/L) | 21.1 ± 6.5 | 78.6 ± 56.8 | 63.8 ± 48.5 | 158.3 ± 167.6 | 0.001 |
| ALP (U/L) | 98.6 ± 39.9 | 112.6 ± 41 | 143.8 ± 6.2 | 239.9 ± 201 | 0.001 |
| Bilirubin (μmol/L) | 10.6 ± 4.9 | 14.1 ± 10.2 | 48.6 ± 60.4 | 27.8 ± 23.2 | 0.001 |
| Albumin (g/L) | 41.5 ± 3.2 | 37.8 ± 4.6 | 27.8 ± 7.9 | 24.7 ± 8.4 | 0.007 |
| WBC ($10^9$/L) | 7.5 ± 2.2 | 6.3 ± 1.7 | 6.12 ± 3.1 | 6.2 ± 1.9 | 0.100 |
| Hemoglobin (g/L) | 127.7 ± 42.5 | 129.2 ± 57.5 | 65.5 ± 55.9 | 62.5 ± 54.5 | 0.704 |
| Platelets ($10^9$/ L) | 250 ± 84.3 | 207.2 ± 56 | 130.8 ± 56 | 217.6 ± 97.4 | 0.113 |
| HBeAg -/+ | 5/5 | 12/9 | 15/1 | 9/1 | 0.024 |

ALT: Alanine transaminase; AST: Aspartate transaminase; ALP: Alkaline phosphatase; GGT: Gamma glutamyl transpeptidase; WBC: White blood cells.

**Table 4 Predisposing factors for advanced hepatitis B virus genotype D liver disease**

| | Variable | Coefficient | 95% CI | *P* |
|---|---|---|---|---|
| Univariate analysis | Age | -0.01 | -0.02 to 0.0005 | < 0.001 |
| | Gender | 0.5 | 0.03 to 1.1 | < 0.001 |
| | AST | 0.2 | 0.09 to 0.3 | < 0.001 |
| | ALT | 0.04 | 0.01 to 0.03 | < 0.001 |
| | ALK | 0.01 | -0.001 to 0.02 | < 0.001 |
| | Albumin | -0.2 | -0.3 to -0.9 | < 0.001 |
| | Bilirubin | 0.08 | 0.0009 to 0.1 | < 0.001 |
| Multivariate analysis | Hemoglobin | -0.05 | -0.08 to -0.03 | 0.001 |
| | Albumin | -0.004 | -0.007 to -0.001 | 0.002 |

CI: Confidence interval; ALT: Alanine transaminase; AST: Aspartate transaminase; ALP: Alkaline phosphatase.

## DISCUSSION

Chronic hepatitis B is an important medical problem in Saudi Arabia. Al-Faleh *et al* in the late 1980's showed that up to 7% of Saudi children were positive for HBsAg[14]. After the introduction of universal vaccination of all Saudi children in 1989, the incidence of hepatitis B infection has declined to as low as 0.3%[15]. In spite of this dramatic decline, the burden of decompensated liver disease secondary to hepatitis B is expected to increase significantly in the next 40 years as the previously infected children start aging.

Hepatitis B genotyping has received immense attention recently and its clinical implications are being investigated extensively throughout the world. This study is the first to show that genotype D is the most prevalent genotype in Saudi Arabia. In this prospective study all regions of the country are represented as well as the entire spectrum of chronic HBV infection ranging from the carrier state to HCC. Our study concurs with reports from other parts of the Middle East like Egypt, Yemen, Turkey, Iran, and Tunisia[16-20], all showing that genotype D is the most common genotype in this region.

HBV genotypes may contribute in part to the wide variation in prevalence rates of HBV infection in different parts of the world through difference in rates of replication and ability to evade immune clearance. However, studies comparing the replication capacity and immune response of the various HBV genotypes have not been performed. Nevertheless, many studies have shown a strong relationship between HBV genotypes and mutations in the precore and core promoter regions that abolish or

diminish the production of HBeAg[23-26]. In our population which is predominantly HBeAg negative (presumably indicating pre-core or core promoter mutation), the majority of patients had genotype D thereby supporting the above observation.

Hepatitis B genotypes have been correlated with various epidemiological, virological, and clinical variables. It has been recently reported from China that patients with genotype B have a lower prevalence of HBeAg than those with genotype C[12]. Several other studies reported a correlation between HBV genotype and HBeAg clearance. Similarly in our study, we found a lower prevalence of HBeAg among our patients with genotype D (28%), suggesting that HBeAg clearance occurred at higher rates among patients with genotype D.

Correlation between the clinical outcomes of patients with HBV and their genotypes has also been reported. One study found that HBsAg carriers with genotype B had lower histological activity scores[23]. Three other studies involving a total of 939 Chinese patients with chronic HBV infection found that genotype C was more prevalent in patients with cirrhosis[27-29]. In our study, while 58.8% in group 1 (HBV with normal ALT) were genotype D, the number of patients with the same genotype across advancing stages of liver disease, in groups 2, 3, and 4 comprised 95.5%, 84.2%, 83.3% respectively ($P = 0.4$). These findings suggest that genotype D does not correlate with advancing liver disease. However, this could be related to the small sample size of the present study, and probably due to the predominance of genotype D in all clinical forms. Further analysis in large-scale longitudinal studies is required to better delineate this relationship.

In our patients with HBV genotype D, HBeAg negativity was found to increase significantly across more advancing stages of liver disease ($P = 0.024$). Previous studies have revealed that HBeAg positive genotype C[6] or genotype A[30] patients were more likely to have active liver disease. Progress of liver disease in relation to HBeAg status has not been reported as yet in the various studies dealing with genotype D[13,19,20]. Although this observation is significant, the association between HBeAg status and genotype D, in terms of severity of liver disease needs to be studied further before any additional conclusions can be derived.

Since predictors of advanced HBV liver disease were described before as a whole or in relation to individual genotypes other than in genotype D[31], our study describes for the first time these predictors in genotype D. Hemoglobin and albumin levels are independent predictors of advanced liver disease. Furthermore, group 4 patients (HCC) with genotype D showed significantly higher biochemical parameters (AST, ALT, and bilirubin) and lower albumin levels compared to early stage liver disease.

The relation between HBV genotype and HCC is inconclusive. One study found that genotype B was associated with hepatocellular carcinoma at an earlier age[28], but this finding was not confirmed by other studies[11,27,32]. Another study in Indian patients reported that genotype D was commonly found to be associated with HCC in patients < 40 years of age[13]. However, in our study, none of our HCC patients with genotype D (10 patients) were < 40 years of age (mean age 61 ± 12).

In Saudi Arabia HCC is the second most common cancer in men[33], and in a country where hepatitis B is endemic this could imply a correlation between the most prevalent genotype (D) and HCC. In our patients, 83.3% of the patients in group 4 (HCC) were genotype D compared to 81.4% of the overall number of recruited patients who expressed the same genotype. This figure did not reach statistical significance ($P = 0.30$). Moreover, since HBV is thought to be directly carcinogenic because of the integration of HBV DNA into the cellular DNA of the host[34], it may also explain the observed lack of correlation between genotype D and the development of HCC.

There were no significant differences between groups in terms of genotypes. This is likely secondary to the fact that the vast majority of patients had genotype D making comparisons with the other relatively rarer genotypes difficult. Along with the limitations imposed by the small sample size across the different groups, the study was also restricted by the absence of histology in groups 1 and 2, thereby possibly misallocating some of these patients into either more or less active groups.

In conclusion, genotype D is the most common genotype in Saudi Arabia. Because of the fact that the vast majority of the patients have genotype D, no correlation could be observed between different genotypes and epidemiological or clinical factors. A large-scale study is required to obtain further information on the role of genotype D and its impact on the progress of liver disease.

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*RAPID COMMUNICATION*

# Hepatitis C virus transmission and its risk factors within families of patients infected with hepatitis C virus in southern Iran: Khuzestan

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## Abstract

**AIM:** To determine whether hepatitis C virus (HCV) infection of index cases increases intrafamilial transmission (sexual and nonsexual contacts) of HCV.

**METHODS:** In a case-control descriptive study we enrolled 300-household contacts of 60 index cases (40 males and 20 females) of HCV infection and 360 pair-matched controls in Ahwaz JundiShapour University Hospitals from August 1, 1998 to September 1, 2003. The control group consisted of first time blood donors referred to the Regional Blood Transfusion Organization. Serum samples and demographic data and a medical history including the existence of risk factors for HCV (after a questionnaire on the risk factors for parenteral exposure) were obtained from each subject. Antibodies to HCV were detected employing a commercially available second-generation enzyme immunoassay (EIA, Abbott Ⅱ). Positive serum specimens were retested using a second-generation recombinant immunoblot assay (RIBA-2) and a polymerase chain reaction for HCV RNA. Data analysis was carried out for intra-household clustering.

**RESULTS:** Only 4 of 300 (1.33%) cases of household contacts without percutaneous risk factors were positive for HCV Ab while the remaining 296 family contacts were negative for anti-HCV. The mean age of the index cases was 28.4 (Std 15.22) years. The anti-HCV prevalences in parents, spouses, children of the index cases were 0.87% (1/115), 3.39% (2/59)) and 0.79% (1/126), respectively. Among couple partners negative for anti-HCV antibodies, the mean duration of the sexual relationship was 6 years. The two-couple partners positive for anti-HCV antibodies married the index cases for longer than 15 years. The prevalence of positive HCV Ab among household contacts (1.33%) was not significantly higher than that in the controls (1%) ($P > 0.06$).

**CONCLUSION:** Intrafamilial transmission of HCV is not the significant transmission route and sexual transmission does not seem to play a role in the intrafamilial spread of HCV infection. Intrafamilial transmission of HCV is possible but occurs at a low rate.

**Key words:** Intrafamilial transmission; Hepatitis C virus; Khuzestan; South-west of Iran

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## INTRODUCTION

One of the most substantial problems in public health is hepatitis C virus (HCV) infection, which affects approximately 1%-5% of the world's population and occurs in all countries. Epidemiological information on HCV is essential for strategic prevention of chronic hepatitis, liver cirrhosis and cancer. The rate of HCV infection differs in particular countries. The prevalence in developed countries amounts to 0.2%-2.2%, while in developing countries it reaches 7%. In some regions or in risk groups the rate of occurrence may be as high as 30%-90%[1,4].

HCV infection is acquired mainly parenterally by transfusion of infected blood, rupture of the continuity of skin or mucous membrane, infected medical equipment despite strict hygienic control, intravenous drug abuse, hemodialysis or organ transplantation. HCV infection is an important cause of post-transfusion hepatitis. Transmission through sexual contacts has been implicated, although this may be a rather inefficient mode of transmission[2,3]. HCV has also been detected in persons in whom no clear risk factor has been defined, and these cases constitute about 40%-45% of HCV infections[4]. According to

**Table 1 Demographic and clinical information on HCV-infected household members**

| Household members | *n* | Age (yr)/sex | Medical history | Clinical liver disease | Transfusion | HCV RNA |
|---|---|---|---|---|---|---|
| Nonsexual household contact (Mother) | 1 | 58/F | None | No | No | Negative |
| Sexual partners of index case | 1 | 43/F | None | No | No | Positive |
| Wife | 1 | 38/F | None | No | | Negative |
| Nonsexual household contact (Daughter) | 1 | 23/F | None | No | No | Positive |

the published data, the prevalence of HCV infection in Iran is 0.59%-0.8%[5]. Mother-to-infant transmission has also been demonstrated[6] but the possibility of other transmission routes has not been thoroughly explored. With the use of RT-PCR or bDNA techniques, HCVRNA has been detected in many systemic fluids other than in blood, including peritoneal effusion, seminal and vaginal secretion, urine, feces and typhoid secretion. At least 20% of hepatitis C patients develop cirrhosis with the associated risk of developing hepatocellular carcinoma (HCC)[7]. Despite primary hepatotropism, HCV can affect tissues and organs such as kidneys, thyroid, salivary glands, eyes, or the hematopoietic and lymphatic systems other than the liver[8]. HCV infection seems to be connected with several autoimmune diseases[9].

To our knowledge little information is available about HCV infection due to contacts of patients infected with HCV in southern Iran: Khuzestan. Because HCV may be transmitted by the non-parenteral routes such as sexual and non-sexual household contacts, this study was undertaken to investigate whether intrafamilial transmission occurs *via* the usual contacts between patients and their household members who are unaware of the potential infectious state of the patients, to determine the prevalence of antibody to HCV in the contacts of HCV positive cases (index patients) and to evaluate the potential risk factors associated with intrafamilial transmission of HCV.

## MATERIALS AND METHODS

### Subjects

During a 5-year period in a case-control descriptive study we enrolled 300 household contacts of 60 index cases (40 males and 20 females) of HCV infection and 360 pair-matched controls in Ahwaz JundiShapour University Hospitals from August 1, 1998 to September 1, 2003. The control group consisted of first time blood donors referred to the Regional Blood Transfusion Organization.

### Methods

Serum samples and demographic data and a medical history including the existence of risk factors for HCV (after a questionnaire on the risk factors for parenteral exposure) were obtained from each subject. The questionnaire consisted of questions regarding demographic variables, household behaviors and extra-familial factors including various potential parenteral exposures to blood or blood products (such as past hospital admission, operation, injuries needing hospital interventions, blood or blood product transfusion), history of parenteral injections and intravenous drips, travel history outside Iran, as well as dental treatment, tattooing and ear piercing. All these factors are known to be associated with HCV infection. All index cases and household contacts and controls answered the questionnaire and had their blood tested for anti-HCV. Antibodies to HCV were detected employing a commercially available second-generation enzyme immunoassay (EIA, Abbott Ⅱ). PCR for HCVRNA and a second-generation recombinant immunoblot assay (RIBA-2) were performed in all index cases and positive serum specimens were obtained from each subject. The Amplicor HCV RNA assay was used to detect HCV RNA in index case serum (Roche Diagnostic Systems). None of our cases (household contacts and index cases) revealed any signs of HBV infection (MEIA, Abbott IMX) or any other causes of acute or chronic liver diseases such as HAV, EBV and CMV infections, autoimmune diseases, alcohol and drug abuse, α1-antitrypsine deficiency, Wilson' s disease, or hemochromatosis. All these index cases were still receiving follow-up at the time of our study.

### Statistical analysis

The data were analyzed statistically using SPSS, version 9. Comparison was made using the Student's *t* test and chi-square test. $P < 0.05$ was considered statistically significant.

## RESULTS

All the 60 index patients gave positive reactions in the second generation anti-HCV EIA. We assumed that most of them were chronically infected with HCV. A total of 300 household contacts of the index patients were subjected to the second generation anti-HCV EIA. Only 4 of 300 (1.33%) cases of household contacts without percutaneous risk factors were positive for HCV Ab while the remaining 296 family contacts were negative for anti-HCV. The mean age of the index cases was 28.4 (Std 15.22) years. The anti-HCV prevalences in parents, spouses, children of the index cases were 0.87% (1/115), 3.39% (2/59)), and 0.79% (1/126), respectively. Among the couple partners negative for anti-HCV antibodies, the mean duration of the sexual relationship was 6 years. The two-couple partners positive for anti-HCV antibodies married the index cases for longer than 15 years. PCR for HCVRNA and second-generation recombinant immunoblot assay (RIBA-2) were performed in household members of two cases, one wife and one daughter (Table 1). The prevalence of positive HCV Ab among household contacts (1.33%) was not significantly higher than that in the controls (1%) ($P > 0.06$).

## DISCUSSION

HCV infection, a world-wide spread liver disease, is most

often asymptomatic in adults. It leads to serious clinical consequences that often occur later. About 50%-70% of mild infections progress to chronic phase and long term observations of natural history of the disease have confirmed that HCV is the risk factor for cirrhosis and primary hepatic carcinoma. However, there is a paucity of data on risk behaviors associated with HCV transmission through household contacts. HCV may be transmitted by the parenteral and other routes similar to that of HIV and HBV. Studies on family members of patients with chronic B hepatitis indicate that this virus can be transmitted both by vertical from mothers positive for hepatitis B surface antigen (HBsAg) and hepatitis B e antigen (HBeAg) and by horizontal (sexual and non-sexual) routes. Spouses and siblings of HBsAg-positive subjects are frequently found to have a high prevalence of serological markers of current or past HBV infection[10,11]. But the epidemiological relevance of intrafamilial transmission of HCV has not been clearly established.

This study estimated (1.33%) HCV seroprevalence among the household contacts of HCV-seropositive index cases, which is lower than the finding of other studies reporting that HCV seroprevalence is 16% and 20% among contacts of HCV-seropositive index patients[12,13]. Our results differ from those in these studies reporting a higher rate of HCV infection in the family members of adult patients with chronic hepatitis C. This difference could be attributed to one or more of the following limitations in studies, i.e. a small-sized study, inadequate duration, intensity of potential contact with adult patients, low infectivity of HCV in blood, genotypes, and unknown sensitivity of the hepatitis C radioimmunoassay used for detecting HCV infection[14]. Although intrafamilial HCV transmission through nonsexual contacts has been recognized as a major route in the Saudi population and elsewhere, the mechanisms underlying such transmissions have not been elucited. Another study has reported an elevated prevalence of 5.7% among household contacts, compared with HCV seroprevalence of 0.5% in the general population[15-17]. There are conflicting data in the literature concerning the role of sexual contacts in the spread of HCV infection. The prevalence of positive anti-HCV in spouses is different and may be a consequence of many factors such as sexual behavior and duration of marriage[18,19].

In this study, we found that spouses of anti-HCV positive patients were more likely to be infected with HCV than other family members and the infection rate increased with duration of marriage. In agreement with other authors[20], we found that positive anti-HCV increased in spouses with their length of marriage. In particular, a significant difference was found in the prevalence of positive anti-HCV between spouses married for more than 15 years and those married for a shorter time. Homology analysis on HCV nucleotide sequence is important in the study of sexual transmission of HCV[21], but we did not perform homology analysis in our studied cases. It is not easy to explain the increased rate of HCV infection in couples married for a longer time. Other authors reported that there is no prevalence in two groups of sexual partners married respectively for 13 and 15 years[3,22]. Marriage usually includes a sexual relationship, but also other kinds of body contact and exposure to the same risk factors (i.e. sharing the same personal tools such as toothbrushes, razors, dental appliances, *etc.*) as suggested by recent findings in Taiwan[20,23]. We did not perform genotyping in our studied cases but in Iran, genotype (1a) has been identified in the majority of chronic HCV patients[24]. HCV genotyping between the index cases and infected family contacts can clarify whether the infection is acquired in or out of the family setting. The HCV seroprevalence among the contacts of HCV-seropositive index patients is lower in our study than in populations elsewhere.

Our data suggest that sexual and nonsexual contacts with HCV-infected hemophilia patients within households are not associated with an increased risk for HCV infection. Further study is needed to assess the extent and the causes of nonparenteral transmission of HCV. Implementation of an education program based on the identified risk factors may reduce the spread of HCV in our area.

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*RAPID COMMUNICATION*

# Evaluation of the role of *H pylori* infection in pathogenesis of gastric cancer by immunoblot assay

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## Abstract

**AIM:** To elucidate the different serological reactions to *H pylori* using the immunoblotting technique for further understanding of its pathogenic role in gastric cancer.

**METHODS:** A total of 54 patients were divided into two groups after upper gastrointestinal endoscopy: normal control group (25 patients) and gastric cancer group (29 patients). Both groups were further divided into *H pylori* (+) and *H pylori* (-) subgroups based on the results of CLO test, Giemsa staining and culture. Sera were further analyzed with the immunoblotting technique (HelicoBlot 2.0, Genelabs Diagnostics, Singapore).

**RESULTS:** The positive rate of the immunoblotting test was as high as 88.9% in the *H pylori* (-) gastric cancer group and only 14.3% in the *H pylori* (-) normal control group with a statistically significant difference.

**CONCLUSION:** The prevalence of *H pylori* infection is higher in gastric cancer patients than in the normal controls, suggesting that *H pylori* may play a role in the pathogenesis of gastric cancer.



**Key words:** Western blot; Immunoblotting; Gastric cancer; *H pylori*; Enzyme-linked immunosorbent assay

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## INTRODUCTION

*H pylori,* a Gram-negative bacterium, is now widely considered as one of the major etiologic factors in the pathogenesis of a great variety of gastrointestinal diseases such as gastritis, peptic ulcers and mucosa-associated lymphoid tissue lymphomas (MALTomas)[1]. There is increasing evidence that cancer of the stomach is also strongly associated with *H pylori* colonization[2-6]. Numerous antibodies against antigens of *H pylori* can be detected by serological analysis using the Western immunoblot technique[7-10]. Among these antibodies to *H pylori*, polypeptides with molecular masses of 116 kDa (against the cytotoxin-associated antigen, CagA), 89 kDa (against the vacuolating toxin antigen, VacA), 35 kDa, 30 kDa, 26.5 kDa and 19.5 kDa are considered as the most specific antibodies used in the diagnosis of *H pylori* infection and their corresponding antigens probably play a pathogenic role in the distinct gastrointestinal diseases. Particularly the antigens CagA and VacA not only seem to have a significant association with peptic ulcer disease but also increase the risk of developing gastric cancer[11-16]. The aim of this study was to elucidate the probable pathogenic role of *H pylori* in gastric cancer and serological stigmata of its remote infection as detected by the immunoblotting technique.

## MATERIALS AND METHODS

### Patients

Between March 1998 and May 2000, 54 consecutive patients (34 women, 20 men; age range: 20-70 years) who had epigastralgia and vague abdominal complaints but no remarkable past medical history of systemic diseases (such as generalized sepsis, uremia or hematologic malignancies) were recruited prospectively in this study. These patients visited the Outpatient Clinic or the Health Management Center of Shin Kong Wu Ho-Su Memorial Hospital for a routine health check-up. During upper GI endoscopy, specimens were taken from the antrum for rapid urease test, Giemsa stain and culture to elucidate the patient's *H pylori* status. When gastric malignancy was suspected, more specimens were taken from the lesion for histological examination. The patients were then divided into a normal control group ($n$ = 25) and a gastric cancer group ($n$ = 29) (Table 1). The normal control group and gastric cancer group were further divided into *H pylori* (+) and *H pylori* (-) subgroups. The *H pylori* (+) subgroup had positive results in at least two of the three tests, while the three tests were

**Table 1 Positive rate (%) of different reaction bands in the two groups of patients**

| | Normal | | CA | | |
|---|---|---|---|---|---|
| | *H pylori* (+) | *H pylori* (-) | *H pylori* (+) | *H pylori* (-) | |
| *n* | 11 (%) | 14 (%) | 11 (%) | 18 (%) | *P* |
| Overall | 100.0 | 14.3 | 100.0 | 88.9 | < 0.0001 |
| 116 kDa | 100.0 | 14.3 | 90.9 | 72.2 | 0.002 |
| 89 kDa | 44.4 | 0.0 | 60.0 | 27.8 | 0.052 |
| 35 kDa | 100.0 | 0.0 | 100.0 | 61.1 | 0.0003 |
| 30 kDa | 100.0 | 7.14 | 81.8 | 58.80 | 0.003 |
| 26.5 kDa | 90.9 | 14.3 | 72.7 | 66.7 | 0.005 |
| 19.5 kDa | 72.7 | 0.0 | 40.0 | 16.6 | 0.238 |

Fisher's exact test was used to test for the different positive rate between CA-*H pylori* (-) and Normal-*H pylori* (-) group.

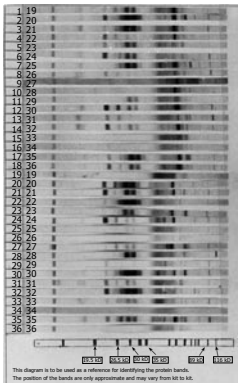

**Figure 1** Example of the immunoblotting reaction bands. A reaction sheet from a group of patients enrolled in the study.

all negative in the *H pylori* (-) subgroup. In the *H pylori* (-) normal subgroup there were 12 female and 2 male patients with a mean age of 34.6 years. In the *H pylori* (+) normal subgroup there were 7 female and 4 male patients with a mean age of 37.2 years. In the *H pylori* (-) cancer subgroup there were 9 female and 9 male patients with a mean age of 58.8 years. In the *H pylori* (+) cancer subgroup there were 6 female and 5 male patients with a mean age of 59 years. In the *H pylori* (-) cancer subgroup, tumors were found in the gastric antrum, angle, corpus and cardia of 6, 3, 5 and 4 patients, respectively. Meanwhile, in the *H pylori* (+) cancer subgroup, tumors were found in the antrum of 5 patients (2 of them had tumor involving antrum and angle), in the antrum and lower corpus of 2 patients, in the angle of one patient, in the corpus of 3 patients, and in the corpus as well as fundus and cardia of one patient. Histopathological studies demonstrated that all the suspicious malignant lesions were adenocarcinoma. In order to analyze the possible link between gastric cancer and remote *H pylori* infection, the sera from patients were analyzed with the immunoblotting technique (HelicoBlot 2.0, Genelabs Diagnostics, Singapore) (Figure 1). Five reaction bands could be recognized with the immunoblot technique: 116 kDa (CagA), 89 kDa (VacA), 35 kDa, 30 kDa, 26.5 kDa and 19.5 kDa. The immunoblotting was considered as positive with the detection of one reaction band of 116 kDa (CagA) and/or 89 kDa (VacA) and/or 35 kDa (major antigens), and/or two other reaction bands (minor antigens, 30 kDa, 26.5 kDa, 19.5 kDa), as recommended by the manufacturer. In addition, sera from the *H pylori* (-) cancer group of patients were further analyzed by enzyme-linked immunosorbent assay (ELISA, Immulite *H pylori* IgG, Diagnostic Products Corporation, Los Angeles, USA), and the two serological methods were compared. The collected data were finally analyzed with the Fisher's exact test.

## RESULTS

The seroprevalence of antibodies to 116 kDa (Cag-A) positive *H pylori* strain was high among the patients enrolled in this study: 100% in the normal *H pylori* (+) control group, 90.9% in the CA-*H pylori* (+) group, and also strikingly high in the CA-*H pylori* (-) group (72.2%). A quite similar finding was observed with the 35 kDa antigen (Table 1). The seroprevalence of antibodies to the third major antigen 89 kDa (VacA) was 44.4% in the normal *H pylori* (+) group, 60% in the CA-*H pylori* (+) group and 35.7% in the CA-*H pylori* (-) group. In the case of minor antigens, the seroprevalence of antibodies to the 30 kDa antigen was 100% in the normal *H pylori* (+) group, 81.8% in the CA-*H pylori* (+) group, and also remarkably high (58.8%) in the CA-*H pylori* (-) group. For the 26.5 kDa antigen, the seroprevalence of antibodies was 90.9% in the normal *H pylori* (+) group, 72.7% in the gastric cancer-*H pylori* (+) group, and 66.7% in the CA-*H pylori* (-) group. For the 19.5 kDa antigen, the seroprevalence of antibodies was 72.7% in the normal *H pylori* (+) group, and 40% in the gastric cancer-*H pylori* (+) group. When the reaction bands were equivocal (neither positive nor negative), they were considered undetermined with a prevalence of 2.2% in the 116 kDa antigen, 10.9% in the 89 kDa antigen, 2.2% in the 35 kDa antigen, 8.7% in the 30 kDa antigen, 3.6% in the 26.5 kDa antigen, and 5.8% in the 19.5 kDa antigen. These equivocal reaction bands might indicate that a low serum concentration of the corresponding antibodies was insufficient to yield a clear-cut reaction with their respective antigens. Analysis of the seroprevalence of antibodies to different antigens yielded the following overall positive rates for immunoblotting test: 100% in the normal *H pylori* (+) subgroup, 14.3% in the normal *H pylori* (-) subgroup, 100% in the gastric cancer-*H pylori* (+) subgroup and 88.9% in the gastric cancer-*H pylori* (-) subgroup, respectively. It should be pointed out that the positive rate for the immunoblotting technique was strikingly higher in the gastric cancer-*H pylori* (-) subgroup than in the normal *H pylori* (-) subgroup and there was a statistically significant difference achieved by Fisher's exact test ($P < 0.05$). This interesting finding denoted that the presence of *H pylori* as a remote infection in both *H pylori* (-) subgroups detected by immunoblotting assay was much more significant in the gastric cancer-*H pylori* (-) subgroup than in the normal *H pylori* (-) subgroup. This important issue might be overlooked if only rapid urease test, Giemsa staining and culture were performed. However, when ELISA was carried out to detect IgG to *H pylori* antigens using sera from these 18 gastric cancer-*H pylori* (-) patients, only 9 of them were positive (50% *vs* 88.9%). Since *H pylori* might not be closely implicated in the development of tumors in the cardiac region, if the four *H pylori* (-)

cancer patients with their tumor localized in the cardia were excluded from statistical analysis, the overall result was identically significant (Table 2).

## DISCUSSION

The role of different *H pylori* antigens in gastrointestinal diseases still remains controversial. In contrast to Western developed countries, different reaction bands in immunoblot assay fail to predict a particular disease in Taiwanese patients[17-21]. Two *H pylori* proteins, VacA and CagA, are virulence factors which may enhance gastric mucosal damage and promote the development of peptic ulcers and gastric mucosa atrophy. By identifying different *H pylori* proteins, immunoblot assay can screen patients at high risk of developing gastrointestinal diseases, such as peptic ulcer and gastric cancer. However, the high seroprevalence of antibodies to CagA-positive *H pylori* strains in Taiwanese patients with various gastrointestinal diseases has rendered the CagA-positive phenotype, an unusable marker for screening patients with a determined disease and immunoblot assay has no predictive and diagnostic value in Taiwanese patients. ELISA may reveal a significant decrease in IgG antibody titers approximately two months after treatment with antimicrobials. In contrast, immunoblot assay may detect IgG antibodies to specific antigens such as CagA and VacA several years after treatment[22-24]. These findings suggest that ELISA is a useful quantitative tool for monitoring eradication of *H pylori* while immunoblot assay is a qualitative method able to demonstrate remote *H pylori* infections which are not detectable by ELISA. The sensitivity and specificity of ELISA may decrease with the decrease in IgG titers. The immunoblotting technique might be recommended as a confirmative test for antibodies detected by ELISA[25,26]. Furthermore, although a high accuracy has been reported in Western countries, commercial ELISA might be unsatisfactory in Asians[27]. Therefore, immunoblot assay may be regarded as a sensitive, non-invasive means for the diagnosis of *H pylori* infection. However, major serological cross-reactions with *Campylobacter jejuni* and bacterial lipopolysaccharide have been found, which might explain the false positive results, while decrease in concentration of antibodies might yield equivocal reaction bands. It is known that *H pylori* colonization causes chronic active inflammation of gastric mucosa which eventually leads to the development of atrophic gastritis, intestinal metaplasia and dysplasia. Eighty-nine percent of *H pylori* (-) patients with gastric adenocarcinoma were proven to have a *positive* immunoblot assay in this study, indicating that these patients might have been infected with *H pylori* in a certain past period of their lifetime. This interesting finding suggests that *H pylori* can be detected in hostile gastric environments such as mucosa atrophy, but its hidden remote infection is still demonstrated in serum by immunoblot assay[28]. Therefore, the role of *H pylori* in the pathogenesis of gastric cancer should be stressed. Further studies are necessary to elucidate the possible link between *H pylori* infection and mechanisms of carcinogenesis.

In conclusion, 88.9% of patients with gastric cancer in *H pylori* (-) subgroup have a positive immunoblot assay for *H pylori* infection. Immunoblot assay can disclose remote *H pylori* infection which might be overlooked if only rapid urease test, Giemsa staining and culture, or ELISA for IgG antibody, is performed.

**Table 2 Positive rate (%) of the different reaction bands in the two groups of patients**

| | Normal | | CA | | |
|---|---|---|---|---|---|
| | *H pylori* (+) | *H pylori* (-) | *H pylori* (+) | *H pylori* (-) | |
| *n* | 11 (%) | 14 (%) | 11 (%) | 14 (%) | *P* |
| Overall | 100.0 | 14.3 | 100.0 | 92.9 | < 0.0001 |
| 116 kDa | 100.0 | 14.3 | 90.9 | 71.4 | 0.006 |
| 89 kDa | 44.4 | 0.0 | 60.0 | 35.7 | 0.04 |
| 35 kDa | 100.0 | 0.0 | 100.0 | 57.1 | 0.002 |
| 30 kDa | 100.0 | 7.14 | 81.8 | 75.1 | 0.01 |
| 26.5 kDa | 90.9 | 14.3 | 72.7 | 71.4 | 0.006 |
| 19.5 kDa | 72.7 | 0.0 | 40.0 | 14.3 | 0.482 |

Four *H pylori* (-) patients with their tumors localized in the cardia were excluded from the analysis. Fisher's exact test was used to test for the different positive rate between CA-*H pylori* (-) and Normal-*H pylori* (-) groups.

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*RAPID COMMUNICATION*

# Evaluation of intraoperative radiotherapy for gastric carcinoma with D2 and D3 surgical resection

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## Abstract

**AIM:** To study the proper sites and doses of intraoperative radiotherapy (IORT) for gastric carcinoma and the effects of this treatment.

**METHODS:** A total of 106 patients with stage Ⅰ-Ⅳ gastric carcinoma who received D2 or D3 radical operation combined with IORT were analyzed. Sixty-seven patients with gastric cancer of the antrum and body underwent distal gastrectomy. The sites of irradiation were at the celiac artery and hepatoduodenal ligment area. Another 39 patients with carcinoma of the cardia and upper part of the gastric body and whole stomach underwent proximal gastrectomy or total gastrectomy. The sites of irradiation for this group were the upper margin of the pancreas and the regional para-aorta. The therapeutic effects (including survival and complications) of these 106 cases received operation combined with IORT (IORT group) were compared with 441 cases treated during the same time period by a radical operation alone (operation group).

**RESULTS:** The radiation dose below 30 Gy was safe. The therapeutic method of the operation combined with IORT did not prolong the survival of patients with stage Ⅰ and Ⅳ gastric cancer, but the 5-year survival rates of patients with stage Ⅱ and Ⅲ gastric cancers were significantly improved. The 5-year survival rates of the stages Ⅲ cancer patients receiving D2 resection combined with IORT were markedly improved, while for those receiving D3 radical resection, only the postoperative 3- or 4-year survival rates were improved ($P$ < 0.005-0.001). The 5-year survival rate for those patients was raised only by 4.7% ($P$ > 0.05).

**CONCLUSION:** The 5-year survival rates of patients with stages Ⅱ and Ⅲ gastric carcinoma who received D2 lymphadenectomy combined with IORT were improved, and there was no influence on the postoperative complications and mortality.

## INTRODUCTION

The fate of patients after surgical removal of a gastric carcinoma is determined to a large degree by regional failure of the operation (e.g. tumor recurrence in the tumor bed or in an adjacent structure). This is true for palliative resections with macroscopic tumor residues (D2) as well as for operations with microscopic involvement of resection margins (D1) or no discernible malignant cells (D0). Recurrent tumors may originate from extension of the primary tumor or from regional lymph node metastases not encompassed by the surgical procedure[1]. It is desirable therefore to develop and study additional tumoricidal measures that might eliminate residual malignant tissues, thereby increasing the chance of regional tumor control[2].

Intraoperative radiotherapy (IORT) as an adjunct to surgical excision of solid malignancies has been used, however, clear-cut evidence of its benefit for tumor control is still deficient. In IORT a large single dose of radiation is given to the area with greatest risk of local failure[3-5]. Based on the available oncologic and radiotherapeutic data, radiation thus applied should be able to control tumor growth. The biologic and logistic advantages of radiation applied directly to the tumor bed at a single dose are obvious. For these reasons any information related to the efficacy of IORT is highly desirable and welcome.

Under these considerations, we have used IORT for gastric cancer and report the clinical results in comparison with that of patients treated by operation alone. The clinical results of IORT for gastric cancer were analyzed based on the histologic findings.

## MATERIALS AND METHODS

### *Research subjects*

A total of 106 patients were treated by radical resection and IORT between 1992 and 1998, including 13 cases in

**Table 1 Clinical data of gastric carcinomas in different stages classified by intraoperative radiotherapy (IORT) and operation alone**

| Groups | Staging | *n* | Location of tumor *n* (%) | | | Operation method *n* (%) | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Antrum | Body | Cardiac | TS | D2 | D3 | DG | PG | TG |
| IORT | | | | | | | | | | | |
| | Ⅰ | 13 | 8 (62) | 2 (15) | 3 (23) | 0 (0) | 9 (69.3) | 4 (30.7) | 10 (76.9) | 3 (23.1) | 0 (0) |
| | Ⅱ | 17 | 12 (70.6) | 2 (11.8) | 3 (17.6) | 0 (0) | 6 (35.3) | 11 (64.7) | 13 (76.5) | 3 (17.6) | 1 (5.9) |
| | Ⅲ | 48 | 25 (52.1) | 6 (12.5) | 8 (16.7) | 9 (18.7) | 9 (18.8) | 39 (81.3) | 30 (62.5) | 5 (10.4) | 13 (27.1) |
| | Ⅳ | 28 | 13 (46.4) | 2 (7.1) | 5 (17.9) | 8 (28.6) | 3 (10.7) | 25 (89.3) | 14 (50) | 6 (21.4) | 8 (28.6) |
| Operation | | | | | | | | | | | |
| | Ⅰ | 70 | 50 (71.4) | 16 (22.9) | 4 (5.7) | 0 (0) | 50 (71.4) | 20 (28.6) | 66 (94.3) | 4 (5.7) | 2 (2.8) |
| | Ⅱ | 67 | 49 (73.1) | 5 (7.5) | 13 (19.4) | 0 (0) | 31 (46.3) | 36 (53.7) | 54 (80.6) | 11 (16.4) | 2 (3.0) |
| | Ⅲ | 244 | 164 (67.2) | 37 (15.2) | 28 (11.5) | 15 (6.1) | 110 (45.1) | 134 (54.9) | 201 (82.4) | 28 (11.5) | 15 (6.1) |
| | Ⅳ | 60 | 36 (60) | 10 (16.7) | 6 (10) | 8 (13.3) | 8 (13.3) | 52 (86.7) | 42 (70) | 4 (6.7) | 14 (23.3) |

TS: Total stomach; D2: Lymph node 2 dissection; D3: Lymph node 3 dissection; DG: Distal gastrectomy; PG: Proximal gastrectomy; TG: Total gastrectomy.

stage Ⅰ, 17 in stage Ⅱ, 48 in stage Ⅲ and 28 in stage Ⅳ. There were 77 men and 29 women, aged from 31-80 years, averaging 52.4 years. Location of the carcinoma was: antrum 58, body 12, cardiac 19, and total stomach 17 cases. Operation method: D2 lymphadenectomy 27, D3 lymphadenectomy 79; distal gastrectomy 17, proximal gastrectomy 67 and total gastrectomy 22 cases. To evaluate the effectiveness of IORT, 441 patients who were treated by operation alone during the same time period were classified histologically, and their survival rates were compared with those of patients treated by IORT. In addition, a comparative study was performed on the survival rates of patients treated by IORT and those treated by operation alone based on the degree of staging (Table 1). Inclusion criteria for IORT[6] were: (a) gastric adenocarcinoma confirmed through preoperative biopsy; (b) age between 25 and 80 years; (c) World Health Organization (WHO) performance status of 0-2; and (d) the availability of IORT facilities.

Patients were excluded if they had serious concurrent illness, active infection, symptomatic ischemic heart disease, congestive heart failure, a recent history of myocardial infarction, or symptomatic arrhythmia. Patients with significant gastrointestinal disturbance that could cloud the interpretation of gastrointestinal toxicity were also excluded. In addition, patients with severe malnutrition or severe nausea, or frequent vomiting were excluded.

Surgery was performed through a median laparotomy and surgeons were allowed to use either total or subtotal gastrectomy (according to the tumor location) combined with D2 and D3 lymphadenectomy. The same two senior surgeons were in charge of the operations during the whole protocol.

### *Radiation location*

After the gastrectomy and lymphadenectomy and before alimentary reconstruction, the local region of the hepatoduodenal ligament and the upper margin of the pancreas or the gastric bed were irradiated in 67 patients who were treated with gastrectomy in the antrum and body of the stomach. The other 39 patients with total gastrectomy underwent removal of the pancreas capsule and extended lymph node dissection including the nodes along the splenic artery. In the patients with body, cardiac and total stomach cancer, the tail and body of the pancreas were moved up to the right side margin of the abdominal aorta and the superior mesenteric vein and the region of the para-aorta received irradiation.

IORT was administered to the tumor bed and celiac axis at the time of gastric resection in those patients whose tumor appeared transmural and who were sufficiently stable to tolerate a transfer to the Radiation Therapy Treatment Room.

A variety of sizes and shapes of the pentagonal treatment cones were prepared so they fit the costal arch adequately and encompassed various anatomic situations of the tumor bed and the high risk lymph node groups noted above.

The field was clearly illuminated by an electric lamp fixed to a telescope attached to the treatment cone. The sterilized cone was inserted into the abdomen inclining degrees so the celiac axis was sufficiently covered.

### *Electronic energy and radiation dose of IORT*

The electronic energy from 6 MeV to 16 MeV was selected. The radiation doses of IORT were selected according to the posibillity of the radicality achieved by surgical operation. A single dose of 10-15 Gy was given to 41 patients who had no clinically undetectable lesions, a single dose of 20 Gy was given to each 27 patients who were suspected to have microscopic residual LN, 25 Gy was given to 37 patients who were suspected to have macroscopic residual LN or direct invasion of adjacent structure, and 30 Gy to one patient who had noncurative surgery because of incomplete excision of metastatic lesions.

## RESULTS

### *Complications*

When IORT is used for gastric cancer, critical organs to which exposure must be avoided, are the pancreas, duodenum and jejunum which must be shielded from radiation. Less than 40% of the pancreas was generally included in the radiation field. Acute and late damage to the pancreas was evaluated by changes in serum amylase and blood glucose levels after IORT. Temporary increases in both serum amylase and blood glucose occurred after IORT, but they returned to preirradiation levels within a week. Neither significant late complications nor deviation

**Table 2 The 5-yr survival rate of patients with operation combined with intraoperative radiotherapy (IORT)**

| Groups | *n* | Stage Ⅰ | Stage Ⅱ | Stage Ⅲ | Stage Ⅳ |
|---|---|---|---|---|---|
| IORT | 106 | 13/13 (100%) | 17/17 (100%) | 29/48 (60.4%) | 4/28 (14.3%) |
| Operation | 441 | 65/70 (92.8%) | 54/67 (80.6%) | 110/22 (45.1%) | 6/6 (10.0%) |
| *P* | | > 0.10 | < 0.001 | < 0.005 | > 0.05 |

**Table 3 The 5-yr survival rate of patients with operation combined with intraoperative radiotherapy (IORT) in stage Ⅲ**

| Groups | *n* | D2 resection | | | | | | D3 resection | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | *n* | 1 yr | 2 yr | 3 yr | 4 yr | 5 yr | *n* | 1 yr | 2 yr | 3 yr | 4 yr | 5 yr |
| IORT | 48 | 9 | 100% | 100% | 87.5% | 87.5% | 60% | 39 | 100% | 100% | 93.1% | 85.5% | 61% |
| Operation | 244 | 114 | 81.6% | 70.4% | 52.3% | 43.3% | 35.7% | 128 | 92.1% | 85% | 66.5% | 62.2% | 56.3% |
| *P* | | | < 0.05 | < 0.001 | < 0.001 | < 0.001 | < 0.005 | | > 0.50 | > 0.10 | < 0.001 | < 0.005 | > 0.05 |

from the usual postoperative course was observed. There was no instance of delayed wound healing. One patient died from cardiac infarction, resulting in a death rate of 0.9% (1/106). Recovery of gut function in all of the patients with IORT was delayed for 24 h.

### *Survival*

Table 2 demonstrates the survival rates based on an analysis of 106 patients treated by IORT and 441 patients treated by operation alone. The survival rate was calculated by the Kaplan-Meier method.

The 5-year survival rate (YSR) for patients treated by operation alone was 92.8% for stage Ⅰ, 80.6% for stage Ⅱ, 45.1% for stage Ⅲ, and 10% for stage Ⅳ. On the other hand, the 5-YSR for patients treated by IORT was 100% for stage Ⅰ and stage Ⅱ, 60.4% for stage Ⅲ, and 14.3% for stage Ⅳ. As shown in Table 2, there was no difference between the 5-YSR of patients in stage Ⅰ, and stage Ⅳ in the two groups. The IORT procedure raised the survival of patients with stages Ⅱ and Ⅲ cancer from 15% to 20%, $P < 0.005$-0.001.

Table 3 demonstrates that the 5-YSR of the stage Ⅲ cancer patients with D2 radical resection combined with IORT was improved as compared with operation alone. The 5-YSR of the stage Ⅲ cancer patients with selective D3 radical resection combined with IORT was not improved as compared to an operation alone. However, the 3- and 4-YSR was significantly improved by IORT combined with operation as compared with operation alone. But the differences of the 3 and 4-YSR between the two groups were statistically significant.

## DISCUSSION

As far as gastric adenocarcinoma is concerned, the prognosis remains poor with a high local failure rate estimated up to 67%[7]. While a lot of trials using systemic chemotherapy are currently being made to decrease the general failure rate in gastric adenocarcinoma, using IORT as a boost to EBRT could be a way to improve local control rate on coeliac area after gastrectomy[2,8]. IORT has been known as a feasible radiation treatment since 1907[9]. Its main advantage is probably to spare normal tissues while delivering a high dose precisely on the tumoral target. Extended lymph node dissection has been used mainly in Japan and the treatment results have improved remarkably as shown in many retrospective studies. But extended lymph node dissection is still controversial in the Western countries because of reported excessive postoperative morbidity and mortality and lack of proof in a recent retrospective study and in randomized controlled trials[1,10]. The Dutch Gastric Cancer Trial and the Medical Research Council (MRC) randomized surgical trial were not able to prove the benefit of extended lymph node dissection.

Due to operating room planning reasons, a great number of patients with gastric adenocarcinoma were not included in the IORT protocol in the reported study, and therefore, no valid comparisons or conclusions can be made. However, it is important to notice that mortality and morbidity rates in patients treated with IORT (1/42 and 3/42) were not excessive when compared with the mortality and morbidity rates reported in the retrospective study of 350 gastric cancer patients treated by surgery alone in the same institution since 1970 (respectively 10.8% and 14%)[11]. Glehen *et al*[12,13] did not detect any acute toxicity of IORT. Concerning the late toxicity of IORT, canine studies provide guidelines for human cancer treatment and the data provided by these canine studies are comparable with the data obtained in an autopsy study which validated the model for clinical use. With a long term follow-up, in the reported study we just detected one case of enteritis, 2 years after irradiation. But this late toxicity was controversial in the literature data because of the reported cases of other enteritis, gastrointestinal bleedings with or without arterioenteric fistulas, vertebral collapses and liver hemangiomas[2,14,15].

Each surgical procedure that does not achieve a complete local excision of the primary tumor in all 3 dimensions (length, width, aboral, and depth of tumor) and of the area of lymphatic drainage is, according to the UICC classification of D1 or D2 resection (i.e. a resection leaving residual microscopic or macroscopic tumor), considered to be a "palliative resection". In addition to procedures that leave the entire tumor *in situ*, any type of operation that leaves residual macroscopic or microscopic tumor at the resection margin, tumor bed, or in the

lymphatic drainage area must also be considered to be a palliative procedure.

The rationale for IORT is to increase the irradiation dose to the tumor without exceeding critical normal tissue tolerance. IORT used as a neoadjuvant therapy, can increase the 5-YSR of patients with advanced gastric carcinoma. Special linear accelerators have been used for the application of high single-dose radiation to the tumor bed[3,4]. Abe *et al*[5] reported 115 gastric cancer patients treated by IORT. The 5-YSR survival of patients in stages Ⅱ through Ⅳ treated by IORT increased by nearly 10%-20%. No difference in the survival of patients was observed between the two groups. IORT did not afford benefit if the lymph node metastases were limited with $n_1$ group or serosal invasion was not found. On the other hand, the 5-YSR for patients treated by IORT increased by nearly 10% when the serosal invasion was observed, and by nearly 18% when $n_2$ and $n_3$ lymph node metastases were found. Ogata *et al*[16] reported that the 5-YSR and 8-YSR for 58 cases treated by IORT were both 100% , and 60% and 48% in control group with stage Ⅱ cancer. The 8-YSR of the stage Ⅲ cancer by IORT was 55%, and 35% in the control group. But these results were controversial. Coquard *et al*[2] reported that the 5-YSR of patients with gastrectomy and local lymph node resection combined with IORT was the same as single expanded lymph node resection. However, the postoperative complications and mortality were lower than that in those who received only gastrectomy. Our results showed that operation combined with IORT is beneficial for patients in stage Ⅱ and stage Ⅲ cancer with a 5-YSR of 14.4%-20%. However, this procedure was ineffective for patients with stage Ⅰ and stage Ⅳ cancer treated with D2 lymphadenectomy combined with IORT, the 5-YSR of stage Ⅱ cancer patients was increased by 60% as compared with D2 lymphadenectomy only by 35.7%. With D3 lymphadenectomy combined with IORT, the 3- and 4-YSR were improved, but it was ineffective for the 1, 2 and 5-YSR. These encouraging results were already reported by Japanese authors[5,17] with a 10%-20% increase in 5-YSR in stages Ⅱ and Ⅲ. However, this has not been reported in Western countries, especially for pN+ patients. But in a shorter follow-up, a local recurrence of under 15% after the use of IORT and EBRT was reported[18,19].

The radiation target area in the region of the upper abdomen includes potential microscopic tumor extensions and known or suspected macroscopic disease[13]. In order to reduce the injury of irradiation to the normal tissues near the stomach, the target area is restricted to the gastric bed. The inferior field border is generally at the level of the L3 to L4 vertebrae, but this depends on the location of the carcinoma and the position of the stomach before treatment. The target area includes the proximal and regional lymphatics, the right and left cardiac nodes, the nodal chains of the lesser and greater curvatures, the suprapyloric and infrapyloric chains, the splenic chains, and the lymph nodes along the hepatic artery. For the cardiac tumors, upper extension of the field to the terminal esophagus and paraesophageal lymph nodes is recommended. If the tumor is unresectable or if, after surgery, macroscopic disease remains, the total irradiation dose can be increased in a coned-down volume, which is restricted to the area of gross disease. With regard to the opitimization of IORT, we developed a new technique that provides a wider irradiation field for patients undergoing total gastrectomy. In these patients, total gastrectomy, splenectomy, removal of the pancreas capsule, and extended lymph node dissection including the nodes along the splenic artery were performed. Then the tail and body of the pancreas were moved up to the right side margin of the abdominal aorta and the superior mesenteric vein. These maneuvers provided a much wider irradiation field, which included the para-aortic lymph nodes. After irradiation, the pancreas was returned to its normal position and an esophagoenterostomy was performed. This method was adopted only in patients without cancer invasion of the pancreas or metastases in the lymph nodes along the splenic artery. The advantage of this method is that it produces a wide irradiation field including para-aortic lymph nodes and does not increase the complications and mortality. Meanwhile, one must be careful to protect the duodenum and jejunum before irradiation as those organs must be shielded from the irradiation field.

With regard to special radiotherapeutic techniques that include the potential of relatively high biologically effective doses, gastric cancer is one of the most convincing indications for intraoperative irradiation. Special linear accelerators have been used for the application of high single doses (15-35 Gy) to the tumor bed. One problem with IORT is to correctly cover tumor sites outside the midline area or under the left hemidiaphgragm. In addition, the maximum tolerable dose administered in one session is limited from 15 to 35 Gy. Abe *et al*[5] reported that doses of irradiation depended on the radicality achieved by the operation. In general, the irradiation doses from 15-30 Gy were applied in curative situations and 30-35 Gy in palliative situations. We selected the doses of IORT according to the residual tumor volume, including clinically undetectable lesions (10-15 Gy), microscopic residual (20 Gy), macroscopic residual (25 Gy), or noncurative surgery (30 Gy). No serious complications occurred in these cases. Our data strongly support the efficacy of IORT for local tumor control.

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*RAPID COMMUNICATION*

# Alterations of biliary biochemical constituents and cytokines in infantile hepatitis syndrome

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## Abstract

**AIM:** To investigate the biliary biochemical constituents and cytokines in infantile hepatitis syndrome (IHS).

**METHODS:** From 42 IHS subjects and 21 controls, serum and biliary biochemical constituents, including total bilirubin (TBIL), direct bilirubin (DBIL), alanine aminotransferase (ALT), gamma-glutamyl transpeptidase (γ-GT), total bile acid (TBA), interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α) both in bile and serum, were assayed. The subjects with IHS were divided into a cholestasis group ($n$ = 21) and a hepatitis group ($n$ = 21).

**RESULTS:** In the cholestasis group, serum TBIL, DBIL, ALT, γ-GT, TBA, IL-6 and TNF-α levels were higher than those in the control ($P$ < 0.01); and also the biliary TBIL, DBIL, γ-GT and TBA levels were lower than those in the control, whereas biliary IL-6 and TNF-α levels were higher than those in the control ($P$ < 0.01). In the cholestasis group, serum IL-6 and TNF-α levels were lower than those in bile ($P$ < 0.01). In the hepatitis group, serum DBIL, ALT, γ-GT, TBA, IL-6 and TNF-α levels were higher than those in the control ($P$ < 0.01 or 140.57 ± 70.32 *vs* 79.06 ± 35.25, $P$ < 0.05), while biliary TBIL, DBIL, γ-GT and TBA levels were lower than those in the control ($P$ < 0.01), and biliary IL-6 and TNF-α levels were higher than those in the control ($P$ < 0.01). In the hepatitis group, serum IL-6 and TNF-α levels were also lower than those in bile ($P$ < 0.01). Serum TBIL, DBIL, γ-GT, IL-6 and TNF-α levels in the cholestasis group were higher than those in the hepatitis group, while biliary IL-6 and TNF-α levels in the cholestasis group were higher than those in the hepatitis group. Biliary IL-6 and TNF-α were found to be more significantly increased than serum IL-6 and TNF-α in IHS ($P$ < 0.01). The biliary IL-6 and TNF-α levels were positively correlated with serum DBIL, TBA and γ-GT levels in IHS subjects.

**CONCLUSION:** Biliary biochemical constituents alter in coincidence with pathological changes in hepatocellular injury. Cholestasis is more serious in IHS patients of cholestasis subtype. Assay of biliary IL-6 and TNF-α levels can be specific and sensitive to determine the inflammatory status of impaired liver in IHS.



**Key words:** Infantile hepatitis syndrome; Biliary biochemical constituents; Biliary cytokines; Interleukin-6; Tumor necrosis factor-α

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## INTRODUCTION

Infantile hepatitis syndrome (IHS), with a morbidity of 1/2500 in live-born infants[1], comprises a series of symptoms, including jaundice, splenohepatomegalia, changes of texture of the liver, hepatic dysfunction in onset mainly in the neonatal period and infancy. It has been reported that the biochemical constituents and cytokines in blood alter when the disorder attacks[2]. However, what changes of those biochemical constituents and cytokines in bile can be, and what relationships between IHS and physiologic jaundice in those constituents can be, remains a puzzle in pediatric practice. In this study, we focused on the alterations of biochemical constituents and cytokines in serum and bile obtained from subjects attacked by IHS.

## MATERIALS AND METHODS

### Subjects

According to the diagnostic criteria[3], IHS was defined as: (1) age < 1 year; (2) jaundice; (3) splenohepatomegalia and changes of texture of the liver; and (4) alteration in hepatic function. Forty-two subjects [29 males and 13

**Table 1 Comparison of serum and biliary biochemical constituents among three groups (mean ± SD, *n* = 21)**

| Group | TBIL (μmol/L) | DBIL (μmol/L) | ALT (nkat/L) | γ-GT (nkat/L) | TBA (μmol/L) |
|---|---|---|---|---|---|
| Serum | | | | | |
| Cholestasis | 188.76 ± 72.88[b,d] | 130.76 ± 48.18[b,d] | 126.80 ± 82.12[b] | 266.20 ± 92.73[b,d] | 118.62 ± 43.58[b] |
| Hepatitis | 125.91 ± 64.28 | 68.67 ± 36.75[b] | 96.11 ± 53.69[b] | 140.57 ± 70.32[a] | 111.89 ± 51.54[b] |
| Control | 124.89 ± 62.07 | 6.19 ± 2.55 | 24.23 ± 7.17 | 79.06 ± 35.25 | 14.50 ± 9.76 |
| Biliary | | | | | |
| Cholestasis | 78.13 ± 58.86[b] | 52.63 ± 42.43[b] | 8.07 ± 4.21 | 167.00 ± 100.06[b] | 180.05 ± 216.98[b] |
| Hepatitis | 89.95 ± 59.12[b] | 63.56 ± 53.39[b] | 8.21 ± 4.15 | 278.55 ± 201.10[b] | 266.63 ± 268.09[b] |
| Control | 252.36 ± 108.81 | 174.08 ± 92.56 | 7.22 ± 3.41 | 783.50 ± 363.28 | 643.63 ± 80.67 |

[a]$P < 0.05$ *vs* control; [b]$P < 0.01$ *vs* control; [d]$P < 0.01$ *vs* hepatitis group.

**Table 2 Comparison of serum and biliary IL-6 and TNF-α among three groups (mean ± SD, *n* = 21, ng/L)**

| Group | Serum IL-6 | Serum TNF-α | Biliary IL-6 | Biliary TNF-α |
|---|---|---|---|---|
| Cholestasis | 63.76 ± 26.67[b,d] | 493.11 ± 137.26[b,d] | 286.92 ± 102.02[b,d,f] | 1625.89 ± 563.41[b,d,f] |
| Hepatitis | 40.81 ± 19.32[b] | 305.30 ± 94.45[b] | 183.64 ± 76.94[b,f] | 1068.55 ± 383.35[b,f] |
| Control | 11.46 ± 6.54 | 214.32 ± 78.40 | 40.11 ± 19.89 | 535.80 ± 168.69 |

[b]$P < 0.01$ *vs* control group; [d]$P < 0.01$ *vs* hepatitis group; [f]$P < 0.01$ *vs* serum.

females; average age 56 d (range: 33-120 d)] suffering from IHS, being divided into cholestasis subtype ($n = 21$) and hepatitis subtype ($n = 21$) according to the color of stool[4], were randomly selected from in-patients in Wuhan Children's Hospital. Another 21 subjects [12 males and 9 females; average age 43 d (range: 23-68 d)], being ruled out from IHS and diagnosed as breast-feeding jaundice, were chosen as controls.

### Methods

In fasting condition, next morning after admission to the hospital, non-anticoagulated venous blood was collected from all patients, and the blood specimens were examined instantly. The bile specimens were collected by infant duodenum drainage tube. The procedure of draining was carried out as mentioned below[5]. Before draining, the subjects were instructed to fast for 4 h and given intravenous transfusion for essential nutrition, and given diazapen intravenously or chloral hydrate orally if restless. Then, the infants were placed at right arm reclining and the head was retained by an assistant; after applying a small amount of liquid paraffin and dispelling the tampon at the end of the tube, the operator inserted the tube through right nasal cavity to the stomach at a depth of 30-35 cm and gastric juice was drained out; then through the pylorus, the tube accessed the duodenum at the depth of 40-45 cm and duodenal juice was drained out. The draining was considered successful if yellow draining juice was obtained, or the head of the draining tube was ascertained in the duodenum by X-ray.

The biliary and serum total bilirubin (TBIL), direct bilirubin (DBIL), alanine aminotransferase (ALT), gamma-glutamyl transpeptidase (γ-GT) and total bile acid (TBA) were assayed by CL-7200 Fully-automated Chemistry Analyzer provided by Shimadzu Co. Ltd. The biliary and serum interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α) were assayed by double antibody Sandwich-ELISA with the apparatus from Micro Reader-3 and the kit from R&D Co. Ltd.

### Statistical analysis

One-way ANOVA (*F*-test, SNK-test) was employed to determine the differences of data among the three groups. Student's *t* test was applied for the differences in same constituent between biliary and serum data. Pearson correlation analysis was adopted to explore the relationships among the data. Statistical analyses were performed using SPSS 12.0 software.

## RESULTS

### Comparison of serum biochemical constituents and cytokines among three groups

Serum TBIL, DBIL, ALT, γ-GT, TBA, IL-6 and TNF-α levels were significantly higher in the cholestasis group than those in controls ($P < 0.01$). Moreover, serum DBIL, ALT, γ-GT, TBA, IL-6 and TNF-α levels were markedly higher in the hepatitis group compared to the controls ($P < 0.01$ or $P < 0.05$); while there was no significant difference in serum TBIL level between the hepatitis and control group. In addition, serum TBIL, DBIL, γ-GT, IL-6 and TNF-α levels in the cholestasis group were markedly higher than those in the hepatitis group ($P < 0.01$), but ALT and TBA levels were not obviously different between these two groups (Tables 1 and 2).

### Comparison of biliary biochemical constituents and cytokines among three groups

Biliary TBIL, DBIL, γ-GT and TBA levels both in cholestasis and hepatitis groups were significantly lower than those in controls ($P < 0.01$), while biliary IL-6 and TNF-α levels in those two groups were notably higher

than those in controls ($P < 0.05$). In contrast to that in the hepatitis group, the value of biliary IL-6 and TNF-α in cholestasis group markedly increased ($P < 0.01$), but TBIL, DBIL, γ-GT and TBA levels in both groups were not significantly different. However, the value of biliary ALT was not obviously different among the three groups (Tables 1 and 2).

### *Comparison between biliary and serum cytokines*

Both in cholestasis and hepatitis groups, the levels of IL-6 and TNF-α in serum were notably lower than those in bile ($P < 0.01$) (Table 2).

### *Relationships between biochemical constituents and cytokines*

Biliary IL-6 was obviously correlated with serum DBIL, ALT and γ-GT with the coefficient of correlation of 0.4621, 0.4152 and 0.5376, respectively ($P < 0.05$). Moreover, the biliary TNF-α was significantly correlated with serum DBIL, ALT and γ-GT with the coefficient of correlation of 0.3972, 0.4309, 0.4713, respectively ($P < 0.05$). However, the serum IL-6 and TNF-α were not correlated with serum TBIL, DBIL, ALT, γ-GT, and TBA.

## DISCUSSION

Serum biochemical constituents, such as TBIL, DBIL, ALT, γ-GT and TBA, are used to monitor liver function in medical practice. Among those constituents, bilirubin is a breakdown product of hemoglobin, and total and direct bilirubin (TBIL and DBIL) are usually measured to screen for or to monitor jaundice caused by liver or gall bladder dysfunction. Alanine aminotransferase (ALT), an enzyme found mainly in the liver, is released into the bloodstream when the liver is damaged or diseased. Gamma-glutamyl transpeptidase (γ-GT), existing in the endochylema of the hepatocyte and epithelium of the intrahepatic bile duct and being mainly synthesized by mitochondria in hepatocytes, is discharged to the duodenum through bile duct. Therefore, rise of γ-GT indicates hepatocytic dysfunction or obstruction of extra-hepatic bile duct. Especially, detection of biliary γ-GT can differentiate extra-hepatic biliary atresia and IHS, for bile not consisting of γ-GT when biliary atresia occurs[5]. Total bile acid (TBA) is an exclusive index reflecting hepatic synthesis, secretion, metabolism and hepatocellular dysfunction, and it has been shown that its specificity, sensitivity and stability tendency in numerical hepatobiliary disorder in liver are all superior to the conventional liver function examinations[6].

Cytokines are small proteins released by cells that have a specific effect on interactions and communications between cells or on behavior of cells, and participate in many pathophysiologic progresses in hepatobiliary disorders. Biliary cytokines are produced by hepatocytes, macrophages and epithelium of bile duct. It has been reported that biliary IL-6 was exclusive for diagnosis of angiocholitis[7] and TNF-α could reflect the extent of angiocholitis[8]. We previously confirmed that under cholestatic condition, biliary IL-6 and TNF-α levels increased and were correlated with hepatocellular impairment and cholestasis in rabbit, thereby indicating that IL-6 and TNF-α could reflect the extent of hepatocellular necrosis and angiocholitis[9].

In this study, we observed that, compared to the controls, almost all biochemical constituents in serum were remarkably increased both in cholestasis and hepatitis groups, while those constituents in bile were mostly decreased, which indicated discharging of those from liver to blood and reduction of those in bile when hepatocyte inflamed and cholangiole was embarrassed. When compared between cholestasis and hepatitis groups, the cholestasis-related indexes TBIL, DBIL and γ-GT in serum increased more obviously in the cholestasis group than those in the hepatitis group, while no significant differences in those biochemical constituents in bile between these two groups were observed. In addition, the ALT and TBA, the indexes related to hepatocellular impairment, were not markedly different between the two groups both in serum and bile, manifesting the feature of cholestasis in IHS.

Following the previous animal experiment, we examined the two related cytokines to explore the role of IL-6 and TNF-α in IHS. We found that the two cytokines notably rose in blood and bile of IHS subjects compared to the controls, thereby indicating the inflammatory status in IHS. Furthermore, IL-6 and TNF-α in the cholestasis group increased more significantly than that in the hepatitis group, elucidating the greater severity in cholestasis. On the other hand, in both cholestasis and hepatitis groups, biliary cytokines were more elevated than serum cytokines, which implied biliary cytokines could be a more sensitive clue for diagnosing hepatic impairment.

From the statistical analysis, it was confirmed that biliary IL-6 and TNF-α had a positive correlation with serum DBIL, ALT and γ-GT, but the serum cytokines had not any correlations with serum biochemical constituents. This result illuminated that as cytokines in blood could be influenced by the state of whole body, assaying biliary inflammatory cytokines might be a specific and sensitive test for monitoring the development of IHS. Thus, the results clearly revealed that biliary biochemical constituents altered in coincidence with pathological changes in hepatocellular injury, which can demonstrate the severity of IHS, especially for cholestasis, and the differences between cholestasis and hepatitis subtype in this disorder. Furthermore, it can be concluded that the test of biliary IL-6 and TNF-α might be a specific and sensitive reference to determine the inflammation status of the impaired liver in IHS.

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*RAPID COMMUNICATION*

# Construction of an oral recombinant DNA vaccine from *H pylori* neutrophil activating protein and its immunogenicity

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**Key words:** *H pylori*; Neutrophil activating protein; DNA vaccine

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## Abstract

**AIM:** To construct a live attenuated *Salmonella typhimurium* (*S. typhimurium*) strain harboring the *H pylori* neutrophil activating protein (HP-NAP) gene as an oral recombinant DNA vaccine, and to evaluate its immunogenicity.

**METHODS:** By genetic engineering methods, the genomic DNA of *H pylori* was extracted as a template. The total length of the HP-NAP gene was amplified by polymerase chain reaction (PCR) and cloned into pBT vector for sequencing and BLAST analysis, then subcloned into a eukaryotic expression vector pIRES followed by PCR identification and restriction enzyme digestion. The identified recombinant plasmid pIRES-NAP was transfected into COS-7 cells for target fusion protein expression, and its antigenicity was detected by Western blotting. Then the recombinant plasmid was transformed into a live attenuated *S. typhimurium* strain SL7207 as an oral vaccine strain, and its immunogenicity was evaluated with animal experiments.

**RESULTS:** A 435 bp product was cloned using high homology with HP-NAP gene in GenBank (more than 98%). With identification by PCR and restriction enzyme digestion, a recombinant eukaryotic expression plasmid pIRES-NAP containing the HP-NAP gene of *H pylori* was successfully constructed. The expressed target protein had a specific reaction with *H pylori*i whole cell antibody and showed a single strip result detected by Western blotting. Oral immunization of mice with recombinant DNA vaccine strain SL7207 (pIRES-NAP) also induced a specific immune response.

**CONCLUSION:** The successful construction of HP-NAP oral DNA vaccine with good immunogenicity may help to further investigate its immunoprotection effects and develop vaccine against *H pylori* infection.

## INTRODUCTION

The discovery of *H pylori* has brought about a revolution in the research of etiological factors of gastrointestinal diseases[1]. It has been confirmed that *H pylori* is the main cause of chronic superficial gastritis, chronic active gastritis and peptic ulcer[2-4], and has a close relation to gastric mucosa-associated lymphoid tissue lymphoma and gastric cancer[5,6]. In 1994, the World Health Organization defined it as a class 1 carcinogen. Although significant progress has been made in treating *H pylori* infection with current triple or quadruple therapy based on antibiotics and proton pump inhibitors, the limitations of pharmacological therapy such as side effects, poor compliance, high cost, and most importantly, rapid emergence of antibiotic resistance have set the stage for the development of less costly and more efficient means to prevent and control *H pylori* infection. Ample precedence from previous experiences suggests that vaccination may be an alternative[7].

DNA vaccine has shown a great potential in protecting against and treating many diseases since it was developed. It can induce complete immune responses, provide heterologous cross protection, and can be easily prepared as a polyvalency vaccine[8]. In addition, the live attenuated *Salmonella typhimurium* (*S. typhimurium*) strain expressing foreign antigens may be a very hopeful new-generation for developing *H pylori* vaccine. Experiments on human body indicate that it has very good endurance and immunogenicity, which can be used to transmit foreign antigens[9]. In our present study, we selected a neutrophil activating protein (HP-NAP), a new major virulence factor of *H pylori* identified more recently, which was termed for its ability to induce adhesion of neutrophils to gastric endothelial cells and to produce reactive oxygen radicals[10]. We attempted to construct a live attenuated

*S. typhimurium* strain harbouring the HP-NAP gene as an oral recombinant DNA vaccine, and to explore its immunogenicity to pave the way for biological treatment of *H pylori* infection.

## MATERIALS AND METHODS

### Materials

The *H pylori* standard strain CCUG 17874, kindly presented by the IRIS Research Center of Italy, was cultured on *Campylobacter* selective agar (Merck, Germany) medium supplemented with 10% defibrillated goat blood containing *Campylobacter* selective antibiotic mixture (Merck), and incubated under microaerobic conditions (50 mL $O_2$, 85 mL $N_2$, 10 mL $CO_2$ and 10% relative humidity at 37℃). The *E. coli* strain DH5α, live attenuated *S. typhimurium* strain LB5000 and SL7207, and COS-7 cell lines conserved in our laboratory, were cultured routinely.

Restriction enzymes including *Xho* I and *Mlu* I, T4 DNA ligase, and *EX Taq*™ DNA polymerase were purchased from TaKaRa Biotechnology (Dalian) *Co*., *Ltd*. Mouse anti-HP-NAP antibody was prepared by our laboratory. Alkaline phosphatase anti-mouse IgG made in horse was purchased from Vector Laboratories. Lipofectamine™ 2000 was purchased from Gibco Corporation. T-A cloning vector pBT was purchased from Shanghai Sangon Biological Engineering & Technologies and Services *Co*., *Ltd*. Eukaryotic expression vector pIRES was purchased from Clontech of BD Biosciences. Other reagents were analytically pure reagents produced in China.

Twenty-four specific-pathogen free male C57BL/6 mice aged 4 wk, were purchased from Sino-British Sippr/Bk Laboratory Animal *Ltd*. of Shanghai.

### Construction of recombinant plasmid pIRES-NAP

According to the nucleotide sequence of the HP-NAP gene in GenBank, we designed a pair of oligonucleotide primers P1 (5′-GTC CTC GAG ATG AAA ACA TTT GAA ATT TTA AAA CAT TTG CAA GCG-3', with *Xho* I restriction site) and P2 (5′-GTC ACG CGT TTA AGC CAA ATG GGC TTG CAA CAT CC-3', with *Mlu* I restriction site) synthesized by Sangon with correct ORF. Genomic DNA of CCUG 17874 was extracted as the template. Four mL of template DNA was added to a 100 mL reaction mixture containing 10 mL 10 × PCR buffer, 0.2 mmol/L each deoxynucleoside triphosphate, 2.5 U of *EX Taq*™ polymerase, and 0.2 mmol/L each primer. PCR was performed with Mastercycler® gradient thermocycler (Eppendorf, Germany) as follows. The initial denaturation cycle was at 94℃ for 5 min, followed by 30 cycles of denaturation at 94℃ for 1 min, primer annealing at 60℃ for 1 min, and DNA chain extension at 72℃ for 1 min. The final cycle was at 72℃ for 10 additional minutes, followed by rapid cooling to 4℃. The purified PCR products were T-A cloned into pBT vectors, then transformed into DH5α competent cells using standard methods. Positive clones were screened by blue/white spot and ampicilin resistance. Single-stranded DNA was prepared from selected clones for sequencing with forward M13 universal primer. Homologous analysis between the cloned HP-NAP gene and related genes in GenBank for neucleotide and deduced amino acid sequence was performed by BLAST. The identified recombinant plasmid pBT-NAP was digested with restriction endonucleases *Xho* I, *Mlu* I and subcloned into the corresponding sites of eukaryotic expression vector pIRES. PCR with primers P1, P2 and double enzyme restriction were performed.

### Assay of HP-NAP fusion protein expression

COS-7 cells were cultured routinely and inoculated into 6-well plates one day before transfection, then co-cultured with a mixture of Lipofectamine™ 2000 and recombinant plasmid pIRES-NAP mixed instantly in definite proportion. After incubation at 37℃ for 24-48 h, the culture was centrifuged to collect supernatant. Western blot analysis was performed to evaluate the immunity of HP-NAP antigen expressed in culture supernatant using mouse anti-HP-NAP as primary antibody and horse anti-mouse IgG as secondary antibody.

### Construction of oral DNA vaccine

Recombinant plasmid pIRES-NAP was transformed into *S. typhimurium* strain LB5000 for methylation decoration, then extracted and transformed by electroporation into ending host bacteria *S. typhimurium* strain SL7207. SL7207 (pIRES-NAP) was grown in LB medium containing 100 mg/mL ampicilin at 37℃ for 60 generations. Identification by PCR amplification and double restriction endonuclease digestion was performed every 10 generations.

### Assay of immunology of oral vaccine

Twenty-four mice were divided into 3 groups (8 mice in each group). LB group consisting of non-immunized mice received LB culture fluid and was used as a control group, *Salmonella* group was immunized merely with attenuated The *S. typhimurium* strain SL7207, the vaccine group was immunized with recombinant strain SL7207 (pIRES-NAP). Prior to immunization, all the mice were left overnight without solid food and 6 h without water. A total volume of 100 μL of 30 g/L sodium bicarbonate was given orally using a special catheter to neutralize the stomach pH. Then the mice in the LB group were lavaged immediately with 200 μL LB fluid. Mice in the *Salmonella* group and vaccine group were lavaged with $1.0 \times 10^9$ c.f.u of *S. typhimurium* strain SL7207 and vaccine strain SL7207 (pIRES-NAP), respectively, in a total volume of 200 μL. At 4 wk after immunization, the mice were sacrificed by terminal cardiac puncture under anesthesia and small intestine juices were collected. Indirect ELISA was performed to evaluate HP-NAP-specific IgG or IgA in serum and intestine juice. Purified HP-NAP was used as a coating antigen in ELISA immunoassay.

## RESULTS

### PCR amplification and homology analysis of HP-NAP gene

A 435 bp gene segment was amplified by PCR, which was consistent with the complete sequence of the HP-NAP gene confirmed by sequencing result (Figure 1). BLAST analysis showed that the nucleotide homology between cloned HP-NAP gene and *H pylori* SS1 strain from Genbank reached 98.2% (427/435), including 3 of C-T

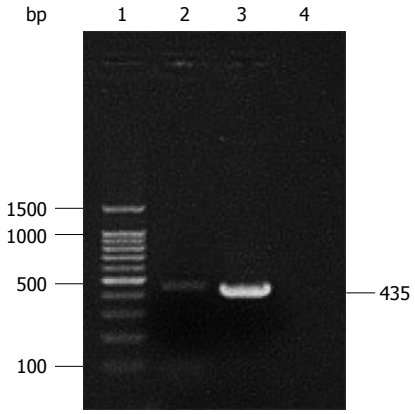


**Figure 1** Electrophoresis of HP-NAP PCR products. Lane 1: 100 bp DNA ladder marker; lanes 2 and 3: PCR products of HP-NAP; lane 4: Blank control.

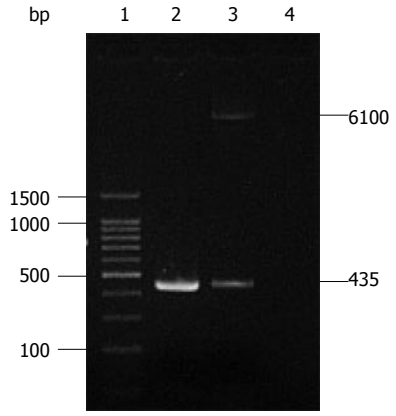


**Figure 2** Identification map of recombinant plasmid pIRES-NAP. Lane 1: 100 bp DNA ladder marker; lane 2: PCR products templated on pIRES-NAP; lane 3: pIRES-NAP digested by endonucleases Xho I and Mlu I; lane 4: Blank control.

and 4 of A-G replacements, and more than 97% (426/435, 425/435) of other common *H pylori* strains such as 5D, 5A, 2B, 2A, RHP901a, DB2 and 1811a. Base replacements of cloned sequence did not significantly affect the translating results. The homology between proteins translated by cloned gene and SS1 strain was 98.6% (142/144).

### *Identification of recombinant plasmid*

A 435 bp target product was cloned as a template on the recombinant plasmid pIRES-NAP using primers P1 and P2. *Xho* I and *Mlu* I enzyme digestion also revealed the target HP-NAP gene in plasmid pIRES-NAP (Figure 2), suggesting that the recombinant plasmid pIRES-NAP and the oral DNA vaccine strain SL7207 (pIRES-NAP) were successfully constructed.

### *Assay of HP-NAP fusion protein*

Western blot analysis results of culture supernatant of COS-7 cells transfected with recombinant plasmid pIRES-NAP are shown in Figure 3. A special simple strip about 17 000 in relative molecular weight was obtained from the supernatant of transfected COS-7 cells, corresponding to the presumed consequence, but no strip was found in the supernatant of non-transfected COS-7 cells as control.

### *Immunology of recombinant strain*

The sera and intestinal juices of the vaccine group immunized with SL7207 (pIRES-NAP) showed positive ELISA results while those of the LB group and *Salmonella* group showed negative ELISA results, indicating that *S. typhimurium* SL7207 (pIRES-NAP) could enable the organism to generate specific mucosal and humoral immunity against the HP-NAP antigen.

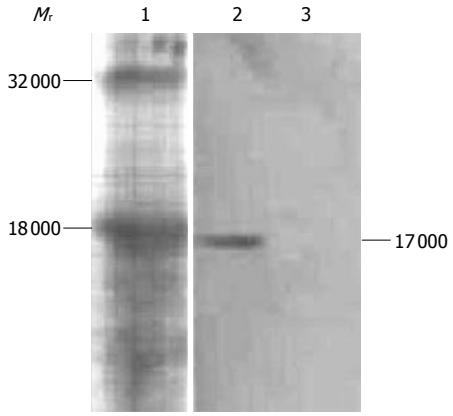


**Figure 3** Western blot analysis of HP-NAP fusion protein expression. Lane 1: Protein standard; lane 2: COS-7 cells transfected with pIRES-NAP; lane 3: COS-7 cells without transfection as control.

## DISCUSSION

Research on the *H pylori* vaccine has been mainly focused on the development of protein vaccines during the past decades, but the preparation and purification of protein antigens is time-consuming and laborious. Since effective immune responses rely on the presence of adjuvants, most of which are toxic to the organism, it is therefore important to develop new *H pylori* vaccines.

Recent advances in immunology and molecular biology have permitted development of DNA vaccines, which have a wide range of applications. Vaccines for such diseases as HIV infection, malaria, and tuberculosis are being developed by using plasmid DNA or viral or bacterial vectors to deliver the genes encoding antigens from pathogens to the host[11]. As live attenuated virus vaccines have come into being for decades, antigenic proteins can be produced *in situ* by the host, engendering cellular and humoral immune responses. But unlike live attenuated vaccines, gene-based vaccines are being designed to deliver only the genes encoding the antigens for the vaccine. The ability of gene-based vaccines to generate cellular as well as humoral responses may be crucial in developing effective vaccines against *H pylori*-induced diseases. Similarly, the ability of gene-based vaccines to generate certain forms of immunogen such as a protein with a particular structure that can be formed only by mammalian cells *in situ*, may be a critical feature of DNA vaccines. It is thought that DNA vaccines can be produced and distributed on a global scale for prevention of diseases such as HIV infection, malaria, and tuberculosis.

*S. typhimurium* can be phagocytized by macrophages and M cells in Peyer node and through mesentery lymph nodes reach the liver and spleen, further stimulating other

organs and tissues to develop mucous membranes, cell and body fluid immunization responses. In the past few years, attenuated *S. typhimurium* as a delivery system has become a new trend to study a new type of oral recombinant live vaccine. Compared with traditional vaccines, live attenuated *S. typhimurium* is used as a new type of vector releasing system for heterologous antigens which does not require antigen purification, and not only protects antigens from degradation and denaturation in the stomach but also expresses adjuvant activity and prevents oral tolerance[12,13].

The development of *H pylori* DNA vaccine is unfolding. Todoroki *et al*[14] have investigated the effect of DNA vaccines encoding *H pylori*-heat shock proteins A and B (pcDNA3.1-hspA and -hspB) on inducing immune responses against *H pylori* in mice. In their study, C57BL/6 mice aged 5 wk were immunized by a single injection of 10 mg of pcDNA3.1-hspA and pcDNA3.1-hspB into intracutaneous tissue. Plasmid DNA lacking the inserted Hsp was injected as a control. Their results demonstrated that DNA vaccines encoding *H pylori*-Hsp could induce a significant immune response against *H pylori* and decrease gastric mucosal inflammation. Miyashita and his colleagues[15] reported that both intranasal and intracutaneous vaccination with pcDNA3.1 encoding *H pylori*-catalase (kat) induces humoral immune responses and suppresses *H pylori* colonization and inflammation of gastric mucosa. Serum IgG and IgA antibodies were induced in mice immunized with intracutaneous pcDNA3.1-kat with suppressed *H pylori* colonization compared to the mice immunized with control DNA, indicating that an effective DNA vaccine can be a new approach against *H pylori* infection in humans with potential foreground.

HP-NAP, a 150 000 dodecameric protein, is released in the medium, most likely after cell lysis, and binds to the bacterial surface, where it acts as an adhesion, mediating its binding to mucin[16] or to polymorphonuclear leukocyte sphingomyelin[17]. Purified recombinant HP-NAP is chemotactic for human neutrophils and monocytes[10] and induces surface expression of $\beta_2$-integrins which are necessary for endothelial trans-migration[18], suggesting that HP-NAP plays a role in the accumulation of these cells at the *H pylori*-infected site. HP-NAP is also a powerful stimulant of the production of reactive oxygen radicals and acts via a cascade of intracellular activation events, including increase in cytosolic calcium ion concentration and phosphorylation of proteins, leading to the assembly of functional NADPH oxidase on neutrophil plasma membrane through a pertussis toxin-sensitive pathway involving extracellular-regulated kinase (ERK) and p38-mitogen-activated protein kinase (MAPK)[19]. The activation of ERK and p38-MAPK is essential for the HP-NAP-induced superoxide anion generation, adhesion and chemotaxis of human neutrophils. Cytokines such as TNF and IFN-$\gamma$ are reported to enhance the production of ROIs induced by HP-NAP and increase the damage of gastric mucosa[20].

HP-NAP has also been shown to increase the synthesis of tissue factor and the secretion of inhibitor-2 of the plasminogen activator in mononuclear cells. By inducing the coordinate expression of cell pro-coagulant and antifibrinolytic activities, HP-NAP might favor fibrin deposition and contribute to the inflammatory reaction of gastric mucosa elicited by *H pylori*[21]. HP-NAP is also capable of crossing epithelial monolayers and inducing activation of the underlying mast cells[22]. These data further support the idea that HP-NAP has an important role in the *in vivo* triggering and maintaining of inflammatory events observed during *H pylori* infection. Once released from the bacterium, HP-NAP would traverse the stomach epithelial layer, reaching the underlying tissue where mast cells reside. The subsequent activation of mast cells by HP-NAP with release of the content of the granules and pro-inflammatory cytokine IL-6, is known to recruit monocytes and neutrophils. Thus, HP-NAP can act at different stages of the inflammatory response by activating mast cells with release of pro-inflammatory molecules able to activate neutrophils and monocytes, and additionally by acting directly on neutrophils and monocytes by promoting their recruitment and activation.

HP-NAP is highly immunogenic in humans. Analysis of serum samples from 35 *H pylori*-infected individuals revealed that 60% of the subjects contain specific antibodies to HP-NAP[2]. Satin and his colleagues[10] immunized 10 mice with purified recombinant HP-NAP by intragastric administration, showing that 80% of them have acquired protective immunity, which is higher than the CagA (70%) group but lower than the supersonic lysate group (90%). These data indicate that this protein is a good vaccine candidate for protecting *H pylori* infection.

In the present study, we successfully amplified and subcloned the HP-NAP gene into the eukaryotic expression vector pIRES, and established the recombinant live attenuated *S. typhimurium* strain SL7207 (pIRES-NAP) as an oral DNA vaccine. BLAST analysis indicated the HP-NAP gene we cloned had a high homology with those in GenBank. The COS-7 cells transfected with recombinant plasmid pIRES-NAP expressing a protein of 17 000 reacted especially with anti-HP-NAP antibody, but no reaction was found in the control group. On the other hand, the serum and intestine fluids from mice immunized with DNA vaccine contained specific antibody to HP-NAP, while those from control groups did not.

In conclusion, HP-NAP DNA vaccine can express target protein with good immunogenicity in eukaryotic hosts, and *S. typhimurium* strain SL7207 (pIRES-NAP) may be a good candidate as a vaccine for prevention and cure of *H pylori* infection. Further study is needed to explore its immunoprotection effects.

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*RAPID COMMUNICATION*

# Randomized clinical trial on seven-day-per-week continuous accelerated irradiation for patients with esophageal carcinoma: Preliminary report on tumor response and acute toxicity

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**Key words:** Esophageal carcinoma; Continuous accelerated irradiation; Conventional irradiation; Toxicity; Overall treatment time

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## Abstract

**AIM:** Tumor response and normal tissue toxicity of seven-day-per-week continuous accelerated irradiation (CAIR) for patients with esophageal carcinoma were evaluated and compared to conventional irradiation (CR).

**METHODS:** Sixty patients with squamous cell carcinoma of the esophagus were randomized into two groups: the CAIR group (30 patients) and the CR group (30 patients). Patients in the CAIR group received radiotherapy (RT) with 2 Gy/fraction per day at 7 d/wk with a total dose of 50-70 Gy (average dose 64.2 Gy). The overall time of irradiation was 3.6-5.0 wk (average 4.6 wk). RT in the CR group was 2 Gy/fraction per day at 5 d/wk with a total dose of 40-70 Gy (average dose 61.7 Gy). The overall time of irradiation was 4.0-7.0 wk (average 6.4 wk).

**RESULTS:** The data showed that the immediate tumor response to RT was better in the CAIR group than in the CR group. Efficiency rates (CR plus PR) were 82.8% (24/29) and 58.6% (17/29), respectively ($P$ = 0.047). In both groups the incidences of esophagitis and tracheitis were insignificant ($P$ = 0.376, 0.959), and no patient received toxicity that could not be tolerated.

**CONCLUSION:** CAIR shortens overall treatment time and is well tolerated by patients. It may be superior to CR in enhancing the local response of tumor, but its remote effect for esophageal carcinoma awaits further follow-up.

## INTRODUCTION

Esophageal carcinoma, especially in China, is one of the most common cancers. Its treatment results are rather dismal, with 5-year survival rates of about 5%-10% for conventional radiotherapy (CR)[1,2]. The poor prognosis is the result of both, local residual disease and early disease relapse. Thus for esophageal carcinoma, local control is the most important factor in prolonging survival[3]. Several animal experiments and clinical investigations have shown that accelerated repopulation of surviving tumor clonogens during a standard course of RT is one of the major reasons for treatment failure in several cancers[4-6]. Some clinical trials of accelerated hyperfractionated RT have been carried out with the aim of overcoming this problem by shortening the overall treatment time. Some reports have already suggested improvement in local control and survival rates, but at the expense of increasing acute toxicity, particularly with the faster schedule[6-8]. In recent years, another RT schedule has been used to treat head and neck cancer. The idea was simple-to continue radiation during the weekends. In this way, the overall treatment time has shortened for about two weeks, giving one fraction per day, seven days a week (including Saturday and Sunday), without any change of the other parameters as time or dose. This schedule was defined as a continuous accelerated irradiation (CAIR) and has been compared to conventional five days treatment in a randomized prospective study for head and neck cancer[9]. Using this RT schedule, patients with esophageal carcinoma were treated and our study aimed to evaluate tumor response and normal tissue toxicity.

## MATERIALS AND METHODS

### Materials

From October 2003 to December 2005, 60 unresectable or medically inoperable patients with esophageal carcinoma were enrolled and randomized into two groups by the sealed envelope method. The project of clinical randomized trial on seven-days-per-week continuous accelerated irradiation (30 patients) *vs* conventional treatment (30 patients), including the criteria for patient eligibility, the diagnostic procedure, the randomization method, the fractionation schemes of treatment techniques, and patient care was approved by the Ethical Committee of Xuzhou Cancer Hospital. All patients received full information concerning the aim of the study, diagnostic and treatment procedures, medical care, risk of acute and late sequelae before they entered the trial. All patients gave informed consent to this study.

Only patients with histologically proven squamous cells of esophageal carcinoma were included in the trial. Additional criteria for eligibility were age $\leqslant$ 75 years, Karnofsky performance status $\geqslant$ 70, white blood cell and hemoglobin levels within normal range, and no prior treatment. The pretreatment evaluation generally included chest radiography, chest CT scan, esophageal barium film, and ultrasound examination of the abdomen, including liver, kidney, spleen, and retroperitoneal lymph nodes, and liver and renal function tests. Based on examinations mentioned above, tumor staging was performed according to the TNM staging system of the 1997 American Joint Committee on Cancer staging system. Patients' characteristics are presented in Table 1 which shows comparable distribution of biological and clinical factors in both groups of the trial.

**Table 1 Patients' characteristics**

| Characteristic | CAIR group | CR group | $\chi^2$ or $t$ | $P$ |
|---|---|---|---|---|
| $n$ | 30 | 30 | | |
| Gender | | | 0.30 | 0.584 |
| Male | 19 | 21 | | |
| Female | 11 | 9 | | |
| Age (yr) | 66.0 ± 8.0 | 70.9 ± 9.4 | 1.781 | 0.083 |
| Length (cm) | 3-10 | 2-12 | | |
| Average (cm) | 5.5 ± 1.8 | 6.2 ± 3.1 | 0.962 | 0.342 |
| Location | | | 0.018 | 0.985 |
| Upper-thoracic | 4 | 6 | | |
| Middle-thoracic | 23 | 19 | | |
| Lower-thoracic | 3 | 5 | | |
| Stage | | | 1.920 | 0.089 |
| Ⅰ | 0 | 2 | | |
| Ⅱ | 17 | 12 | | |
| Ⅲ | 13 | 16 | | |
| WBC ($\times 10^9$/L) | 6.4 ± 1.9 | 6.1 ± 2.0 | 0.443 | 0.660 |
| HGB (g/L) | 138.4 ± 14.4 | 131.4 ± 17.1 | 1.411 | 0.166 |

### Methods

**Radiation methods:** Radiation source was 6MV-X-ray linear accelerator. For the design of the radiation fields for all patients, a three-field approach was used: one anterior and two posterior oblique portals.The length of the field should cover clinical tumors with 3 cm extended margin at both ends of the lesion. The width of the fields was adjusted to cover gross tumors with 2 cm margins to include the subclinical lesions. RT in CAIR group was 2 Gy/fraction per day for 7 d/wk with a total dose of 50-70 Gy (average dose 64.2 Gy), the overall time of irradiation was 3.6-5.0 wk (average 4.6 wk). RT in CR group was 2 Gy/fraction per day for 5 d/wk with a total dose of 40-70 Gy (average dose 61.7 Gy), the overall time of irradiation was 4.0-7.0 wk (average 6.4 wk).

**Tumor response and acute radiation reactions evaluated:** All patients received esophageal barium examination before, during, and at the end of RT. At the end of RT, the tumor response to RT was evaluated. A complete response (CR) was the disappearance of the mass shadow, no narrowing observed in the esophageal lumen, and no, or slight rigidity of the esophageal wall remains without residual ulceration. A partial response (PR) was a > 50% reduction in tumor bulk but < 100% resolution of the disease and a residual shallow ulcer with a diameter of < 1.5 cm, despite the disappearance of the mass shadow. A minor response (MR) was definite improvement in the barium esophagogram but with < 50% regression, with a large residual ulcer crater and/or narrowing of the esophageal lumen, regardless of the residual state of the mass shadow. No change (NC) was no improvement in the X-ray findings, with a deep and large residual ulcer or complete obstruction of the esophageal lumen, regardless of the residual state of the mass shadow[10]. Acute radiation toxicity was evaluated by the Radiation Therapy Oncology Group (RTOG) toxicity criteria.

### Statistical analysis

Statistical analysis was done by SPSS (Version 10.0). $t$-test, Chi-square test, or Wilcoxon-$W$ test were used to compare the patients' characteristics, tumor response, and normal tissue toxicity to RT of both groups.

## RESULTS

### Early tumor response

One patient in the CAIR group interrupted RT because of multi-metastasis and another patient in CR group died from cardiac muscle infarction in the schedule. Within three months after RT, the patients that completed the schedule planed were evaluated by criteria as described above. In the two groups, efficiency rate (CR plus PR) was 82.8% (24/29) and 58.6% (17/29), respectively and, accordingly, the inefficiency rate (MR plus NC) was 17.2% (5/29) and 41.4% (12/29), respectively. The difference in tumor response to RT was statistically significant, the efficiency rate in the CAIR group was higher than the CR group ($P$ = 0.047). The immediate response of the two groups of patients to RT are listed in Table 2.

### Acute radiation reactions

Table 3 shows the acute radiation reactions during the treatment course and up to three months after RT. We found that acute radiation esophagitis and tracheitis in both groups was mainly grade Ⅰ-Ⅱ and the difference between the two groups was not statistically significant. No patient received treatment resulting in intolerable acute

**Table 2 Tumor response to RT *n* (%)**

| Group | CR | PR | MR | NC | $\chi^2$ | *P* |
|---|---|---|---|---|---|---|
| CAIR | 8 (27.6) | 16 (55.2) | 4 (13.8) | 1 (3.4) | 4.08 | 0.047 |
| CR | 6 (20.7) | 11 (37.9) | 12 (41.4) | 0 | | |

radiation reactions in either group.

### *Impact on blood cell and hemoglobin level*

In the CAIR group, the total number of white cells declined below normal level in 5 patients within two weeks after beginning RT. In three of them, white cell counts normalized by medical intervention. In three patients of the CR group, total number of white cell declined below normal level. In both groups, there were two whose patients, total number of white cells did not increase right up to the end of RT. Furthermore, all patient's hemoglobin level had no statistically significant change during the treatment course.

## DISCUSSION

Esophageal carcinoma is one of most common malignant diseases in China. The prognosis for patients with esophageal carcinoma is extremely poor. The five years survival rate is 5%-10% for CR alone. The poor prognosis is the result of both local treatment failure, seen in up to 80% of cases, and early disease dissemination[3,10]. Thus, for esophageal carcinoma, local control is at present the most important factor in prolonging survival. Accelerated repopulation of tumor cells during conventionally fractionated radiotherapy is a proposed reason of failed local control in head-and-neck tumors. In the clinical setting, one goal of treatment is to limit the extent of tumor cell regeneration that occurs during a course of fractionated RT. There is radiobiological rationale and convincing evidence from a number of clinical studies that a therapeutic gain may be achieved, at least for head and neck cancers, when conventional fractionation is modified by reduction in size of dose per fraction with the increase in total dose, reduction of overall treatment time, or both[9,11]. Rapid repopulation of tumor clonogens is able to compensate about 0.6 Gy/d[5], beginning after a lag period, which on average, in head and neck tumors is about 3-4 wk from the inception of therapy[12]. Thus, shortening overall treatment time should limit the extent of accelerated tumor repopulation, and therefore one may expect an increase in the probability of tumor control for given total dose. Since treatment time is thought to have little or no influence on the response of late reacting normal tissue, a reduction in overall treatment time would not be expected to affect the incidence and severity of late normal tissue injury (provided the size of dose per fraction is not increased and the interfraction interval is sufficient for repair to be completed). These concepts became a basic rationale for the development of various altered fractionation strategies as an alternative to conventional fractionation[13,14]. Simultaneously with reduced treatment time schedules, multiple fraction per day regimens have been used[15-17]. They allow a higher total dose to be given within the tolerance of late responding normal tissues.

**Table 3 Grade of acute reaction (RTOG) of esophagus and bronchus according to fractionation regimen *n* (%)**

| Groups | Ⅰ | Ⅱ | Ⅲ | Ⅳ | *Z* | *P* |
|---|---|---|---|---|---|---|
| Esophagitis | | | | | 0.855 | 0.376 |
| CAIR | 6 (20.7) | 19 (65.5) | 4 (13.8) | 0 | | |
| CR | 11 (37.9) | 13 (44.8) | 5 (17.2) | 0 | | |
| Tracheitis | | | | | 0.051 | 0.959 |
| CAIR | 14 (48.3) | 10 (34.5) | 5 (17.2) | 0 | | |
| CR | 13 (44.8) | 13 (44.8) | 3 (10.4) | 0 | | |

One modality currently in use to achieve this goal is the concomitant boost schedule designed by Maciejewski B *et al*[18] on carcinoma of the head-and-neck. Their data indicated when dose per fraction of 2.0 Gy given once-a-day at 24 h intervals, an analysis of severe mucosal reactions shows significant difference between CAIR group and CR group. Developed severe mucositis was 48% of patients and 5%, respectively. Their conclusion was that the accelerated treatment, using daily fractions of 2.0 Gy, 7 d per week, gives unacceptable toxicity. When dose per fraction was lowered from 2.0 Gy to 1.8 Gy, the overall rate of acute mucosal reactions decreased to 10% as reported by Skladowski K *et al*[9]. Both the 3 years local control rate and the 3 years survival rate was improved.The 3-year local tumor control was 82% in the CAIR group and 37% in the CR group, and 3 years overall survival was 78% and 32%, respectively.

In our trial, dose per fraction was 2.0 Gy in both groups. The data showed that acute radiation esophagitis and tracheitis in both groups was mainly grade I-II, and the difference between the two groups was not statistically significant. No patient received treatment resulting in acute radiation reactions that could not be tolerated in in either group. The immediate response of two group patients to RT was statistically significant. The efficiency rate (CR plus PR) was 82.8% (24/29) and 58.6% (17/29), respectively, the CAIR group was significantly better than the CR group ($P = 0.047$). The overall treatment time has been shortened by two weeks, as given in the result section.

The present trial demonstrates that seven-day-per-week continuous accelerated irradiation provides significant therapeutic benefit for patients with esophageal carcinoma with regard to both response and toxicity to RT. Fractions of 2.0 Gy could keep acute radiation esophagitis and tracheitis on a tolerable level (different from Maciejewski B). The results of our trial suggest that local control in patients with esophageal carcinoma might be improved by CAIR compared to CR methods, when using dose escalation by continuous accelerated irradiation (with sufficiently long interfraction intervals) and a shorter overall treatment time.

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*RAPID COMMUNICATION*

# Application of laparoscopy in diagnosis and treatment of massive small intestinal bleeding: Report of 22 cases

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## Abstract

**AIM:** To investigate the diagnostic and therapeutic value of laparoscopy in patients with massive small intestinal bleeding.

**METHODS:** Twenty-two patients with massive small intestinal bleeding and hemodynamic alteration underwent laparoscopic laparotomy in our unit from December 2002 to April 2005. Post pathologic sites were found, laparoscopy- or laparoscopy-assisted part small intestinal resection including pathologic intestinal site and enteroanastomosis was performed in all these patients.

**RESULTS:** The bleeding sites were successfully detected by laparoscopy in all these 22 patients. Massive small intestinal bleeding was caused by jejunum benign stromal tumor in 8 cases, by jejunum potential malignant stromal tumor in 5 cases, by jejunum malignant stromal tumor in 1 case, by Mechel's diverticulum in 5 cases, by small intestinal vascular deformity in 2 cases, and by ectopic pancreas in 1 case. A total of 16 patients underwent laparoscopy-assisted enterectomy and enteroanastomosis of small intestine covering the diseased segment and 6 patients received enterectomy of the diseased segment under laparoscope. No surgical complications occurred and the outcome was satisfactory.

**CONCLUSION:** Laparoscopy in diagnosis and treatment of massive small intestinal bleeding is noninvasive with less pain, short recovery time and definite therapeutic efficacy.



**Key words:** Small intestine; Bleeding; Laparoscopy; Meckel's diverticulum; Stromal tumor

## INTRODUCTION

Massive small intestinal bleeding is rare, accounting for 0.4% of all cases of gastrointestinal bleeding. So far there has been no effective method for its definite diagnosis due to its nonspecific clinical symptoms and signs, thus its treatment is a challenge for clinical surgeons[1-3]. From December 2002 to April 2005, 22 patients with suspected massive small intestinal bleeding admitted to our department received laparoscopy for definitive diagnosis and treatment. The outcomes were satisfactory.

## MATERIALS AND METHODS

### *Clinical data*

Twenty-two patients with suspected massive small intestinal bleeding (13 males and 9 females) were included in the present study. Their age ranged from 16 to 61 years with a mean age of 38.6 years. The course of the disease ranged from 20 d to 10 years with a mean of 36.6 mo. The patients had recurrent massive bleeding, ranging from 3 to 15 times prior to admission with a mean of 5 times. The chief complaints were bright red or catsup-like bloody stools with concurrent abdominal pain in 4 cases. On the day of admission, the amount of bleeding was more than 1200 mL with concurrent hemodynamic changes. Hemoglobin ranged from 40.6 g/L to 66 g/L with a mean of 50.6 g/L. Massive small intestinal bleeding was suspected after bleeding from gastric duodenum, colon and rectum was excluded. Double contrast of barium and air examination of the alimentary tract were performed in 6 patients, of them 1 had small intestinal tumor and 1 had Meckel's diverticulum. Small intestine enteroscopy was carried out in 5 patients, of them 2 had small intestinal bleeding due to Mechel's diverticulum and 1 had small intestinal stromal tumor (Figures 1A and B). Emission CT scanning (ECT) of the abdominal cavity was performed in 6 patients, of them 2 had a small intestinal bleeding due to Mechel's diverticulum and small intestinal tumor (Figure 2). Digital subtraction angiography (DSA) was conducted

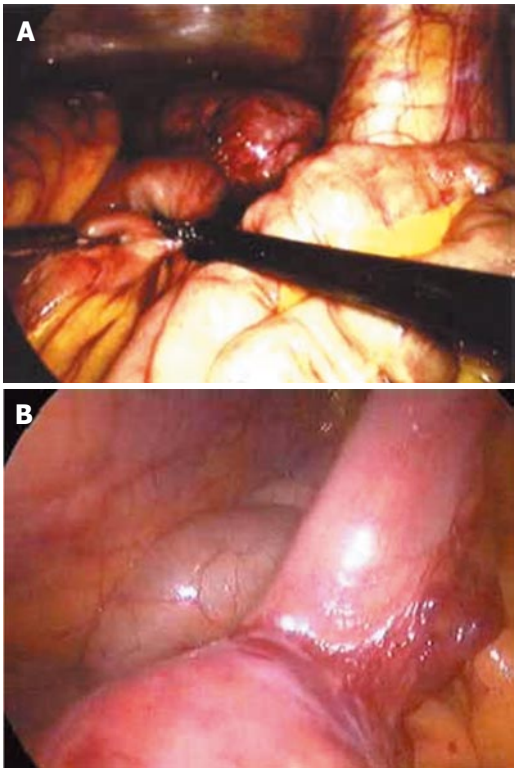


**Figure 1** Laparoscopic laparotomy showing small intestinal bleeding (**A** and **B**).

in 3 patients, of them 1 had a jejunum tumor and 1 had the contrast medium in blood vessels of terminal ileum flowing into the intestinal cavity where pathological changes were not defined.

### *Surgical procedures*

Before operation, shock was treated with blood transfusion until hemoglobin level reached 90 g/L or above. After the blood pressure became normal, emergency laparoscopic laparotomy was performed under general anesthesia with the patients at a head-down position. A transverse incision (1 cm) was made 0.5 cm inferior to the umbilicus to establish pneumoperitoneum with a pressure of 13 mmHg. A hole was made inferior to the umbilicus to insert the laparoscope of 10 mm at 30℃ to examine the abdominal viscera. Under the guidance of a laparoscope, a second and third holes of 5 mm were made at the level of umbilicus on the midlines of right and left clavicles. The second hole was used to check the whole small intestine from the part of the ileum and cecum to Treitz's ligament with a non-impairing laparoscopic bowel clamp, during which the proximal segment of intestinal tract from the boundary of the hematocele was observed and accumulated blood was squeezed out. The recurrent hematocele showed the definite bleeding site. When failing to reach the bleeding site, two alternative methods were used: perioperative small intestine enteroscopy to check the transparency of the intestinal wall with the help of a light source at the top of the enteroscope to localize the bleeding site, thus the small intestine near the hematocele was clamped out of the abdominal wall for incision and perioperative enteroscopy (Figure 3). After the bleeding site was localized, the third hole was dilated to make a 5 cm incision through the left rectus abdominis muscle to clamp the diseased intestinal segment, followed by resection of the diseased intestinal tract according to the laparotomy procedures (Figures 4A and B). In some cases, removal of the diseased small intestine and enteroanastomosis were performed under a laparoscope by transverse dilation of the hole inferior to the the umbilicus.

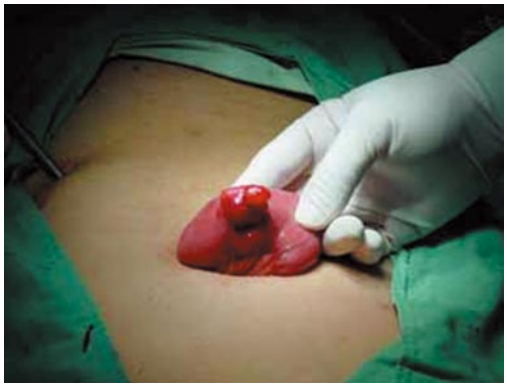

**Figure 2** An oval stromal tumor causing small intestinal bleeding. Laparoscopic laparotomy found there is an oval stromal tumor on the up segment of jejunum, with clear borderline and smooth surface, having no adhesion with other tissue around it. Expansive abdominal excision was performed to draw out the tumor for resection.

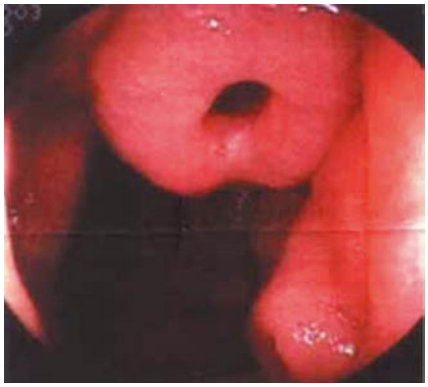

**Figure 3** Electric intestinal endoscopy showing a benign jejunum tumor. A deep ulcer sunken on the top of the tumor could be seen.

## RESULTS

The bleeding sites in these 22 patients were successfully found, of them 2 received laparoscopy combined with perioperative enteroscopy, 4 underwent perioperative incision of the small intestinal tract under laparoscope combined with perioperative enteroscopy under laparoscope. Massive small intestinal bleeding was caused by benign jejunum stromal tumor in 8 cases, by potential jejunum malignant stromal tumor in 5 cases, by malignant jejunum stromal tumor in 1 case, by Mechel's diverticulum in 5 cases, by small intestinal vascular deformity in 2 cases with 1 case having 2 sites of jejunum vascular dysplasia with concurrent intestinal mucous ulcer (the two sites were 10 cm and 40 cm from Treitz's ligament respectively) and by ectopic pancreas in 1 case. A total of 16 patients underwent laparoscopy-assisted enterectomy

and enteroanastomosis of small intestine covering the diseased segment and 6 patients received enterectomy to remove the diseased segment under laparoscope. The operative duration ranged from 45 min to 180 min with a mean of 90 min. After operation, all patients recovered passing flatus through the anus and taking food within 2 d. No surgical complications occurred and the mean postoperative hospitalization time was 6.5 d. Phone call follow-up was conducted for 2-24 mo with no recurrent alimentary tract bleeding. The surgical outcome was satisfactory.

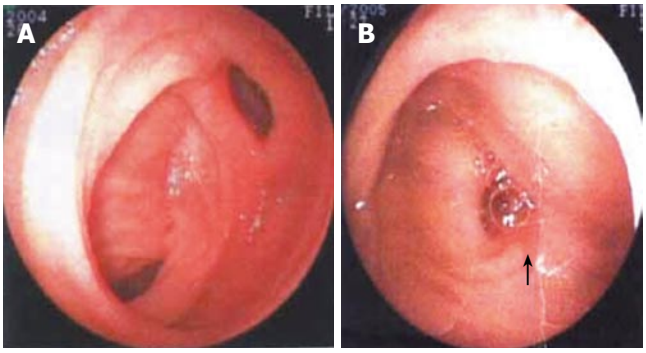


**Figure 4** Double balloon enteroscopy showing the clamped diseased intestinal segment (**A**) and resected diseased intestinal tract (**B**). The double-balloon enteroscope was pushed 200 cm into the ileum through anus. Diverticulum was found in the ileum 90-100 cm away from the ileocecal valve, at the opening of which a 1.2 cm × 1.0 cm ulcer was observed. The ulcer had thin covering of lichenoid substance, but no active bleeding. No other abnormalities were found. Meckel's diverticulum was diagnosed.

## DISCUSSION

The small intestine is about 3-5 m long, occupying three fourths of the whole gastrointestinal tract. The ansa intestinalis is circuitous overlapping active peristalsis and its location varies greatly in the abdominal cavity. Since massive small intestinal bleeding lacks specific clinical symptoms and signs, it is difficult to diagnose and locate it by routine examinations[4-6]. Small intestine enteroscopy is the most specific method for its diagnosis but its application is limited because this examination is time-consuming, extremely unpleasant, and causes bleeding and perforation with a high false positive rate[7-13]. Recently, a capsule endoscope is under clinic experiment, but it cannot perform biopsy and make pathological diagnosis[10-13]. Stromal tumor is the most frequent cause of small intestinal bleeding[1-3,5], Mechel's diverticulum and vascular conditions are the second frequent cause of small intestinal bleeding[1,4,6]. False positive tumor may not be shown on X-ray imaging of the whole alimentary tract because the bleeding foci often grows in exogenesis[14,15]. During the active stage of small intestinal bleeding, DSA can find the contrast medium flowing from the tumor site into the intestinal tract, showing local shadow with a slightly high density and embolism treatment can be conducted during diagnosis[15]. $^{99m}$Tc-sestamibi is sensitive to mild intestinal bleeding , thus marking erythrocytes for gastrointestinal bleeding imaging, while it has no diagnostic value in the resting phase of bleeding or the bleeding being less than 0.05 mL/min[16]. At present, the diagnosis of massive obscure gastro-intestinal bleeding is usually made by exposure laparotomy, which is invasive with a false positive rate of 5%. Besides, patients with massive small intestinal bleeding are often weak with poor conditions and unstable vital signs, which prevent them from undergoing a major surgical operation.

Laparoscopy can clearly, directly and conveniently observe the whole intestinal serosa and mesentery and the small intestinal conditions can be managed with its assistance[11,16-20]. From December 2002, we have tried to use laparoscopy to manage obscure gastrointestinal bleeding in patients with massive small intestinal bleeding. The outcomes showed that laparoscopy could find the bleeding site of massive obscure gastro-intestinal bleeding. It is noninvasive with less pain and short recovery time. We believe that laparoscopy has a promising prospect in diagnosis and treatment of acute massive small intestinal bleeding and can be used as a routine method for the management of massive small intestinal bleeding[11,16,18,20]. Since intestinal stromal tumor and ectopic pancreas that cause small intestinal bleeding are generally small, examination followed by laparotomy cannot find the bleeding foci. Perioperative small intestine enteroscopy in combination with removal of hematocele can avoid the disadvantages of enteroscopy, such as time-consuming, extreme unpleasantness and complications of bleeding and perforation. Laparoscopy can find the bleeding foci, showing the advantages of noninvasive surgery. For those whose bleeding site is not defined by laparoscopy, perioperative enteroscopy of small intestine generally can reach the definite bleeding foci, deserving wide promotion[11,17,19,20].

In the present study, small intestinal bleeding occurred, leading to insufficient blood volume, the average hemoglobin level was 50.6 g/L. Once laparoscopy is accepted by patients, the laparoscope equipment and surgical appliances should be prepared as fast as possible for immediate surgery when shock takes a favorable return. The patient should lie on his/her left side at the head-down position. Firstly, parenchymatous viscera should be generally examined, followed by examination of the whole small intestine. This part of the ileum and cecum has a relatively stable location in the abdominal cavity and thus is easily exposed. Cecum should be used as the landmark during laparoscopic exploration, which starts from the terminal ileum with each 10 cm as one segment to the Treize's ligament. One patient had 2 sites with small intestinal vascular deformity so that exploration of the whole small intestine segment by segment was emphasized to avoid missing any focus. The laparoscopic exploration of small intestinal hemangiomas or vascular deformity should be more careful. The intestinal wall should be carefully explored for local prominence, pitting, overlapping and abnormal mesentery. The suspected bleeding segment should be palpated carefully with clamps to feel its hardness, flexibility, and activity. In case of active massive bleeding, intestinal peristalsis is active and the blood often accumulates in the distal bleeding segment which is dark blue under laparoscope. The suspected foci can be confirmed if emptied, blocked and reformed

hematocele is found. The time-consuming examination is mainly due to repeated enteroscopy. One patient with a history of 3-year bleeding had no positive laparoscopic findings. Repeated examinations had no other positive findings. A slightly hard intestinal wall of this part was touched during exploration, which was ectopic pancreas confirmed by pathological biopsy.

After the bleeding site was found by laparoscopy, laparoscopy-assisted enterectomy and enteroanastomosis were performed, during which an exploratory incision about 5 cm in length was made at the umbilicus level on the midline of the left clavicle to remove the diseased intestinal segment. The resected part of the small intestine should be 5 cm longer than the bleeding site that may result in a fast and reliable excision with light contaminations in the abdominal cavity. Enterectomy and enteroanastomosis can be performed under laparoscope in those whose bleeding sites are adjacent to the Treize's ligament, thus the diseased segment can be conveniently removed[1,2,11,16-20].

In conclusion, laparoscopy in diagnosis and treatment of massive small intestinal bleeding is noninvasive with less pain, short recovery time and definite therapeutic efficacy and has rather good clinical application prospects.

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*CASE REPORT*

# Pravastatin: A potential cause for acute pancreatitis

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## Abstract

Acute pancreatitis (AP) secondary to drugs is uncommon, with an incidence ranging from 0.3% to 2.0% of AP cases. Drug-induced AP due to statins is rare, and only 12 cases have thus far been reported. In this case report, we report a case of a 50-year-old female on pravastatin therapy for 3 d prior to developing symptoms of AP. The common etiological factors for AP were all excluded. The patient was admitted to the intensive care unit secondary to respiratory distress, though she subsequently improved and was discharged 14 d after admission. Although the incidence of drug-induced AP is low, clinicians should have a high index of suspicion for it in patients with AP due to an unknown etiology. Clinicians should be aware of the association of statins with AP. If a patient taking a statin develops abdominal pain, clinicians should consider the diagnosis of AP and conduct the appropriate laboratory and diagnostic evaluation if indicated.



**Key words:** Drug-induced pancreatitis; Acute pancreatitis; Statins; Pravastatin.

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## INTRODUCTION

Acute pancreatitis (AP) secondary to drugs is uncommon, with an incidence ranging from 0.3% to 2.0% of AP cases[1]. The literature on drug-induced AP consists mostly of case reports, though there have been reviews analyzing the association of various drugs with AP[1-3]. The following drugs have been definitely associated with AP in many of the reviews: azathioprine, chlorothiazide, hydrochlorothiazide, estrogens, furosemide, sulfonamides, tetracycline, L-asparaginase, sulindac, valproic acid, didanosine, salicylates, aminosalicylates (mesalamine, sulfasalazine), calcium, sodium stibogluconate, pentamidine, vinka alkaloids, and metronidazole. There are many other drugs which have been implicated as having probable or possible associations with AP, including 6-mercaptopurine, methyldopa, ACE inhibitors, clozapine, rifampicin, cyclosporine, and many other drugs.

There have been 12 cases of AP associated with statins described thus far[4-14]. In four of the cases presented, reintroduction of the statin led to a recurrent attack of AP[4-6,12]. In this case report, we report a case of AP associated with pravastatin therapy. In view of the magnitude of use of statins in prevention of coronary artery disease, even an infrequent occurrence is worth reporting.

## CASE REPORT

A 50-year-old female presented to our hospital with right upper quadrant abdominal pain, nausea, and vomiting for 1 d. Four days prior to admission, she was started on 10 mg pravastatin by mouth daily, though the patient stopped this medication the day prior to admission as she attributed her symptoms to the new medication. Her lipid panel one month prior to admission showed a total cholesterol level of 262 mg/dL, triglyceride level of 268 mg/dL, high-density lipoprotein (HDL) level of 52 mg/dL, and low-density lipoprotein (LDL) level of 156 mg/dL. She was also treated for hypertension with 10 mg enalapril by mouth daily for the past 18 mo, and 25 mg hydrochlorothiazide by mouth daily for 6 years. She took 2.5 mg olanzapine by mouth daily for the past year for severe anxiety, and a combination of 500 mg/1 mg metformin and rosiglitazone (Avandamet™) in the past year for type Ⅱ diabetes mellitus. She had osteoarthritis of both knees, and took 325 mg/37.5 mg one to two tablets of acetaminophen/tramadol (Ultracet™) as needed for pain. Of note, she was on atorvastatin 2 years prior to admission for a period of 3 d, though this was discontinued secondary to generalized body pain. Laboratory tests were not performed at that time. She had a cholecystectomy 16 years prior to admission. The common etiological factors for AP such as alcoholism, trauma to the abdomen, HIV disease, hypertriglyceridemia, and hypercalcemia were all excluded.

**Table 1 Laboratory values**

| | Admission | 48 h |
|---|---|---|
| Amylase (U/L) | 914 | 280 |
| Lipase (U/L) | 1613 | 261 |
| WBC ($mm^3$) | 26300 | 21500 |
| HCT (%) | 53.9 | 42.4 |
| LDH (IU/L) | 389 | 645 |
| Glucose (mg/dL) | 495 | |
| AST (IU/L) | 18 | |
| ALT (IU/L) | 28 | |
| Calcium (mg/dL) | 10.2 | 6.5 |
| $PO_2$ (mmHg) | | 76 |
| BUN (mg/dL) | | 17 |
| Base deficit | | -1 |
| Fluid sequestration (L) | | 2.8 |
| Total cholesterol (mg/dL) | 202 | |
| Triglycerides (mg/dL) | 118 | |

On physical examination, her blood pressure was 158/104 mmHg, heart rate was 131 beats per minute, respiratory rate was 30/min, temperature was 98 degrees Fahrenheit, and $SpO_2$ was 93% breathing room air. Her abdominal examination revealed hypoactive bowel sounds, with diffuse tenderness without rebound tenderness or guarding.

Laboratory values are included in Table 1. Three out of five Ranson criteria were fulfilled on admission, and two out of six criteria were fulfilled after forty-eight hours.

She was admitted to the intensive care unit, and given isotonic intravenous fluids and meperidine for pain control. CT scan of the abdomen showed inflammatory changes within the pancreas, though no discrete peripancreatic fluid collections were noted. Abdominal ultrasound did not show biliary ductal dilatation. Magnetic resonance cholangiopancreatography showed a normal pancreatico-biliary system. She developed respiratory distress, though this resolved after support with BIPAP and diuretic therapy. She improved significantly following this, and was discharged 14 d after admission.

Of note, she was restarted on enalapril during the admission and continued to take enalapril without any adverse effects. She was not restarted on pravachol or hydrochlorothiazide. Olanzapine and metformin/rosiglitazone were also restarted without any adverse effects. The patient was last seen 4 mo after discharge, and her hypertension and diabetes were well controlled on enalapril, metformin, rosiglitazone, atenolol, and glipizide. She also continued to take olanzapine without any adverse effects.

## DISCUSSION

Statin-induced AP is rare and only 12 cases have thus far been reported in the literature[4-14]. Although AP is a rare side effect of statin therapy, there seems to be a strong association between statins and AP, as there have been four cases where reintroduction of the statin has led to a recurrence of AP[4-6,12]. In our case report, the patient was on pravastatin for three days prior to symptom onset, and the outcome was favorable. A rechallenge test involving documenting AP development during treatment with a drug, its disappearance after stopping the drug, and recurrence after reintroduction of the drug, was not performed in this patient due to ethical issues. This would be the most reliable evidence that pravastatin caused AP in this patient.

No data about a potential mechanism for statin-induced AP are available at this time. In previously published cases of statin-induced pancreatitis, the duration of statin treatment until the onset of AP varied from 8 h to 7 years, though the vast majority of patients presented within 6 mo of introduction of the statin[4-14]. Generally, the outcome is favorable in statin-induced AP, though there was a fatality in one case after a four-month hospital stay[13].

A number of medications that our patient took are known to be associated with AP. Thiazide diuretics[15], ACE inhibitors[16], atypical antipsychotics[17], biguanides[18], and acetaminophen[19] have been associated with AP. However, continuation of all of the above medications, with the exception of the thiazide diuretic, did not precipitate AP.

In conclusion, though the incidence of drug-induced AP is low, clinicians should have a high index of suspicion for it in patients with AP due to an unknown etiology. A diligent review of medications should be performed, focusing on drugs that have been associated with drug-induced AP[1-3]. Clinicians should be aware of the association of statins with AP. If a patient taking a statin develops abdominal pain, clinicians should consider the diagnosis of AP and conduct the appropriate laboratory and diagnostic evaluation if indicated.

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*CASE REPORT*

# Carcinoid of the ampulla of Vater: Morphologic features and clinical implications

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## Abstract

Carcinoids involving the ampulla of Vater are rare lesions that may produce painless jaundice. The published data indicate that these tumors, in contrast to their midgut counterparts, metastasize in approximately half of cases irrespective of primary tumor size. Therefore, radical excision in the form of pancreaticoduodenectomy is recommended regardless of tumor size. As with other gastrointestinal carcinoid tumors, biological treatment with octreotide analogues can be applied to symptomatic patients. Tumor-targeted radioactive therapy is a newly emerging treatment option. We here report case of a carcinoid tumor of the ampulla of Vater presenting as painless jaundice in a 65-year old man and review the relevant literature, giving special attention to the morphologic features, clinical characteristics, and treatment modalities associated with this disease process.



**Key words:** Ampulla of Vater; Histopathologic features; Clinical characteristics; Radical excision

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## INTRODUCTION

Carcinoid tumors belong to the family of neuroendocrine tumors, which usually grow slowly with distinct biological and clinical characteristics. The incidence of these tumors is approximately 2.5 in 100 000 people per year. The appendix is the most common location, accounting for roughly 60% of all cases, followed by the ileum, the rectum and the stomach. Approximately 2% of cases involve the duodenum, 1% the biliary tract, and 0.6% the pancreas[1]. Carcinoid tumors that involve the ampulla are an extremely rare clinical entity, with 93 reported cases[2-7]. We report another case of a carcinoid tumor of the ampulla of Vater presenting as painless jaundice. The relevant literature is reviewed, giving special attention to the morphologic features, clinical characteristics, and treatment modalities associated with this disease process.

## CASE REPORT

A 65-year old otherwise healthy man presented with a 2-wk history of intractable pruritus. Review of systems was notable for dark urine. He denied clay-colored stool, diarrhea, flushing or dyspnea. Physical examination was remarkable for mildly icteric sclerae. There were no palpable abdominal masses, adenopathy, or café-au-lait spots. Laboratory studies indicated a markedly elevated level of alkaline phosphatase (712 U/L). Total bilirubin was 29 mg/L. Abdominal computed tomography (CT) scan revealed significant dilatation of both the common bile duct (CBD) and the pancreatic duct, without any obvious periampullary mass, retroperitoneal adenopathy or liver lesions (Figure 1).

Endoscopic retrograde cholangiopancreatography (ERCP) showed a prominent major ampulla with normal overlying mucosa. The CBD measured 17 mm with an abrupt "shoulder" in the region of the ampulla (Figure 2). The ampullary obstruction was relieved with temporary biliary stent placement. Subsequent endoscopic ultrasonography (EUS) identified a 23 mm × 27 mm well circumscribed, round, hypoechoic mass in the region of the ampulla adjacent to the biliary stent, which was distinct from the pancreatic tissue and the duodenal wall (Figure 3). EUS-guided fine needle biopsy was suggestive of a carcinoid tumor. Positron emission tomography (PET) scan confirmed a lesion of increased metabolism in the vicinity of the pancreatic head, and excluded distant metastases. Urinary 5-hydroxyindoleacetic acid (5-HIAA), and serum carcinoembryonic antigen (CEA), CA19-9, serotonin, somatostatin and chromogranin A levels were all within normal limits.

A pylorus-preserving pancreaticoduodenectomy was performed. Pathology revealed a $T_3$, N1 ampullary carcinoid extending into the peripancreatic fat, with 7/13 positive lymphnodes. Immunohistochemically, tumor cells expressed chromogranin A and synaptophysin, and focally serotonin and somatostatin, which were stained negative

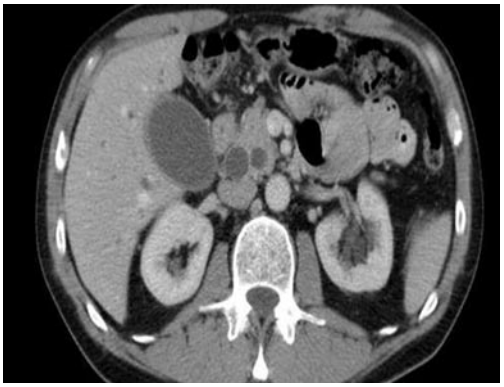

**Figure 1** The characteristic "double duct sign" on abdominal CT is indicative of obstruction at the level of the ampulla.

for insulin, glucagon, and gastrin. The patient recovered uneventfully and was discharged to home on the seventh postoperative day. The follow-up plan consists of structural studies and biochemical markers every 3 mo up to 1 year and every 6 mo thereafter. At 9 mo he remained asymptomatic with no radiographically or biochemically detectable disease.

## DISCUSSION

In 1888 the first case of a carcinoid tumor was reported as a lesion of the ileum[8]. Oberndorfer coined the term *karzinoide* ("carcinoma-like") in 1907 to distinguish these presumptively benign neoplasms from the malignant cecal adenocarcinomas[9]. Carcinoids have been detected in the entire gastrointestinal tract, from the esophagus to the rectum, as well as in extra-gastrointestinal locations, such as the bronchus, the testis, the ovary and the larynx. In contrast to the relative rarity of duodenal carcinoids, those of the ampulla of Vater represent a medical curiosity with only 93 cases reported in the world literature.

Indicative of their location, ampullary carcinoids cause jaundice as the leading symptom in approximately two-thirds of cases. Forty percent of patients present with abdominal pain and few with pancreatitis, weight loss, or malaise[2-7]. Every fourth patient has von Recklinghausen's disease[2]. The etiology is not clear, but it is likely that mutation of the NF-1 tumor suppressor gene may predispose to the development of ampullary carcinoids. In contrast to midgut carcinoids, foregut carcinoids are very rarely associated with carcinoid syndrome. In the largest reported series of 73 ampullary carcinoid cases, a history of flushing, diarrhea, or asthma was found in only 2 patients who also had extensive liver metastases[2]. Walton *et al*[4] have described a "variant" form of carcinoid syndrome that may occur in association with gastric or other foregut carcinoid tumors, which may be hidden by the elaboration of other hormonally active substances, such as adrenocorticotropic hormone, parathyroid hormone, calcitonin, gastrin, vasoactive intestinal peptide, growth hormone, or insulin, but responds to treatment with antihistamines.

Immunohistochemistry is of principal importance in accurately diagnosing ampullary carcinoids. Makhlouf *et al*[7] compared the immunohistochemical features of 12 ampullary and 53 duodenal carcinoids and demonstrated that the former group always expresses chromogranin A, but almost never expresses gastrin which has been identified in 56% of cases of duodenal carcinoids. Apart from its role in immunohistochemical diagnosis, chromogranin A has proven particularly useful as a tumor marker for monitoring disease response and progression in patients with gastrointestinal carcinoids. Analyzing data from 301 such patients, Janson *et al*[10] found that serum chromogranin A level > 5000 μg/L (normal value: 0-76 μg/L) is an independent predictor of poor survival.

Surprisingly, in the case of ampullary carcinoids, tumor size does not correlate with metastatic potential. In the case review by Hatzitheoklitos *et al*[2], metastasis is present in 46% of ampullary carcinoids > 2 cm, in 50% of tumors between 1-2 cm, and in 66% of tumors < 1 cm. Makhlouf *et al*[7] have reported two tumors measuring less than 2 cm demonstrating metastases, as well as a 5 cm tumor without any evidence of metastatic disease. These data indicate that carcinoids involving the ampulla of Vater metastasize in approximately half of the cases regardless of tumor size.

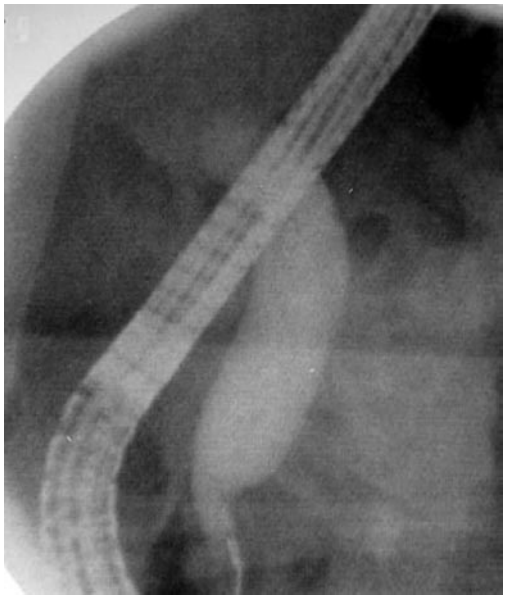

**Figure 2** ERCP showing a markedly dilated CBD with an abrupt "shoulder" in the region of the ampulla, suggestive of a periampullary mass.

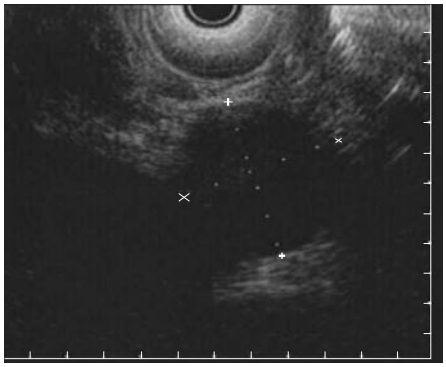

**Figure 3** EUS identifying a 23 mm x 27 mm well circumscribed, round, hypoechoic mass in the region of the ampulla, which is distinct from the duodenal wall.

This is opposed to the classic teaching regarding midgut and hindgut carcinoids, in which the incidence of metastasis is felt to be a function of tumor size and is significantly higher with larger tumors.

Accordingly, the size of ampullary carcinoids cannot predict node-positive status, and therefore cannot determine the extent of operation. Should every patient with this tumor undergo a pancreaticoduodenectomy? The small number of cases and reported follow-up in the literature are not sufficient to answer this question definitively. Instead, each case should be individualized realizing that the goal is complete tumor removal. Given the propensity of ampullary carcinoids smaller than 2 cm to show nodal involvement, and considering the safety of pancreaticoduodenectomy in experienced hands, radical excision should be the treatment of choice to completely extirpate the tumor-bearing tissue. It should be observed that in multiple reported cases, long-term survival has been achieved by local excision of the ampulla[2]. These recommendations, however, were made during a period of surgical history when the operative mortality rate for pancreaticoduodenectomy was high, and therefore are not applied currently.

In cases of liver metastases, surgical resection or other cytoreductive techniques, such as radiofrequency ablation and chemoembolization, have been shown to improve hormone-mediated symptoms, quality of life and survival in certain groups of patients[11]. Patients with slowly growing carcinoid tumors do not generally benefit from cytotoxic chemotherapy. Somatostatin analogues can induce a symptomatic and biochemical response, but more recent studies have also indicated a cytostatic effect[11]. Tumor-targeted radioactive treatment with $^{90}$yttrium and $^{177}$lutetium coupled to a somatostatin analogue is currently under clinical evaluation[12]. Preliminary data indicate interesting clinical potentials.

In summary, carcinoids of the ampulla of Vater are rare tumors. They can cause symptoms mainly secondary to their periampullary location. Up to 25% of patients have von Recklinghausen's disease. Carcinoid syndrome is uncommon, unless hepatic metastasis is present. Chromogranin A is an important tumor marker. Determination of histopathology is of utmost importance and involves specific immunohistochemical staining. Aggressive operative extirpation is the cornerstone of treatment and provides the only chance for cure. Biological treatment with somatostatin analogues can be applied in symptomatic patients with slowly growing neoplasms. Tumor-targeted radiotherapy has been introduced recently with promising results. Future therapy will be based on specific tumor biology and treatment will be customized for each individual patient.

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*CASE REPORT*

# Retroperitoneal fibrosis: A rare cause of both ureteral and small bowel obstruction

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## Abstract

Retroperitoneal fibrosis (RPF) is a rare condition of unclear etiology. It can cause ureteral obstruction. We present the unique case of a 54 years old female, who initially presented with spontaneous perforation of the cecum. Upon exploring the abdomen, the classical glistening white, unyielding retroperitoneal fibrosis was encountered. A right hemicolectomy was performed. Subsequently, the patient presented with bilateral ureteral obstruction, and later on with small bowel obstruction. Ureteral obstruction was treated with stents, and small bowel obstruction was treated with bypass. To our knowledge no case of idiopathic RPF presenting with features of both bilateral ureteral and small bowel obstruction has been reported in the literature.



**Key words:** Retroperitoneal fibrosis; Ureteral obstruction; Small bowel obstruction; Surgery for retroperitoneal fibrosis

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## INTRODUCTION

Retroperitoneal fibrosis (RPF) is a rare condition of unclear etiology. It is believed to be immune-related. About two-thirds of the cases are thought to be idiopathic. We present a case of idiopathic RPF in a 54 years old female who developed bilateral hydronephrosis and then small bowel obstruction due to extrinsic compression from retroperitoneal fibrosis.

To our knowledge no case of idiopathic RPF presenting with features of both bilateral ureteral and small bowel obstruction has been reported in the literature. We recommend early surgical intervention in patients with advanced RPF who are unresponsive to steroids.

## CASE REPORT

A fifty four years old African-American female came to the emergency room with a five-day history of abdominal pain. She described it to be sharp in nature and localized mostly in the middle of the abdomen. She graded pain to be seven on a scale of zero to ten. The pain had no radiation or shifting. The patient also complained of nausea and three episodes of vomiting which was bilious in nature. The patient passed her last bowel movement three days ago and it was normal. She denied fever or shaking chills. Her past medical history was significant for hypertension, type Ⅱ diabetes mellitus and asthma.

Her past surgical history was significant for laparoscopic cholecystectomy done 6 years ago. On physical examination, she was afebrile and her vital signs were stable. She was awake and alert. She had normal heart sounds, and bilateral breath sounds. Her abdomen was soft, and had generalized and reboud tenderness. Her white blood cell count was 18000 cells/mm$^3$; the hemoglobin was 14 mg/dL and platelet count was 253 cells/mm$^3$. Her serum chemistry and coagulation tests were normal. Her flat and upright abdominal radiographs were normal. CT of the abdomen and pelvis showed extravasation of oral contrast and inflammation around the ascending colon (Figure 1). She was brought to the operating room and an exploratory laparotomy was performed via a midline incision. On entering the peritoneal cavity, dense intra-abdominal adhesions were encountered at the hepatic flexure. The classical glistening white, unyielding retroperitoneal fibrosis was encountered. The plaque predominantly encased both kidneys. It extended over the renal pelvis and upper ureters on both sides up to the level of aortic bifurcation and encased the great vessels in the midline. A 1 cm × 2 cm perforation was found in the cecum. A right hemicolectomy was performed. Pathology report showed fibrinous adhesions and inflammatory reaction around the site of perforation. No evidence of any tumor was seen. Patient's post-operative course was uneventful. She started a clear liquid diet on post-operative d 2, and was gradually advanced to a regular diet. The

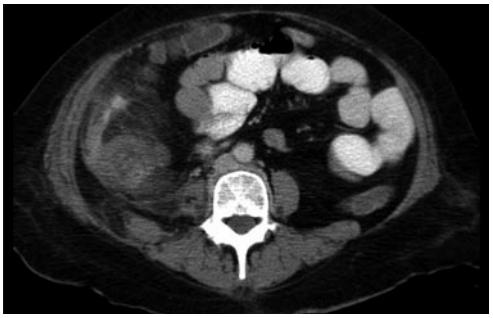

**Figure 1** CT of Abdomen showing inflammation around right colon and extravasation of oral contrast.

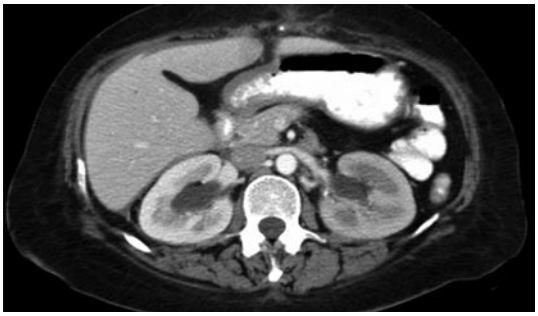

**Figure 2** CT of abdomen showing bilateral hydronephrosis.

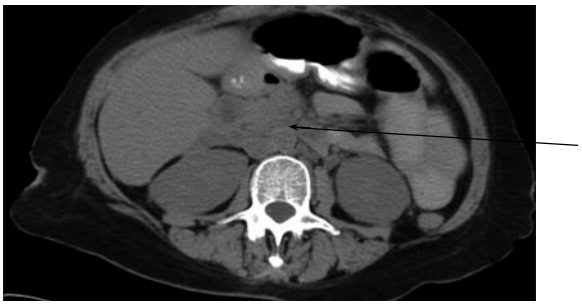

**Figure 3** CT of abdomen showing retroperitoneal fibrosis, encasing aorta and inferior vena cava.

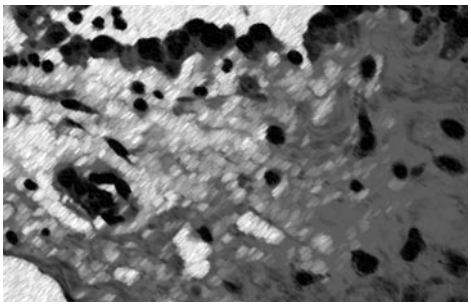

**Figure 4** Hematoxylin and Eosin Staining showing fibrinous adhesions and inflammatory reaction.

patient was diagnosed with retroperitoneal fibrosis and, hence, received oral steroids. The patient was discharged home on post-operative d 6. She was examined in clinic 4 wk after surgery and reported no complaints.

Three months after surgery, the patient presented to the emergency room with complaints of severe back pain. Her examination was normal. Her laboratory tests were normal. CT scan of the abdomen showed marked hydronephrosis of the right kidney. She underwent cystoscopy which showed narrowing of the right proximal ureter. A stent was placed in the right ureter. The stent was removed 3 wk after placement.

She presented to emergency room after 9 mo with complaints of back pain. Her physical examination and laboratory work was normal. CT of abdomen and pelvis showed bilateral hydronephrosis (Figure 2). A cystoscopy was performed which showed a normal bladder. Retrograde pyelogram showed normal ureters until the proximal part of both ureters, where tortuosity and dilation of the renal pelvis on both sides was noted. Bilateral ureteral stents were placed.

Eight months after bilateral stent placement, she presented to the hospital with complaints of severe abdominal pain. The pain was localized to the middle of the abdomen. It was sharp in intensity and had no radiation and no shifting. The pain was associated with repeated bouts of vomiting. On physical examination, she was afebrile and her vital signs were stable. She was awake and alert. Her abdomen was soft and not distended. She had tenderness in the right upper quadrant. Her white blood cell count was 34000 cells/$mm^3$, and the hemoglobin was 13 mg/dL. All other laboratory values were in the normal range. Abdominal radiographs showed multiple air fluid levels. CT of her abdomen showed multiple dilated loops of the small bowel and thickening of the bowel wall. Transition point appeared to be somewhere between the jejunum and the ileum. The colon was normal (Figure 3). An exploratory laparotomy was performed through midline incision. On opening the abdomen, the findings were that of severe fibrotic reaction with almost a frozen abdomen on the right lower aspect. Once again, classical glistening white, unyielding retroperitoneal fibrosis was encountered. Careful lysis of adhesions was done and the left upper quadrant was explored, whereby dilated loops of small bowel were found. Lysis of adhesions was attempted in the right lower quadrant but was encountered with enormous amount of bleeding. It was felt that the obstruction was caused by the external compression from retroperitoneal fibrosis. A decision was made to bypass the dilated segment of the jejunum into the colon without interfering with the frozen abdomen on the right side. The ligament of Treitz was identified and the dilated small segment was seen up to about 200 cm of the jejunum. The last portion of jejunum which entered into the frozen part of the abdomen was identified. A 10 cm long segment was anastomosed with the transverse colon which was free of adhesions. Side-to-side anastomosis was done using a GIA-75 and a TA60. Irrigation of the abdomen was carried out. The abdominal cavity was closed. The postoperative course was uneventful. On postoperative d 2, the patient's nasogastric tube was discontinued. A clear liquid diet was started on postoperative d 3 and was gradually replaced with regular diet. The patient was discharged home on post operative d 9. Pathology report showed fibrinous adhesions and inflammatory reaction (Figure 4).

## DISCUSSION

Classical idiopathic retroperitoneal fibrosis, also known as Ormond's disease, was described in 1948[1]. The typical retroperitoneal fibrosis is a fibrotic process encasing the abdominal vessels and the ureter with an epicenter at the level of L4-5[2]. RPF has an inciting agent in one-third of the cases, while the remaining two-thirds have no known cause[2].

CT scan of the abdomen is the imaging investigation of choice. Cases with ureteric obstruction can be successfully treated with steroids alone[3], and occasionally with tamoxifen[4], but ureterolysis may still be necessary in advanced or unresponsive cases. This case is unusual because the patient did not respond to oral steroids and had symptomatic ureteral and small bowel obstruction due to retroperitoneal fibrosis. We recommend early surgical intervention in patients with advanced RPF who are unresponsive to steroids.

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# Meetings

## MAJOR MEETINGS COMING UP

First Biennial Congress of the Asian-Pacific Hepato-Pancreato-Biliary Association
March, 2007
Fukuoka, Japan
http://www.congre.co.jp/1st-aphpba

American College of Gastroenterology
Annual Scientific
20-25 October 2006
Las Vegas, NV

14th United European Gastroenterology Week, UEGW
21-25 October 2006
Berlin, Germany

APDW 2006: Asian Pacific Digestive Week 2006
26-29 November 2006
Lahug Cebu City, Philippines

## EVENTS AND MEETINGS IN THE UPCOMING 6 MONTHS

Falk Symposium 151: Emerging Issues in Inflammatory Bowel Diseases
24-25 March 2006
Sydney - NSW
Falk Foundation e.V.
symposia@falkfoundation.de

10th International Congress of Obesity
3-8 September 2006
Sydney
Event Planners Australia
enquiries@ico2006.com
www.ico2006.com

Easl 2006 - the 41st annual
26-30 April 2006
Vienna, Austria
Kenes International

Prague hepatology 2006
14-16 September 2006
Prague
Foundation of the Czech Society of Hepatology
veronika.revicka@congressprague.cz
www.czech-hepatology.cz/phm2006

12th International Symposium on Viral Hepatitis and Liver Disease
1-5 July 2006
Paris
MCI France
isvhld2006@mci-group.com
www.isvhld2006.com

Falk Symposium 152: Intestinal Disease Part I, Endoscopy 2006 - Update and Live Demonstration
4-5 May 2006
Berlin
Falk Foundation e.V.
symposia@falkfoundation.de

Falk Symposium 153: Intestinal Disease Part II, Immunoregulation in Inflammatory Bowel Disease - Current Understanding and Innovation
6-7 May 2006
Berlin
Falk Foundation e.V.
symposia@falkfoundation.de

ILTS 12th Annual International Congress
3-6 May 2006
Milan
ILTS
www.ilts.org

Internal Medicine: Gastroenterology
22 July 2006-1 August 2006
Amsterdam
Continuing Education Inc
jbarnhart@continuingeducation.net

6th Annual Gastroenterology And Hepatology
15-18 March 2006
Rio Grande
Office of Continuing Medical Education
cmenet@jhmi.edu
www.hopkinscme.net

World Congress on Gastrointestinal Cancer
28 June 2006-1 July 2006
Barcelona, Spain
c.chase@imedex.com

International Conference on Surgical Infections, ICSI2006
6-8 September 2006
Stockholm
European Society of Clinical Microbiology and Infectious Diseases
icsi2006@stocon.se
www.icsi2006.se/9/23312.asp

7th World Congress of the International Hepato-Pancreato-Biliary Association
3-7 September 2006
Edinburgh
Edinburgh Convention Bureau
convention@edinburgh.org
www.edinburgh.org/conference

Society of American Gastrointestinal Endoscopic Surgeons
26-29 April 2006
Dallas - TX
www.sages.org

Digestive Disease Week 2006
20-25 May 2006
Los Angeles
www.ddw.org

Annual Postgraduate Course
25-26 May 2006
Los Angeles, CA
American Society of Gastrointestinal Endoscopy
www.asge.org/education

American Society of Colon and Rectal Surgeons
3-7 June 2006
Seatlle - Washington
www.fascrs.org

## EVENTS AND MEETINGS IN 2006

10th World Congress of the International Society for Diseases of the Esophagus
22-25 February 2006
Adelaide
isde@sapmea.asn.au
www.isde.net

Falk Symposium 151: Emerging Issues in Inflammatory Bowel Diseases
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Easl 2006 - the 41st annual
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Vienna, Austria
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International Gastrointestinal Fellows Initiative
22-24 February 2006
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www.cag-acg.org

Canadian Digestive Disease Week
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Falk Seminar: XI Gastroenterology Seminar Week
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European Multidisciplinary Colorectal Cancer Congress 2006
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Falk Symposium 152: Intestinal Disease Part I, Endoscopy 2006 - Update and Live Demonstration
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6-7 May 2006
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United European Gastroenterology Federation
www.uegw2006.de

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Asia Pacific Obesity Conclave
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Hepatitis 2006
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mangosee.com/mangosteen/hepatitis2006/hepatitis2006.htm

World Congress on Gastrointestinal Cancer
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International Conference on Surgical Infections, ICSI2006
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5th International Congress of The African Middle East Association of Gastroenterology
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71st ACG Annual Scientific and Postgraduate Course
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27-31 October 2006
Boston, MA
AASLD

New York Society for Gastrointestinal Endoscopy
13-16 December 2006
New York
www.nysge.org

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*EDITORIAL*

# Biofeedback therapy for dyssynergic defecation

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## Abstract

Dyssynergic defecation is one of the most common forms of functional constipation both in children and adults; it is defined by incomplete evacuation of fecal material from the rectum due to paradoxical contraction or failure to relax pelvic floor muscles when straining to defecate. This is believed to be a behavioral disorder because there are no associated morphological or neurological abnormalities, and consequently biofeedback training has been recommended for treatment. Biofeedback involves the use of pressure measurements or averaged electromyographic activity within the anal canal to teach patients how to relax pelvic floor muscles when straining to defecate. This is often combined with teaching the patient more appropriate techniques for straining (increasing intra-abdominal pressure) and having the patient practice defecating a water filled balloon. In adults, randomized controlled trials show that this form of biofeedback is more effective than laxatives, general muscle relaxation exercises (described as sham biofeedback), and drugs to relax skeletal muscles. Moreover, its effectiveness is specific to patients who have dyssynergic defecation and not slow transit constipation. However, in children, no clear superiority for biofeedback compared to laxatives has been demonstrated. Based on three randomized controlled studies in the last two years, biofeedback appears to be the preferred treatment for dyssynergic defecation in adults.



**Key words**: Biofeedback; Constipation; Pelvic floor dyssynergia; Dyssynergic defecation; Functional defecation disorders; Randomized controlled trials

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## INTRODUCTION

Chronic constipation is a common self-reported bowel symptom that affects 2%-30% of people in Western countries and has considerable impact on health expenses and quality of life[1]. Most patients respond either to fiber-fluid supplementation or to judicious use of laxatives[1]. Among the non-responders, outlet dysfunction type constipation seems particularly common; it affects up to 50% of referrals to a tertiary care center[2].

Patients with outlet dysfunction can be divided into those with structural causes for obstructed defecation and patients with a functional defecation disorders[3,4]. Possible structural causes for obstructed defecation include stricture, neoplasia, rectocele, enterocele, and Hirschprung's disease. Functional defecation disorders include dyssynergic defecation (i.e., paradoxical contraction or failure to relax the pelvic floor and anal muscles during defecation) and inadequate defecatory propulsion (i.e., insufficient intra-rectal pressure due to inadequate contraction of abdominal wall muscles during defecation); both may lead to inadequate emptying of the rectum[4]. It is unclear whether idiopathic megarectum is associated with dyssynergic defecation. Functional defecation disorders are believed to be more common than obstructed defecation and approximately as common as slow transit constipation; however, the true prevalence of these subtypes of constipation has not been documented. Functional defecation disorders may coexist with slowed transit through the colon. Dyssynergic defecation is commonly considered to be a form of maladaptive behavior because there is no discernable neurological or anatomical defect and because it can be eliminated by behavioral training[4].

Diagnostic criteria for functional defecation disorders[4] include those for functional constipation[5], namely two or more of 6 symptoms present for the last 3 mo with an onset more than 6 mo in the past; the symptoms are straining, lumpy or hard stools, sensation of incomplete evacuation, sensation of anorectal obstruction/blockage, or manual maneuvers to facilitate defecation on more than 1/4 of bowel movements, or less than 3 bowel movements per week. To meet criteria for functional defecation disorders, the patient must also undergo

objective diagnostic testing and demonstrate at least two of three abnormalities: impaired evacuation of the rectum, inappropriate contraction or less than 20% relaxation of the pelvic floor muscles, and inadequate propulsive forces during defecation[4].

An exhaustive explanation of the diagnostic work up of these patients is beyond the scope of this review. However, most normal subjects can easily evacuate a 50 mL water-filled balloon from the rectum. Additional anorectal testing includes anorectal manometry, anal electromyography (EMG) and evacuation proctography (defecography)[4]. Anorectal manometry provides a comprehensive assessment of anal pressures, rectoanal reflexes, rectal pressures, sensation and compliance. Several types of recording devices are available, but perfused catheters and balloon probes are among the most commonly used. A paradoxical increment in anal pressure on straining efforts is a distinctive feature of dyssynergic defecation[4]. An increment in muscle motor activity on straining may be demonstrated by means of EMG either by intra-anal electrodes or by electrodes taped to the peri-anal skin.

Defecography is a radiographic test providing morphological and functional information on the ano-rectum. Several parameters may be assessed, such as pelvic floor descent, anorectal angle, rectocele, and rectal prolapse. Failure of the anorectal angle to become more oblique on straining provides indirect evidence of defective pelvic floor relaxation and impaired evacuation of contrast material is also suggestive of dyssynergia[4]. There must be manometric, EMG, or radiologic evidence for inappropriate contraction or failure to relax the pelvic floor muscles on straining to diagnose dyssynergic defecation according to Rome Ⅲ criteria[4].

Evaluation of colon transit by means of radiopaque markers is not relevant to the diagnosis, but it may be performed to additionally test for slow transit constipation.

Patients with functional defecation disorders are often unresponsive to conservative medical management, and the surgical division of the pubo-rectalis muscle (which has been proposed for the treatment of dyssynergic defecation) has resulted in poor benefit and an unacceptable risk of anal incontinence[1,6]. Treatment with botulinum toxin injection may provide temporary improvement, but it remains an investigational treatment. Therefore, behavioral treatment is a logical choice for these disabled patients[4].

Biofeedback is a conditioning treatment where information about a physiologic process (contraction and relaxation of a muscle) is converted to a simple visual or auditory signal to enable the patient to learn to control the disordered function. Biofeedback is considered appropriate when specific pathophysiological mechanisms are known and the voluntary control of responses can be learned with the aid of systematic information about functions not usually monitored at a conscious level[7]. As early as 1979, Thomas Almy and John Corson, in an enthusiastic editorial about the biofeedback treatment of fecal incontinence, pioneered the extension of behavior therapy to functional defecation disorders[8]. However, the first paper dealing with the subject included only a small number of subjects and was not published until 1987 due to the preference for conservative, drug-oriented therapy[9].

## BIOFEEDBACK TECHNIQUES FOR TREATING DYSSYNERGIC DEFECATION

Paradoxical increases in anal pressure and electromyographic (EMG) activity during straining is easily detected[10,11]. Anal pressure may be measured by means of water-perfused catheters, solid state transducers or balloon catheters[10]. No single technique seems superior to the others, and the choice relies on the researcher's training and experience.

Anal EMG may be recorded either by intra-anal probes or by peri-anal EMG electrodes stuck to the skin[10,11]. The EMG activity used in biofeedback training is the averaged activity of large numbers of muscle cells rather than the activity of small groups of muscle cells innervated by a single axon. This averaged EMG activity is recorded with large electrodes on the skin or the mucosa of the anal canal rather than with needle electrodes. Averaged EMG recorded in this way is proportional to the strength of contraction of the underlying muscles.

Defective expulsion is commonly investigated by asking the patient to defecate a 50-mL water-filled rectal balloon; patients with functional defecation disorders usually fail this test[11]. Some patients also have a higher threshold for perceiving the urge to defecate[10], but the clinical significance of this sensory dysfunction is ill-defined, in contrast to the relevance of rectal sensory impairment in fecal incontinence[12]. Ano-rectal imaging studies (defecography, ultrasonography, and pelvic floor MRI) may also help to characterize the physiological dysfunction responsible for outlet dysfunction, but they do not seem to influence treatment outcome[4,13].

Biofeedback training protocols vary among different centers[10,11]. In the next paragraph, a standard biofeedback protocol is described and differences in biofeedback procedures are outlined. A mainstay of behavior therapy is to first explain the anorectal dysfunction and discuss its relevance with the patient before approaching the treatment[3,11]. Most protocols would then include training the patients on a more effective use of the abdominal muscles to improve pushing effort. Patients are next shown anal manometry or EMG recordings displaying their anal function and are taught through trial and error to relax the pelvic floor and anal muscles during straining[10,13]. This objective is first pursued with the help of visual feedback on pelvic floor muscle contraction, accompanied by continuous encouragement from the therapist. When the patient has learned to relax the pelvic floor muscles during straining, the visual and auditory help are gradually withdrawn[10,13]. Another retraining option is to simulate defecation by means of an air-filled balloon attached to a catheter, which is slowly withdrawn from the rectum while the patient concentrates on the evoked sensation and tries to facilitate its passage[3,11]. In the next phase of training, the patient is taught to defecate the balloon by bearing down, without the assistance of the therapist. Some centers also add a balloon sensory retraining to lower the urge perception threshold[14]. The number of

training sessions is not standardized, but 4 to 6 sessions are frequently provided. Individual training sessions last 30 to 60 min.

Therapeutic sessions are professionally demanding and a highly trained and motivated therapist is essential. No study has addressed the necessary training required for an individual to administer biofeedback therapy. Particularly, it is unclear whether the adequate provider should be either physician, psychologist, or nurse. Experience varies among centers, but the low cost reimbursement provided for behavior therapy is likely to influence future choices.

Controlled studies systematically comparing different biofeedback protocols to each other are lacking. However, a recent meta-analysis showed that in open label studies, the mean success rate with pressure biofeedback was slightly greater than with EMG biofeedback (78% *vs* 70%)[13]. No differences were found between anal *vs* peri-anal EMG recording. In addition, adding balloon feedback did not seem to influence the therapeutic outcome[13]. However, the majority of studies in the last ten years have utilized EMG biofeedback rather than pressure feedback even in the absence of scientific evidence[13]. There are no standardized protocols, and centers use different combinations of laboratory EMG training, home EMG training, and balloon feedback, depending on the researcher's experience.

## EFFICACY OF BIOFEEDBACK TREATMENT FOR DYSSYNERGIC DEFECATION

### *Uncontrolled studies*

In 1987 Bleijenberg and Kuijpers[9] were the first to report the efficacy of EMG biofeedback treatment combined with oatmeal porridge defecation in 10 patients affected by spastic pelvic floor syndrome, later redefined as functional defecation disorder[4]. Treatment was a complete success in 7 patients and a partial success in two others. This open label trial stimulated a number of uncontrolled studies to investigate the efficacy of behavior therapy in functional defecation disorder[7]. Therapeutic outcome varied greatly among centers with success rates ranging from 18% to 100% of patients studied[15,16].

A major drawback to assessing this literature was the huge variance in inclusion criteria, outcome criteria, follow-up intervals, and therapeutic protocols[13,17]. Additional limitations were small sample size (often no more than 30 subjects studied) and lack of any control group[17]. However, the majority of uncontrolled studies in adults reported a favorable outcome in about two thirds of patients, without side effects[7,11,13,17]. Coexisting morphological abnormalities of the pelvic floor, namely rectocele, intussusception and abnormal perineal descent, seemed not to influence behavior treatment outcomes[18]. Researchers were unable to identify any functional variable that could predict treatment outcome, but anxious patients appear to be less likely to succeed[13,17].

Although the majority of published uncontrolled studies reported beneficial effects of treatment, a series of studies from the St. Mark's group cast doubts on whether biofeedback training has specific value in the treatment of functional defecation disorder: these investigators reported similar benefits of biofeedback therapy in patients, irrespective of whether they had slow whole gut transit or functional defecation disorder[19,20] (See below for contrasting views). They also suggested that the autonomic innervation of the colon may influence the outcome of biofeedback treatment[21].

### *Controlled studies*

Randomized, controlled trials were first performed in the pediatric population. In 1987 Wald *et al*[22] compared pressure biofeedback therapy with mineral oil in a group of 55 encopretic children; 16 of whom showed evidence of functional defecation disorder. Although a trend toward greater improvement in the biofeedback group was evident, the difference in success rate did not reach significance. In another controlled study[23], a well-defined pediatric population of 43 children with functional defecation disorder was randomized to receive either biofeedback therapy plus conventional care (laxatives) or conventional treatment only. All children had fecal impaction and encopresis. The biofeedback group did significantly better than the conventional one, with about half of patients showing successful symptoms resolution at one year follow-up compared to 16% in the conventional-care-only group. The clinical benefit was correlated with normalization of defecation dynamics. Similar benefits were reported in another controlled study[24] in the pediatric population, but the follow-up was too short (3 mo) to draw firm conclusions.

In contrast to the successful studies described above, the largest randomized, controlled study in pediatric constipation (192 children), which compared laxatives plus EMG biofeedback therapy to laxatives alone, failed to show any benefit from biofeedback[25]. A criticism of this study was that not all the subjects had functional defecation disorder. However, a similar failure to show superior efficacy with biofeedback was reported in a controlled study considering a smaller sample of children with both functional defecation disorder and encopresis[26]. In both studies, improved defecation dynamics were reported in biofeedback-treated patients, but this did not translate into greater symptom improvement.

In the adult population, four controlled studies were published prior to 2005. Two of these studies compared different biofeedback techniques to each other[27,28] and two studies compared EMG biofeedback to simulated defecation[19,29].

Heymen *et al*[27] compared intra-anal EMG biofeedback to (1) a combination of EMG and intra-rectal balloon distension training, (2) EMG and home trainers, and (3) a combination of all three techniques. All groups showed significant improvement from pretreatment, but no significant differences were found among treatment strategies. Glia *et al*[28] found peri-anal EMG biofeedback to be superior to pressure biofeedback combined with balloon defecation training. However, neither Glia *et al* nor Heymen *et al* had sufficient sample size to provide a meaningful analysis.

Bleijenberg *et al*[29] found an intra-anal EMG biofeedback to be superior to balloon defecation training (90% *vs*

60% improved). Although the sample size was too small to draw reliable conclusions, subjects who failed balloon defecation training were then given biofeedback training, yielding an 80% success rate.

The St. Marks group[19] studied a series of 60 adults with functional constipation unresponsive to conservative management and randomized them either to EMG and rectal balloon biofeedback or to abdomino-pelvic muscular coordination training and balloon feedback. After only two unsatisfactory sessions, patients who were judged unable to respond, were switched to the alternative treatment. At the end of treatment, approximately 50% of patients in both groups rated their symptoms as significantly improved. The outcome did not correlate with colon transit time, the presence of functional defecation disorder, or other functional and clinical variables. In addition, the St. Marks group recently reported biofeedback to be no more effective than bowel training and education for fecal incontinence in a large, controlled, randomized study[30]. These results challenge both the effectiveness of behavior therapy and the claim that retraining makes a specific contribution to the treatment of constipation other than education and/or psychotherapy.

To determine whether biofeedback is equally effective in slow transit constipation and dyssynergic defecation and also whether the benefits are due to education alone, we conducted an open study on 52 patients with slow transit constipation (objectively documented) who were unresponsive to conservative measurements[3]. Thirty-two of them showed evidence of dyssynergic defecation, 6 formed a mixed group who satisfied some but all criteria for dyssynergic defecation, and 12 had slow transit only. All patients received 5 weekly sessions of a biofeedback protocol, including improved use of the abdominal muscles to strain, anal EMG and balloon biofeedback to teach relaxation of the pelvic floor on straining, and simulated defecation.

Functional ano-rectal and clinical parameters were evaluated both before and after behavior therapy. After six months, 71% of patients with functional defecation disorder and slow transit reported satisfaction with treatment *versus* 8% in the slow transit only group. The results were well maintained at follow-up 2 years later[3]. Patients' satisfaction was correlated with improved rectal emptying as demonstrated by successful balloon expulsion and reductions in dyssynergia at manometry. A significant increase in rectal pressure on straining was also evident. Interestingly, biofeedback training resulted in a significant decrement in the threshold volume of balloon distention required to produce a sensation of urge to defecate, although no specific sensory retraining had been provided. Treatment success was predicted by pelvic floor dyssynergia, milder constipation, and less frequent abdominal pain at baseline.

This study allowed us to conclude that biofeedback therapy is specifically indicated for dyssynergic defecation and that retraining works through teaching patients to relax the pelvic floor and anal muscles during straining. Since biofeedback therapy is time- consuming, dedicated trained personnel are not easily found, and drug treatments (laxatives, muscle relaxants) are cheaper and more easily available, we were in strong need of a randomized, controlled study to prove that biofeedback is more effective than laxatives or placebo. This need was reinforced by the recent statement of the American College of Gastroenterology's Chronic Constipation Task Force that osmotic laxatives, namely polyethylene glycol and lactulose, are effective in improving stool frequency and consistency in all patients with chronic constipation[31].

Recently, three randomized, controlled studies coming from different centers have provided satisfactory answers to this question. The first of them[32] compared 5 weekly sessions of biofeedback to a commonly prescribed osmotic laxative (polyethylene glycol [PEG] in incremental dosage (14.6-29.2 g/d) given in combination with 5 weekly counseling sessions. Patients with normal transit constipation secondary to dyssynergic defecation were randomized either to the biofeedback (54 patients) or to the laxative group (55 patients). Follow-up assessment extended up to 12 mo in the laxative group and to 24 mo in the biofeedback group. Satisfaction with treatment, symptoms of constipation, and pelvic floor physiology were assessed at pretreatment, every six months in the first year, and at 24 mo. At six months, major clinical improvement was reported by 80% of patients in the behavior group *versus* only 20% in the PEG group. Biofeedback benefits were well sustained for the whole two-year follow-up interval. Clinical benefits correlated well with objective evidence of a reduction or elimination of paradoxical contractions of the pelvic floor during straining. The only clinical variable that correlated with treatment outcome was digital facilitation of defecation, which predicted failure; anorectal physiology could not predict outcome. Interestingly, laxatives consumption other than PEG was significantly decreased in the biofeedback group compared to the PEG group at 6-12 mo follow-up, while bowel frequency was significantly increased in both group compared to baseline.

Rao *et al*[33] conducted a randomized trial comparing biofeedback to sham feedback (relaxation therapy) and to standard medical care (diet, exercise, and laxatives). A significantly greater proportion of subjects receiving biofeedback (88%) reported more than a 20% improvement in global satisfaction and stool frequency on a visual analog scale compared to subjects receiving sham biofeedback (48%), but not when compared to standard care (70%). The authors also reported a significant improvement in favor of the biofeedback group to normalize the dyssynergic pattern and improve on a defecation index, with trends in favor of biofeedback subjects reducing balloon expulsion time and decreasing colonic marker retention compared to alternative treatment groups.

In a third randomized controlled trial, Heymen *et al*[34] randomly assigned 84 constipated subjects with dyssynergic defecation to receive either biofeedback ($n$ = 30), diazepam ($n$ = 30), or placebo ($n$ = 24). An important feature of this study was that all subjects were trained to do pelvic floor muscle exercises to correct pelvic floor dyssynergia during 6 biweekly 1-h sessions, but only the

biofeedback patients received EMG feedback. All other patients received pills (muscle relaxant or placebo) 1-2 h before attempting defecation. Biofeedback was superior to diazepam by intention-to-treat analysis (70% *vs* 23% reported adequate relief of constipation), and also superior to placebo (38% successful). In addition, biofeedback patients had significantly more unassisted BMs compared to placebo, with a trend favoring biofeedback over diazepam. Biofeedback patients also reduced pelvic floor EMG during straining significantly more than diazepam patients.

### *Limitations of biofeedback training*

The negative outcomes reported in controlled studies have been in the pediatric population. These poorer outcomes may be due to the inclusion of children whose constipation was not due to functional defecation disorder, since it is known that patients with other etiologies for their constipation respond poorly to biofeedback. In addition, biofeedback training requires complex cognitive processing and sustained attention that may be beyond the abilities of younger children. Finally, the quality of the therapist-patient relationship and the skills and experience of the therapist seem to influence the success of behavior therapy, and there is currently a shortage of trained personnel to provide this form of treatment.

In conclusion, a series of controlled studies have now shown that functional defecation disorder, one of the most frequent and disabling subtypes of adult constipation, can be treated effectively with biofeedback training. This form of treatment is more effective than laxatives, and it has no known adverse effects. Although this training is relatively expensive to provide, it produces improvements that are sustained for up to two years. For these reasons, we may conclude that biofeedback training is the treatment of choice for functional defecation disorder.

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*EDITORIAL*

# Insulin resistance and hepatitis C

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## Abstract

Insulin resistance is the major feature of the metabolic syndrome and depends on insulin secretion and insulin sensitivity. In chronic hepatitis C, insulin resistance and type 2 diabetes mellitus are more often seen than in healthy controls or chronic hepatitis B patients. Hepatitis C virus (HCV) infection promotes insulin resistance, mainly by increased TNF production together with enhancement of suppressor of cytokine (SOC-3); both events block PI3K and Akt phosphorylation. Two types of insulin resistance could be found in chronic hepatitis C patients: "viral" and "metabolic" insulin resistance. Insulin resistance in chronic hepatitis C is relevant because it promotes steatosis and fibrosis. The mechanisms by which insulin resistance promotes fibrosis progression include: (1) steatosis, (2) hyperleptinemia, (3) increased TNF production, (4) impaired expression of PPARγ receptors. Lastly, insulin resistance has been found as a common denominator in patients difficult-to-treat like cirrhotics, overweight, HIV coinfected and Afro-American. Insulin resistance together with fibrosis and genotype has been found to be independently associated with impaired response rate to peginterferon plus ribavirin. Indeed, in genotype 1, the sustained response rate was twice (60%) in patients with HOMA $\leqslant$ 2 than patients with HOMA > 2. In experiments carried out on Huh-7 cells transfected by full length HCVRNA, interferon alpha blocks HCV replication. However, when insulin (at doses of 128 μU/mL, similar that seen in the hyperinsulinemic state) was added to interferon, the ability to block HCV replication disappeared, and the PKR synthesis was abolished. In summary, hepatitis C promotes insulin resistance and insulin resistance induces interferon resistance, steatosis and fibrosis progression.



**Key words:** Diabetes mellitus; Homeostasis model of assessment; Fibrosis; Steatosis; Sustained response; Peginterferon; Ribavirin

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## INTRODUCTION

Insulin resistance emerges as a very important host factor in patients with chronic hepatitis C, mainly because it has been related to steatosis development, fibrosis progression and non-response to peginterferon plus ribavirin. IR appears as a consequence of the inability of insulin to induce the effect on glucose metabolism and an abnormally large amount of insulin is secreted to achieve a biological response. The hyperinsulinemic state induces several abnormalities in the liver, endothelium, and kidneys, and is the main feature in the metabolic syndrome. Obesity and metabolic syndrome are frequently found in western countries. In Spain, the prevalence of obesity has been found in around 13% and more than half of the population showed increased body mass index. In chronic hepatitis C, overweight and obesity showed a distribution similar than healthy people. Although insulin resistance has been found to be strongly associated with body mass index, in some circumstances it depends on the virus C. Two types of insulin resistance could be defined in patients with chronic hepatitis C: "metabolic" insulin resistance and "viral" insulin resistance.

## MATERIALS AND METHODS

Insulin, after binding with it's receptor, induces the phosphorylation of receptor substrates in the liver and muscles and induces several steps toward the transactivation of GLUT-4 that increases glucose uptake by cells and the storage as glycogen and inhibits the net production of glucose by the liver, blocking glycogenolysis and neoglycogenesis. Moreover, insulin promotes lipids storage inhibiting lipolysis. When insulin is not able to induce glucose uptake, pancreatic beta-cells increase insulin production and the hyperinsulinemic state avoids hyperglycemia. Insulin secretion increases when insulin sensitivity decreases until a threshold in which insulin secretion did not induce improvement in insulin sensitivity, and diabetic state emerges. Several factors have been proposed as mediators in the islet adaptation: glucose, glucagon-like peptide 1, autonomic nervous system, and

free fatty acids[1].

The best method to measure insulin sensitivity is the hyperinsulinemic euglycemic clamp. This method measures the quantity of glucose required to maintain normal glycemic levels during insulin infusion. Briefly, a perfusion of 60 mU/kg per minute of insulin was started and every 5 minutes glucose levels were measured. To avoid hypoglycaemia, glucose (20%) was infused to maintain levels between 90 mg/dL and 100 mg/dL. The whole-body glucose uptake (M value) depends on the glucose infused in the last 30 min of the test. Lower M value (lower glucose requirements) are associated with insulin resistance. However, the clamp method is expensive, labor-intensive and uncomfortable for practical use in clinical medicine. A simple mathematical model named Homeostasis Model for Assessment [HOMA = fasting insulin (mUI/mL) * fasting glucose (mmol/L)/22.5] has been proved useful in the measurement of insulin sensitivity in euglycemic patients. However, this measurement is not useful once the blood glucose level begins to increase because it lacks accuracy. Normal plasma insulin concentrations are not standardized in healthy controls. Thus, HOMA has not been standardized yet. In previous reports HOMA < 2 has been considered "completely" normal, and higher than 4 as prediabetic state.

## INSULIN RESISTANCE IN CHRONIC HEPATITIS C

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 been considered for long-time responsible of the appearance of insulin resistance and type 2 diabetes in patients with chronic liver diseases. Taking together available data from several studies about the prevalence of type 2 diabetes mellitus in hepatitis C, we found that in cirrhotic patients ($n$ = 2152 cases) the prevalence of type 2 diabetes was 27.3%, higher than the 17.5% in non-cirrhotic hepatitis C patients ($n$ = 1016 cases). Hyperinsulinemia in liver cirrhosis has been reported to be due to diminished hepatic insulin extraction by liver dysfunction and not to pancreatic hypersecretion. C-peptide and insulin are secreted in equimolar quantities. More than 50% of insulin is degraded in the liver at first pass, whereas C-peptide is degraded in the kidneys[2]. Simultaneous measurements of C-peptide and insulin revealed that both insulin resistance and insulin secretion contribute to glucose intolerance in patients with chronic hepatitis C[3]. In spite of insulin resistance correlated with liver fibrosis, it has been found higher in patients with chronic hepatitis C and mild fibrosis (F0-F1) than in healthy controls matched by age, sex, visceral obesity and body mass index[4]. In a cohort of patients with chronic hepatitis C and chronic hepatitis B matched by age, sex, body mass index and fibrosis stage HOMA index was found statistically higher in hepatitis C[5], this result confirms previous reports showing higher insulin resistance in chronic hepatitis C than in others hepatopathies[6]. Thus, hepatitis C virus induces insulin resistance independently of body mass index or fibrosis stage. Moreover, in a transgenic animal model, HCV core protein has been found able to induce insulin resistance, steatosis and type 2 diabetes mellitus. The main mechanism seems to be the over production of TNF. This cytokine phosphorylates serine residues of insulin-receptor substrates 1 and 2 and enhances the production of suppressor of cytokines (SOC3). The SOC-3 substance inhibits the phosphorylation of Akt and phosphatidyl inositol 3 kinase (PI3K). All these impairments in the intracellular signaling of insulin could block the transactivation of GLUT-4, avoiding the uptake of glucose by cells. Moreover, in transgenic animal models unable to express SOC-3, in spite of core HCV protein expression, insulin resistance does not develop. Indeed, in transgenic mice, TNF correlates with hyperinsulinemic state and the blockade of TNF production by anti-TNF drugs as infliximab also avoid insulin resistance appearance. Therefore, mechanisms inducing insulin resistance by hepatitis C virus include: TNF production, serine phosphorylation of IRS, and over expression of SOCs. Furthermore, over production of TNF in patients with chronic hepatitis C has frequently been seen[7], and correlated with higher fibrosis progression and impaired antiviral response to interferon alpha. Besides, in humans the risk for the development of insulin resistance and further appearance of DM is greater in patients with higher TNF production.

The development of type 2 diabetes mellitus depends on environmental, genetic and diet-related factors. Type 2 diabetes mellitus is more often seen in patients with chronic liver diseases than in general population[8]. Data supporting an association between diabetes mellitus and hepatitis C include: (1) Cross-sectional studies that found increased prevalence of diabetes mellitus in patients with chronic hepatitis C, or higher prevalence of hepatitis C in patients with diabetes mellitus. In a recent meta-analysis taking together available data about the prevalence of DM in patients with chronic liver diseases, the prevalence was higher in chronic hepatitis C than chronic hepatitis B. In non-cirrhotic chronic hepatitis C, DM was found in 17.5% and in chronic hepatitis B in 8.7% while in cirrhotics the prevalence was 27.3% in hepatitis C versus 15.2% in cirrhotics B (Figure 1). Besides, anti-HCV was more often detected in patients with diabetes mellitus (4.9%) than in the general population (0.77%)[9]. (2) case-cohort studies to analyze the development of diabetes mellitus during follow-up in patients with hepatitis C against uninfected patients, including follow-up after orthotopic liver transplantation. In a cross-sectional survey including 9841 persons, Mehta *et al* found that HCV-positive persons who were older than 40 years had an increased risk for type 2 diabetes mellitus higher than 3 times compared with persons without HCV-infection, while no difference was seen in HBV infection[10]. In a cohort of 1084 patients followed-up for 9 years 548 cases developed diabetes mellitus. The presence of hepatitis C was associated with a greater development of diabetes, but solely in high-risk diabetes patients[11]. Besides, in a cohort of 2327 cases, hepatitis C infection increased three times the rate of diabetes mellitus appearance during follow-up in patients aged between 35 and 49 years[12]. Thus, HCV infection could promote type 2 diabetes in high-risk populations.

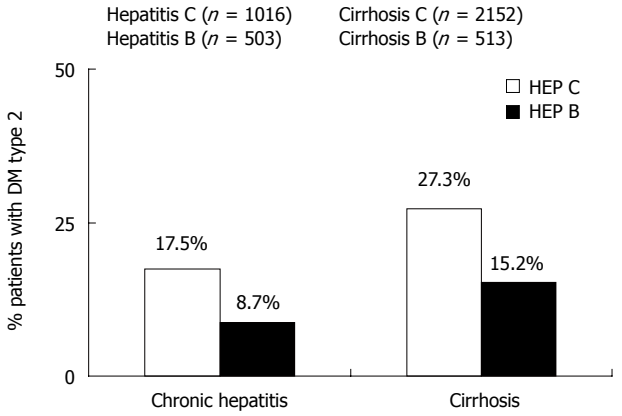


**Figure 1** Prevalence of type 2 diabetes mellitus in patients with chronic hepatitis C and chronic hepatitis B.

Recently, in a Spanish study including 525 chronic hepatitis C patients treated with peginterferon plus ribavirin were followed-up after treatment and the incidence of altered baseline glucose and the appearance of diabetes type 2 was greater in non-responders than in sustained responders even after multivariate analysis including confounding variables as previous diabetes type 2 in relatives, older than 40 years and male sex. Thus, hepatitis C virus clearance induces a decrease in insulin resistance index during a short-time follow-up and decrease the incidence of diabetes type 2 in long-term follow-up[13].

Hepatocyte steatosis is a common feature found in the liver of patients with chronic hepatitis C. The mechanisms implied in steatosis appearance seem to be genotype-dependent. In patients infected by genotype 3, steatosis emerges as a cytopathic effect of the virus while in genotype 1 steatosis seems to be an expression of the metabolic syndrome. In genotype 3, steatosis degree correlated with liver and serum HCV load[14]. In genotype 1, steatosis depends on leptin levels and insulin resistance. Recently, insulin resistance has been found implied in the development of steatosis in 331 non-diabetic genotype 1 patients. Insulin resistance together with gender and gammaglutamyltranspeptidase (a surrogate marker of TNF levels) were independently associated with moderate/severe steatosis in this cohort[15]. Insulin resistance is the main pathogenic factor in the development of steatosis in chronic hepatitis C, both viral insulin resistance and metabolic insulin resistance could be implied in the development of steatosis.

The main deleterious effect of insulin resistance in chronic hepatitis C is the ability to promote fibrosis progression. High serum glucose levels have been found associated with an increased rate of fibrosis progression, greater even than overweight[16]. Mean HOMA index increases with the stage of fibrosis[17] and could help to differentiate stages of fibrosis. Recently, Sud *et al*[18], proposed an index to predict fibrosis containing age, cholesterol, gammaglutamyl transpeptidase and alcohol consumption together with HOMA. The mechanisms by which insulin resistance promotes fibrosis progression include: (a) steatosis, (b) hyperleptinemia, (c) increased TNF production, and (d) impaired expression of PPARγ receptors. (a) hepatocyte steatosis induces fibrosis progression. In a cohort of patients with chronic hepatitis C, the presence of steatosis in the first biopsy was associated with higher fibrosis progression rate, irrespective of genotype[19]. Besides, steatosis is more often seen in patients with advanced fibrosis[20]. (b) Hyperleptinemia has been usually found in patients with insulin resistance, also hepatic stellate cell showed specific leptin receptors, thus leptin could play a role in the activation of HSC and fibrosis progression[21,22]. (c) TNF production is enhanced in hepatitis C and has been implicated in the development of insulin resistance, also, TNF levels were related to fibrosis progression, owing to the ability to activate HSC and promote collagen deposits. Moreover, TNF could inhibit PPARs activity[23]. (d) In patients with hepatitis C an impaired expression of PPARγ receptors has been found[24]. PPARs agonist inhibits inflammation and fibrosis progression by blocking the activation of redox-sensitive transcription factor NFκB and TGFβ1[25].

In HIV-infected patients insulin resistance depends on host, virus and drugs-related factors. Adverse metabolic effects have been found in all antiretroviral drug classes. Protease inhibitors (PI) and nucleoside reverse transcriptase inhibitor (NRTI) therapy induces insulin resistance during treatment[26]. Insulin resistance has been found increased in HIV+/HCV+ co-infected patients. Factors implied in the development of insulin resistance in HIV infected patients include: HCV infection, PI and/or NRTI based-therapy, age, HIV and cytokine dysregulation induced by chronic infection and genetic predisposition[27]. Moreover, the development of type 2 diabetes mellitus was twice in co-infected patients versus HIV non-HCV infected[28]. Duong *et al*[29] reported a higher HOMA in co-infected patients than in HIV-infected cases without hepatitis C. However, in a large study no differences were seen between co-infected or not co-infected patients. In a recent study, including 127 co-infected patients that underwent antiviral therapy (peginterferon plus ribavirin) and 85 hepatitis C, insulin resistance index was higher in co-infected patients, in spite of lower body mass index and lower baseline glucose levels. Besides, in a genotype-dependent manner, insulin resistance was strongly associated with steatosis development and impaired the sustained response rate in genotype 1 co-infected patients. No association between insulin resistance and steatosis or sustained response was seen in genotype 2 or 3 coinfected patients[30]. Lastly, history of diabetes in relatives, increased body mass index and HCV-infection have been found as independent variables associated with diabetes development in HIV patients, supporting the hypothesis that HCV infection promotes diabetes in high-risk patients.

## INSULIN RESISTANCE AND RESPONSE TO PEGINTERFERON PLUS RIBAVIRIN

In healthy volunteers, after the first injection of interferon alpha insulin resistance could be detected[31]. Besides in patients with chronic hepatitis C, interferon alpha induces insulin resistance in the first two weeks, mainly owing to a decrease of hepatic glucose uptake[32]. This effect has been related to the pro-inflammatory repertoire of cytokines induced by interferon. However it's a transitory effect because insulin resistance was not found at month three

of therapy[33] or at the end of therapy[34]. In patients with chronic hepatitis C receiving peginterferon plus ribavirin insulin resistance measured by HOMA, decreased in patient with HCV RNA clearance at mo 6, but not in non-responders. At the end of follow-up sustained responders showed a significantly lower HOMA in comparison with baseline insulin resistance index. However, in relapser patients, the HOMA index increased and the levels at the end of follow-up were not different from the baseline. These data support a connection between HCV replication and insulin resistance, and HOMA decreased when the virus was eradicated. Besides, the incidence of diabetes type 2 is different in cured patients than in non-responders, supporting a better control of insulin resistance after hepatitis C virus clearance[13].

Genotype and viral load have been found as the most important viral factor influencing peginterferon plus ribavirin response. Host factors include genes as HLA, weight, body mass index, hepatocyte steatosis, age, Afro-American ethnicity and fibrosis. Several genes and polymorphisms as HLA-B44 has been found related to the possibility of curing the disease[35]. On the other hand, features of metabolic syndrome as overweight, steatosis and fibrosis have been reported as independently associated with response. The influence of steatosis in the chance of curing hepatitis C has been controversial for long time. Steatosis impairs SVR rate in patients with genotype 1, but not in genotype 3. Thus, the possibility of finding steatosis as independent variable depends on the balance between genotypes included in the cohort. In fact, in a multivariate analyses including steatosis together with fibrosis, body mass index, genotype and insulin resistance, either steatosis or body mass index were not found independently associated with response but insulin resistance, genotype and fibrosis[32]. In genotype 1, sustained response rate was 32% in patients with insulin resistance (HOMA > 2) against 60% in patients with HOMA lower than 2[36]. However, in a recent cohort including 331 non-diabetic genotype 1 chronic hepatitis C patients, steatosis but not insulin resistance was found independently associated with response. Steatosis and insulin resistance showed a great colineality in genotype 1 patients and could be the expression of the same mechanisms. Thus, measure insulin resistance seems to be easier and comfortable than study steatosis in a liver biopsy. Besides, in 52 patients from UK also treated with peginterferon plus ribavirin, HOMA index was significantly higher in non-responders than patients with sustained response[37]. Thus, insulin resistance emerges as the most important host factors in the prediction of response in non-diabetic patients treated with the best available option peginterferon plus ribavirin. Interestingly, insulin resistance has been found as a common denominator to the majority of features associated with difficult-to-treat patients: patients with cirrhosis, obesity, co-infected by HIV and Afro-American. Unresolved question is whether insulin resistance is a marker of very difficult-to-cure or a pathogenic mechanism able to block antiviral activity of the interferon. Peginterferons induce their antiviral activity via extracellular receptor binding. The interferon alpha signalling pathway involves the activation of Janus kinase (Jak1) and tyrosine kinase (Tyk2), initiated by the binding of peginterferon alpha-2 to the interferon heterodimeric receptor complex (IFNAR1/IFNAR2), which leads to activation of their downstream substrates, signal transducers and activators of transcription (STAT 1 and STAT2). Activated STAT then assemble as a multimeric complex and translocate into the nucleus where they bind to interferon alpha-2-stimulated response elements in the promoters of interferon alpha-2-stimulated genes[38]. Recently, in a replicon model using Huh-7 cells transfected by full length HCVRNA, interferon alpha blocks HCV replication. However, when insulin (at doses of 128 μU/mL, similar that seen in the hyperinsulinemic state in patients with metabolic syndrome) was added to interferon, the ability to block HCV replication disappeared, and the PKR synthesis was abolished[39]. In this experiment, a blockage of PI3K by LY294002 avoided the interference of insulin, supporting that interferon resistance induced by insulin is mediated by PI3K (Figure 2). Moreover, in genotype 1 patients obesity has been found able to increase intrahepatic synthesis of SOC-3, inhibiting the interferon signalling and promoting interferon resistance[40]. Thus, hyperinsulinemia and obesity induce interferon resistance blocking intracellular signalling.

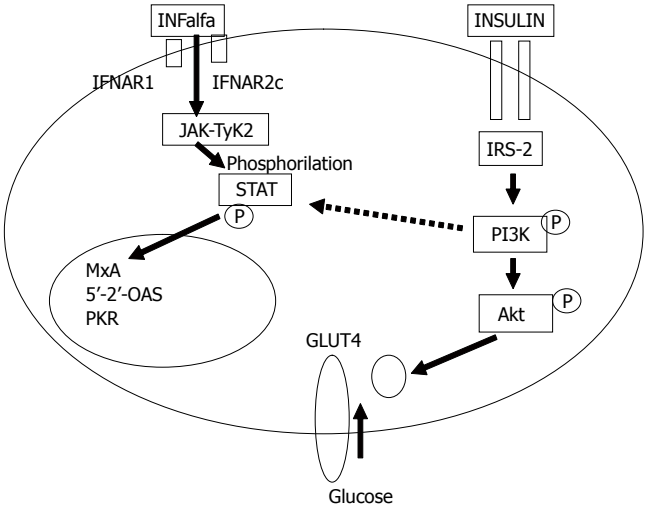


**Figure 2** Interaction between insulin and peginterferon alpha signalling pathway. The phosphatidil-inositol-3-kinase (PI3K) activated by insulin seems to be responsible for the block of STAT 1 translocation avoiding antiviral effect of interferon. Dotted lines represent inhibition, continuous lines represent activation.

## CONCLUSIONS

In summary, hepatitis C promotes insulin resistance and insulin resistance induces interferon resistance, steatosis and fibrosis progression in a genotype-dependent manner. In genotype 1 insulin resistance decreases sustained response rate, and increase the risk for the development of steatosis and fibrosis progression, in both, coinfected HCV+/HIV+ and in hepatitis C. However, the impact of insulin resistance in non-1 genotype seems not achieve enough importance to impair sustained response, probably due to the high sensitivity to peginterferon. The treatment of insulin resistance, decreasing hyperinsulinemia, could improve sustained response rate in genotype 1 patients with chronic hepatitis C when treated with peginterferon

plus ribavirin. A multicenter Spanish trial (TRIC-1) is ongoing to analyze the effect of triple therapy with peginterferon alfa-2a plus ribavirin and metformin versus standard therapy and placebo in patients with chronic hepatitis C infected by genotype 1 and showing insulin resistance.

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*EDITORIAL*

# The "return" of hepatitis B

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## Abstract

There has been a significant advance in the treatment of chronic Hepatitis B virus (HBV) infection and the following drugs were approved for therapy: Conventional interferon (IFN), pegylated interferon alfa-2a (PEG IFN α2a), lamivudine, adefovir and entecavir. Compared to nucleoside analogues IFN induces higher rates of sustained remission and HBsAg loss. Conventional IFN in lower doses (1, 5-3 MIU) tiw for 4-6 mo has similar efficacy in comparison to "standard IFN therapy". Longer IFN treatment is a significant factor for long-term remission in HBeAg-negative CHB, but the higher actual IFN dose is not such a factor. PEG IFN is superior to conventional IFN. There is no significant difference between PEG IFN α2a at doses 90 mcg/wk and 180 mcg/wk in HBeAg-positive patients. These results provide a rational for further clinical trials with lower doses PEG IFN α2a given in prolonged course as maintenance or intermittent treatment. Serious new problems arose after the introduction of nucleoside/nucleotide analogues in clinical practice. The most important ones are drug-resistance and the high rates of relapse after treatment discontinuation. Therapy should only be recommended if the expected benefit exceeds significantly the abstain from treatment. The choice of therapy should take into account the patient's age, co-morbidity, severity of liver disease and the risk of drug-resistance. New antivirals significantly suppress HBV-replication, but have no effect on cccDNA in hepatocytes, and after the treatment discontinuation viral relapses occurs. At the present level of knowledge it is impossible "to eradicate the virus" The realistic treatment goal is to achieve durable response by clearance of HBeAg, sustained decrease of serum HBV DNA levels, normalization of ALT, improvement of liver histology and stopping of liver fibrogenesis. The competition between IFN based therapy and nucleoside or nucleotide analogues still remains. IFN can cure the liver disease while nucleotide analogues only suppress the viral replication during therapy and can reduce the liver fibrosis. Treatment should be prolonged for 24-mo or longer by using maintenance or intermittent treatment course with the lowest effective IFN and PEG IFN doses. Nucleoside/nucleotide analogues are a promising treatment option, but additional data for treatment duration and long-term post-treatment outcome are necessary.



**Key words:** Chronic Hepatitis B virus infection; Interferon; Pegylated interferon; Low-dose therapy; Cyclic treatment

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## INTRODUCTION

Hepatitis B virus (HBV) infection is a global health problem. This infection is especially endemic in Asia, South Pacific Region, sub-Saharan Africa and South America[1]. It is estimated that over 350 million people worldwide are chronically infected with HBV and up to one million die annually due to HBV-related complications, including hepatocellular carcinoma (HCC)[2]. In China and sub-Saharan Africa HCC associated with HBV is one of the leading causes of cancer in men[1].

In the mid-1980s, IFN became the first approved therapy for CHB. In addition effective vaccines against HBV became available and there was a flicker of hope that the problem can be solved. Over the years it became clear that the chronic HBV infection is much more severe and difficult to treat disease than previously supposed. The positive impact of routine infant HBV-immunization programs is beyond doubt, but chronic HBV-infection still remains an important public health issue.

During the last decade much was learned about the HBV genome organization, viral replication cycle, role of host immune response and natural course of chronic HBV infection[3]. The emergence of human immunodeficiency virus (HIV) infection on the other hand facilitated the search for effective and safe antiviral agents. Several new drugs were found to be quite promising candidates for the treatment of CHB. All these findings led to the 'return' of research interest in the treatment of CHB. A large number of clinical trials with new long-acting pegylated interferons (PEG IFN) and antiviral agents were performed. As a result of this intensive clinical research now we have five approved drugs for the treatment of CHB: Conventional IFN, PEG IFN α2a as well as three nucleoside or

**Table 1 Treatment response at the end of 24-wk follow-up after 24-wk therapy with IFN α2a 4.5 MIU tiw, 90 mcg/wk, 180 mcg/wk and 270 mcg/wk PEG IFN α2a (Adapted from W Cooksley *et al*[12])**

| Response at the end of the 24-wk follow-up | 4,5 MU IFN (*n* = 51) | 90 μg PEG IFN α2a (*n* = 49) | 180 μg PEG IFN α2a (*n* = 46) | 270 μg PEG IFN α2a (*n* = 48) |
|---|---|---|---|---|
| HBV DNA < 500 000 cp/mL | 13 (25%) | 21 (43%) | 18 (39%) | 13 (27%) |
| Loss of HBeAg | 13 (25%) | 18 (37%) | 16 (35%) | 14 (29%) |
| HBeAg seroconversion | 13 (25%) | 18 (37%) | 15 (33%) | 13 (27%) |
| Normal ALT | 13 (25%) | 21 (43%) | 16 (35%) | 15 (31%) |
| Combined response[1] | 6 (12%) | 13 (27%) | 13 (28%) | 9 (19%) |

[1]HBeAg-negative, HBV DNA < 500 000 cp/mL, Normal ALT.

nucleotide analogues (lamivudine, adefovir and entecavir). Furthermore, several new drugs are under investigation in phase Ⅱ and Ⅲ clinical trials: emtricitabine, tenofovir, clevudine and telbivudine. Nevertheless, the treatment of chronic HBV infection is still a challenge due to the low rates of durable response with currently available therapies, especially in HBeAg-negative patients. The main problems with current treatments now are associated with suboptimal efficacy, poor tolerability, and/or emergence of resistance.

There are no clinical trials comparing directly the efficacy of all discussed therapies. Furthermore, it is difficult to compare the results of clinical trials in CHB due to the different sensitivity of the assays used for detection of serum HBV DNA over the years. In the early 1980's the virological response was measured only by serological assays (HBeAg and anti-HBe Ab). Later it became clear that the presence and absence of HBeAg is not an accurate indicator of replicative and nonreplicative infection and low-sensitive hybridization assays (detection limit of approximately $10^6$ copies/mL) were developed for the measurement of serum HBV DNA[3]. In the last years the majority of laboratories moved from hybridization techniques, through bDNA, to high-sensitive PCR (Roche Amplicor monitor) and RT PCR assays with lower detection limits of $10^3$ copies/mL and < 100 copies/mL, respectively.

## CURRENTLY APPROVED THERAPIES FOR CHRONIC HEPATITIS B

In spite of the mentioned difficulties, the treatment results of the approved therapies for CHB have been summarized by M Osborn and A Lok[4,5]. Available data suggest that higher rates of sustained remission and HBsAg loss can be achieved with interferon based treatment in comparison to nucleoside analogue therapy.

### *IFN and pegylated interferons in HBeAg-positive CHB: Standard and low-dose treatment schedules*

Conventional IFN has been used in CHB patients for 20 years. IFN has both antiviral and immunomodulatory effects[6], but only 1/3 of HBeAg-positive patients achieve HBV DNA and HBeAg loss after 4-6 mo course of IFN at a dose of 5 MIU daily or 9-10 MIU thrice weekly[7]. Subsequently, 6 to 12 mo post therapy HBsAg loss was found only in 8% of HBeAg-positive patients[7]. These treatment doses and durations have been validated as a "standard IFN therapy" for HBeAg-positive CHB by both EASL and AASLD treatment guidelines[8,9]. However, some studies in HBeAg-positive patients suggested that conventional IFN, given in lower doses (1, 5-3 MIU thrice weekly) for 4-6 mo, is with similar efficacy compared to "standard IFN therapy"[10,11]. This low-dose therapy is much better tolerated than higher IFN doses. Although treatment with low-dose IFN has not been generally accepted, these results are quite interesting especially in the light of new-coming data for the efficacy of PEG IFNs. A phase Ⅱ clinical trail showed that PEG IFN α2a is superior to conventional IFN in HBeAg-positive CHB[12]. In addition no significant difference was found in PEG IFN α2a efficacy given in dose of 90 mcg/wk in comparison to dose of 180 mcg/wk (Table 1)[12].

Having in mind that the majority of the side effects of PEG IFN α2a are dose-related, these results provide a rational for further clinical evaluation of lower dose of PEG IFN α2a as well as for new clinical studies with individualization of treatment schedule by PEG IFN tapering therapy. This might be a possibility to achieve better tolerability and lower treatment costs without losing the treatment efficacy. Further investigations are needed to confirm or reject this hypothesis.

### *IFN and pegylated interferons in HBeAg-negative CHB: standard and low-dose treatment schedules*

Early clinical trials in HBeAg-negative CHB, with 5-10 MIU of conventional IFN for 4-6 mo reported high (60%-90%) end-of-therapy response (ETR), but only 10% sustained response (SR) due to frequent relapses after the therapy[13]. Similarly to HBeAg-positive patients, HBeAg-negative subjects are sensitive to lower doses (1,5-3 MIU) of conventional IFN, but the relapse rate after 6-mo treatment course with low dose was also high[14,15].

Mathematical models of chronic HBV infection indicate that, although the half-life of HBV is short (< 1 d), the half-life of hepatocytes is relatively long (10-100 d) or even higher for the infected hepatocytes[16]. Therefore viral suppressive therapy might be continued for 1-10 years for viral elimination[16]. Subsequently, a longer course with conventional IFN was reported to improve the SR, which was found to be 11% and 22% after 6 and 12 mo of conventional IFN therapy, respectively[17]. Only the longer IFN course was identified as a significant factor of long-term remission, while the higher actual IFN dose was

not[17]. An Italian study showed a 30% SR after a 24-mo maintenance course with 6 MIU IFN tiw[18]. However, this prolonged treatment was associated with poor tolerability of IFN and high incidence of dose-related adverse events. In a recent pilot study we found similar SR by using approximately 2-year cyclic therapy with low-dose (1,5 MIU) conventional IFN therapy[19]. Taken together, this findings suggest that prolonged treatment (24-mo or longer) with conventional IFN in low-dose (1, 5-3 MIU) given as a maintenance or intermittent treatment course is appropriate therapeutic approach for HBeAg-negative CHB.

Recently, the efficacy of IFN-based therapy in CHB was improved by the introduction of PEG IFNs. In HBeAg-negative patients a 36% SR (combined biochemical and virological) was found at 6 mo after the 12-mo therapy with PEG IFN α2a[20]. However, 24-mo after the end of the treatment only 41% of patients with normal ALT levels 6-mo post therapy remained with combined biochemical and virological response[21]. Our results with cyclic (re-induction) treatment[19] provide a rational for further clinical trials with low-dose intermittent PEG INF therapy in responders of 12-mo PEG IFN α2a treatment. We have just initiated such a study aiming to test the potential of this treatment approach for reducing the relapse rate in end-of-treatment PEG IFN responders.

### *Lamivudine*

Lamivudine was the first introduced nucleoside analogue in clinical practice. Twelve-month course with lamivudine induces HBeAg-seroconvertion in 17% to 22% of the patients[22-24]. HBeAg-seroconvertion rates increased to 50% after continuous treatment for 5 years, but this was associated with increasing rates of drug-resistant mutations (up to 70%) after 5-year therapy[25]. One-year lamivudine therapy suppressed viral replication in 65% to 90% of HBeAg-negative patients[26,27]. However more than 90% of HBeAg-negative responders at the end of 12-mo course relapsed after treatment discontinuation[27]. Extended treatment duration was associated with decreased response rate due to drug-resistance[28]. The main concerns with lamivudine treatment are the selection of drug-resistant mutations and the very high relapse rate after treatment discontinuation. Both drug-resistance and relapse are associated with risk of hepatic flare and liver failure. Due to these reasons it is not recommended to use lamivudine as a first line therapy, especially in young patients. It should be also stressed that recent studies show PEG IFN α2a monotherapy to induce higher SR rate in both HBeAg-negative and HBeAg-positive subjects in comparison to 1-year course with lamivudine[20,29]. Combined therapy with lamivudine plus PEG IFN α2a is with efficacy, similar to that of PEG IFN α2a monotherapy[20,29]. According to the current knowledge there is no biological rational for further use of this combination, but the consequent treatment with antivirals and IFN-based treatment is a possible treatment approach.

### *Adefovir*

Recently, adefovir and entecavir were approved in the United States. Adefovir is effective in both HBeAg-positive and HBeAg-negative patients[30,31]. However, only 8% of the patients had a SR one year after treatment discontinuation[32]. Adefovir effectively suppress lamivudine resistant mutants which is a significant advantage of this drug. Because of the high rates of drug-resistance, patients on prolonged lamivudine therapy need to be closely monitored. If virological breakthrough occurs due to drug-resistance, adefovir can be administrated in addition to lamivudine. Resistance to adefovir is uncommon during 2-year therapy, but emerge later in the course of treatment to 30% after 5-year therapy. Thus drug-resistance will be an increasing concern with longer adefovir treatment duration. Nephrotoxicity is another disadvantage of this drug.

### *Entecavir*

Entecavir is more potent than lamivudine in suppressing HBV replication with significantly higher rates of biochemical and histological responses both in HBeAg-negative and HBeAg-positive patients[33,34]. No entecavir-resistance was observed after 2 year therapy among nucleoside analogue naïve patients[35]. However, the presence of lamivudine resistance increases the likelihood of entecavir resistance[35,36]. Long-term data with entecavir still are not available.

## NEW PROMISING ANTI-VIRAL AGENTS

Many new antivirals are under evaluation for the treatment of CHB. Emtricitabine (FTC) and tenofovir are licensed for use in HIV infection.

### *Emtricitabine*

FTC is closely related structurally to lamivudine and therefore they share similar mutational sites[37]. A recent study in HBeAg-positive and HBeAg-negative patients found that 48 wk of treatment with emtricitabine 200 mg daily resulted in significant histological, virological, and biochemical improvement[38]. The results of emtricitabine treatment were quite similar to published data for lamivudine. The incidence of YMDD mutations in patients, receiving emtricitabine 200 mg daily, is 12% and 19% at treatment wk 48 and 96, respectively[37,38]. The role of emtricitabine as a monotherapy may be limited due to its structural similarity to lamivudine and the risk of development of drug resistance.

### *Tenofovir*

Tenofovir is an acyclic nucleotide inhibitor of HBV polymerase and HIV reverse transcriptase with close chemical similarity to adefovir[37]. Antiviral activity of tenofovir against HBV is found to be greater than the one of adefovir 10 mg in lamivudine resistant patients[37,39]. Furthermore, the N236T mutation that confers resistance to adefovir is sensitive to tenofovir[37]. Phase Ⅲ clinical trials are under way to determinate the long-term safety and efficacy of tenofovir.

### *Clevudine*

Clevudine is a nucleoside analog of the unnatural β-L configuration. A recent randomized, double-blind study

found no significant difference between 24-wk therapy with emtricitabine plus clevudine and emtricitabine alone[40]. However, there was a significantly greater virologic and biochemical response at wk 24 after the end of treatment in the emtricitabine plus clevudine arm[40]. Further studies are needed to assess the long-term efficacy and safety of this drug.

### *Telbivudine*

Telbivudine (LdT) is another antiviral agent, which is under clinical investigation in CHB. A phase Ⅱ study in 104 HBeAg-positive patients compared different therapeutic schedules for 52 wk[41]. The telbivudine-treated patients exhibited significantly greater virological and biochemical response in comparison to lamivudine[41]. Results from the combined regimens (LdT plus lamivudine) were similar to those obtained with LdT alone[41]. These data support the ongoing phase Ⅲ evaluation of telbivudine for the treatment of CHB.

## ADVANTAGES, DISADVANTAGES AND TREATMENT LIMITATIONS OF CURRENT THERAPIES

Substantial progress has been made in the treatment of chronic hepatitis B, but quite serious new problems arose after the introduction of nucleoside/nucleotide analogues in clinical practice. The most important of them is the selection of drug-resistant mutations. Drug-resistance usually is accompanied by virologic breakthrough during therapy and increased ALT levels after initial normalization[35]. In some patients this may cause severe hepatic flare with liver failure and death[35]. In addition, resistance to one antiviral drug may confer resistance to other agents and may limit future treatment options[35]. Another important problem is the high rate of relapse after therapy.

With regards to the limited long-term efficacies of the approved therapeutic regimens, therapy should only be recommended if the expected benefit exceeds significantly abstain from treatment[35]. The choice of therapy should take into account the patient's age, co-morbid medical conditions, the severity of the liver disease and the risk of drug-resistance[4].

Interferon based therapy is associated with more durable response and the absence of drug-resistant mutations[42]. However, IFN is effective mainly in a subgroup of young patients with high ALT levels and low viral load[7,37]. At present it is generally accepted that subjects with normal or slightly elevated ALT (< 2x ULN) are not indicated for antiviral therapy[8,9]. These patients should be strictly monitored at 3-mo intervals. Low serum HBV DNA levels (HBV DNA < 30 000 000 copies/mL) are an important predictor of response to both conventional IFN and PEG IFN therapy[37]. With regards to this the initiation of IFN should be avoided in patients with very high viral load and especially if ALT is not markedly elevated. If possible the start of IFN therapy should be postponed for the moment, when serum HBV DNA and ALT levels predict higher likelihood of treatment response. High baseline ALT levels are the most important predictor of response to lamivudine and adefovir as well[42]. However, it should be mentioned that the high levels of ALT also are a predictor of spontaneous remission in HBeAg-positive CHB. So the decision to start nucleoside analogue should balance between the benefits of this treatment and the risk of drug resistance and hepatic flare.

Interferon based treatment is also limited by significant disadvantages in terms of injection-based application, poor tolerability, potentially severe side-effects, contraindications and relatively high cost. Flu-like symptoms, fatigue, bone marrow suppression thyroid disorders, irritability and depression are the most common adverse effects[5]. Patients should be closely monitored with monthly clinical and laboratory examinations. Approximately one-third of subjects may require dose reduction and 5% may discontinue therapy prematurely due to the adverse events.[5] Furthermore, IFN and PEG IFN are contraindicated in decompensated cirrhosis as well as in subjects with autoimmune disorders. They are also ineffective in immunosupressed patients. In fact nucleoside or nucleotide analogues are the only available treatment option in decompensated cirrhosis and in immunosupressed subjects.

## TREATMENT GOALS

Sensitive HBV DNA assays revealed that HBV replication might persist even after HBsAg seroconvertion and this finding changed our treatment concept. Although the new antiviral agents significantly suppress HBV-replication, there is a pool of covalently closed circular DNA (cccDNA), resistant to the available antiviral treatment. This cccDNA serves as a template for viral transcription, so viral relapses occurs once antiviral medications are discontinued[4]. At the present level of knowledge it is impossible "to eradicate the virus" The realistic treatment goal now is to achieve durable response by clearance of HBeAg, sustained decrease in serum HBV DNA levels with normalization of ALT, improvement of liver histology and stopping of liver fibgogenesis[4].

In conclusion the competition between IFN based therapy and nucleoside or nucleotide analogues treatment still remains. IFN can cure the liver disease while nucleotide analogues only suppress the viral replication and can reduce the liver fibrosis. In the future IFN treatment in HBeAg-negative CHB might need to be prolonged for 24-mo or even longer by using maintenance or intermittent treatment course with the lowest effective IFN or PEG IFN doses. Nucleoside/nucleotide analogues are a promising treatment option, but additional data for treatment duration and long-term post-treatment outcome are necessary.

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*REVIEW*

# Pathophysiology of pulmonary complications of acute pancreatitis

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## Abstract

Acute pancreatitis in its severe form is complicated by multiple organ system dysfunction, most importantly by pulmonary complications which include hypoxia, acute respiratory distress syndrome, atelectasis, and pleural effusion. The pathogenesis of some of the above complications is attributed to the production of noxious cytokines. Clinically significant is the early onset of pleural effusion, which heralds a poor outcome of acute pancreatitis. The role of circulating trypsin, phospholipase A2, platelet activating factor, release of free fatty acids, chemoattractants such as tumor necrsosis factor (TNF)-alpha, interleukin (IL)-1, IL-6, IL-8, fMet-leu-phe (a bacterial wall product), nitric oxide, substance P, and macrophage inhibitor factor is currently studied. The hope is that future management of acute pancreatitis with a better understanding of the pathogenesis of lung injury will be directed against the production of noxious cytokines.



**Key words:** Acute pancreatitis; Cytokines; Acute respiratory distress syndrome; Complications of pancreatitis; Pleural effusion; Interleukins

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## INTRODUCTION

Acute pancreatitis (AP), an acute inflammatory process of the pancreas in its severe form, is complicated by the development of multi-organ dysfunction syndrome (MODS) with a mortality of 15%-20%. Regardless of etiology, once AP is initiated the inflammatory events within the acinar cells will progress to a generalized systemic inflammatory response syndrome (SIRS). Amongst the systemic complications, pulmonary complications are the most frequent and potentially the most serious. Recognition of these complications and their pathology may lead to more rapid diagnosis and better therapies. The following is a brief summary of the current researches on each of the possible pulmonary complications ranging from hypoxemia to acute respiratory distress syndrome (ARDS). Hypoxemia may occur without radiological abnormalities in 75% of cases. There is a direct correlation between hypoxemia noted early in the course of AP and mortality. Pleural effusion, once thought to be a marker of AP, is now a noted poor prognostic sign. Atelectasis, a frequent radiological complication, is attributed to a decrease in the quantity of pulmonary surfactant. The most dangerous complication of the pulmonary system is ARDS. The pathophysiology of ARDS and most of the other pulmonary complications is multifactorial. Activated trypsin causes damage to pulmonary vasculature and increases endothelial permeability. Active circulating phospholipase $A_2$ ($PLA_2$) is known to remove fatty acids from phospholipids. One of the main components of surfactant is the phospholipid, dipalmitoylphosphatidylcholine. Many recent studies have assessed the role of platelet activating factor (PAF) which stimulates polymorphonuclear cells (PMNs) regulating the interaction between PMNs and endothelial cells facilitating migration of activated WBC into interstitial spaces. Based on the observation a PAF inhibitor Lexipafant has undergone double blind randomized studies. Another major advance in the understanding of the pathophysiology of pulmonary complications is the explosion of knowledge of cytokines. There are pro-inflammatory cytokines released from the pancreas such as tumor necrosis factor-alpha (TNF-$\alpha$), interleukin (IL)-1, IL-6, and IL-8. PMNs also contribute to release of cytokines. Our aim was to help the clinician understand the importance of the many studies looking at the effect of inflammatory modulators in decreasing the severity of AP. Today's experimental medicine is likely to become a therapeutic modality in the near future.

## PATHOPHYSIOLOGY OF PULMONARY COMPLICATIONS OF ACUTE PANCREATITIS

AP is an acute inflammatory process of the pancreas with

variable involvement of peripancreatic organs and/or remote organ systems in different degrees. In 10%-20% of AP, it presents in its severe form, which is frequently complicated by the development of MODS with a mortality rate of 15%-20% or more[1,2]. Recent studies have documented two peaks of mortality in patients with AP, an early mortality due to the effects of SIRS and MODS, and a late mortality caused by the effects of MODS combined with pancreatic sepsis following pancreatic necrosis[3,4]. About 30% of patients with fatal AP have been diagnosed first at autopsy[2,5], indicating how often we miss the diagnosis and how fast the disease can progress after the onset. Regardless of its etiology, the clinical course follows a pattern of interaction between inflammatory events within the pancreas which initiate a generalized systemic inflammatory response. The mortality and severity of the disease appear to be influenced by events occurring subsequently to the pancreatic injury as a result of release of cytokines and other mediators.

Severe AP is associated with MODS and/or local complications such as necrosis, abscess, pseudocyst, or pancreatic ascites. Fortunately, AP is usually mild.

Pulmonary complications of AP occur in almost 75% of cases, ranging from hypoxemia to ARDS[6,7]. The local and systemic complications are listed in Table 1. Of the systemic complications, acute respiratory failure is perhaps the most serious one. In the last two decades, our understanding and management of severe AP have substantially improved. The traditional concept that AP is entirely due to the release of activated proteases from an injured pancreas has been considerably changed. The present view is that the activation of trypsin by intracellular zymogens, trypsin activating chymotrypsinogen, proelastase and prophospholipase A plays an initiating role, but other chains of events predominate in precipitating complications[6-8].

Recent studies using secretagogue-induced models of pancreatitis, caerulein- induced AP in rats, sodium taurocholate- induced AP, choline deficient ethionine-supplemented diet- induced AP in mice and free fatty acid- induced AP in dogs, as well as a number of other models have suggested that pancreatitis is a disease that evolves in 3 phases. The initial phase is characterized by intrapancreatic digestive enzyme activation and acinar cell injury which involves the first few hours of AP. The second phase characterized by an intrapancreatic inflammatory reaction and varying degrees of acinar cell necrosis involves approximately 12-72 h. Finally, the third phase which involves the rest of the progression of AP is characterized by further progression of the pancreatic injury and the appearance of extrapancreatic changes including SIRS and ARDS[9].

It is during phase three that pulmonary insult occurs. In this review, we chiefly focus on the many pulmonary complications of AP, their pathogenesis, and clinical significance. This review is an extension of our previous review and other excellent recent reports on the topic[10-13]. The literature search was done by Medline search using the terms 'pancreatitis', 'pleural effusion', 'hypoxemia', and 'ARDS' in combination searches in the English language.

Pulmonary complications of AP for ease of explanation are divided into three stages. Stage 1 deals with pulmonary manifestations without any noticeable changes radiologically, stage 2 emphasizes radiologic changes observed and stage 3 discusses ARDS. Following these stages is a discussion on the players in the pathophysiology of the pulmonary complications of AP.

**Table 1 Complications of acute pancreatitis**

| | |
|---|---|
| Ⅰ | Pancreatic |
| | 1 Necrosis-sterile *vs* infected |
| | 2 Pseudocyst-infection/rupture/hemorrhage |
| | 3 Abscess |
| Ⅱ | Local-extrapancreatic |
| | 1 Involvement of contiguous organs (intraperitoneal hemorrhage, GI bleeding, thrombosis of splenic vein, bowel infarction) |
| | 2 Pancreatic ascites |
| | 3 Obstructive jaundice |
| Ⅲ | Systemic |
| | 1 Pulmonary |
| | a Early arterial hypoxia |
| | b Atelectasis, pneumonia, pleural effusion, mediastinal abscess |
| | c ARDS |
| | 2 Cardiac: shock, pericardial effusion, EKG changes, arrhythmias |
| | 3 Hematologic: DIC, TTP/HUS |
| | 4 Gastrointestinal: GI bleeding (portal-splenic vein thrombosis, colonic infarction) |
| | 5 Renal: azotemia, oliguria |
| | 6 Metabolic: hypocalcemia, hyperglycemia, hypertriglyceridemia, acidosis, elevation of free fatty acids |
| | 7 CNS: psychosis, pancreatic encephalopathy, Purtscher's retinopathy |
| | 8 Peripheral: fat necrosis (skin and bones), arthritis |
| | 9 Miscelaneous: rhabdomyolysis |

## STAGE 1: HYPOXEMIA WITH NO RADIOLOGICAL ABNORMALITIES

Tachypnea, mild respiratory alkalosis, and hypoxemia are seen in almost two thirds of patients with AP during the first 2 d of admission to the hospital[14]. In these patients, physical examination is essentially normal, chest radiographs rarely demonstrate abnormalities (11%), and clinical signs if present, seldom indicate the severity of the hypoxemia[6]. Ranson and colleagues[15] observed that 52% of patients have arterial oxygen tensions (PaO2) < 71 mmHg. Imrie and co-workers[6] showed that 45% of patients have severe arterial hypoxemia (PaO2 < 60 mmHg) and indicated that a PaO2 of < 52.5 mmHg is associated with a mortality of more than 30%. A study conducted several years ago identified that the incidence of pulmonary insufficiency in acute edematous pancreatitis is < 10%, increasing to 47% for acute sterile necrosis and 74% for infected necrosis[16,17]. Ranson *et al*[18] reported that respiratory insufficiency characterized by an arterial pO2 < 75 mmHg and drawn within 48 h of admission is observed in 58%. Recently Lankisch and associates[19] studied a group of 204 patients with AP and demonstrated that 63% of the patients have an arterial pO2 < 70 mmHg, and 30% have a pO2 < 60 mmHg, showing that hypoxemia is present early in AP patients. The major cause of hypoxia is ventilation and perfusion mismatch,

which results in a right to left intrapulmonary shunting of up to 30% of cardiac output[20] The most important precipitating factor for MODS during the first week of AP is perhaps the failure to promptly recognize and treat hypoxia and hypovolemia[21]. The incidence of respiratory insufficiency is not related to the etiological factors of AP, severity of the disease, age of the patient, admission serum amylase, serum calcium, the amount or the nature of fluid administered (colloid solutions or blood), or the estimated fluid sequestration, but is seen more often in patients experiencing their first attack of AP.

### *Clinical significance*

If pulmonary infiltrates and severe hypoxemia develop concurrently, mortality rate may be as high as 56%[16]. In two studies, Imrie and coworkers[6,22] correlated the overall mortality with the degree of hypoxemia. They found that patients with arterial pO2 below 70 mmHg have a mortality of 3% to 5.9% and when the arterial pO2 decreases below 60 mmHg the mortality rate increases to about 14%. Marked respiratory insufficiency in 22% of the cases of AP is associated with a 60% mortality from uncontrollable hypoxemia[23]. These studies suggest that respiratory insufficiency early in AP represents a risk for future ARDS especially if it is persistent and refractory to oxygen therapy.

## STAGE 2: HYPOXEMIA WITH RADIOLOGICAL ABNORMALITIES

### *Pleural effusion*

In 33% of all patients with AP, respiratory complications are clinically or radiographically detectable[16]. Pulmonary infiltrates or atelectasis (15%), pleural effusions (4%-17%), and pulmonary edema (8%-50%) are the pulmonary manifestations of AP in this category. Mortality and morbidity are significantly higher as compared to Stage 1. Some of these patients require ventilatory assistance. A direct relationship to prognostic factors is observed in this stage.

Presence of pleural effusion is currently considered an indication of severe pancreatitis and not just a marker of the disease[24]. Pancreatic ascites and pleural effusion are rare complications of both chronic and acute pancreatitis, and are associated with a mortality rate of 20% to 30%[25]. Mediastinal pancreatic pseudocysts and acute fluid collections are rare complications of pancreatitis resulting in an increase in morbidity and mortality[26]. Pleural effusions in AP are usually small, occasionally bloody, and are characterized by high amylase (up to 30 times greater than corresponding serum value), protein (> 30 gm/L), and lactic acid dehydrogenase (ratio) (> 0.6 serum value) levels[27]. The majority of pleural effusions (68%) are left-sided, 22% are bilateral and 10% are right-sided only. Two main causes of pleural effusion are transdiaphragmatic lymphatic blockage or pancreaticopleural fistulae secondary to leak and disruption of the pancreatic duct or pseudocyst caused by an episode of acute pancreatitis. The leak or disruption is more likely to lead to a pleural effusion if the duct disruption is posteriorly into the retroperitoneum. The pancreatic enzymes can track up into the mediastinum and then rupture into the pleural cavity either left side or bilaterally and so create a connection between the pancreatic duct and the pleural cavity. Pancreatic pleural effusions may be massive and require treatment[28].

Close to 1/3 of all patients with an internal pancreatic fistula present with pleural effusion[29]. Formation of a posterior mediastinal cyst indicates that a patient is at increased risk of developing pleuropulmonary complications including acute and chronic pleural effusions which can be sympathetic in origin or result from the development of a fistulous tract into the pleural space[30]. Dyspnea and hypoxia, chest pain, atelectasis, cough, and ARDS can develop, worsening the prognosis[26].

Treatment of pleural effusion is usually at first conservative. Pleural effusions which become symptomatic often require thoracentesis, tube thoracotomy, endotracheal intubation, ICU admission, parenteral alimentation, and administration of octreotide. When the intraabdominal etiology is resolved, pleural effusions often resolve as well. Chronic effusions often require drainage of the pseudocyst or abscess or excision of the fistulous tract[27].

### *Atelectasis*

Consolidation of lung tissue and atelectasis are frequent radiological observations. In cerulein-induced experimental pancreatitis-associated lung injury, it has been observed that there is a decrease in the production of pulmonary surfactant[31]. A decrease in the quantity of pulmonary surfactant may be a significant cause of atelectasis formation in dependent lung regions. Cerulein-induced pancreatitis appears to cause a decrease in the strength of the muscles of the diaphragm as well as a decrease in the endurance of these muscles[32]. Because the primary lesion is found at the alveolar interstitial level, it is probable that this decreases lung compliance and increases the work of breathing, leading to fatigue of the diaphragm more quickly[32].

There is mounting evidence that the proinflammatory cytokines are the agents behind the systemic complications of AP[33-35]. Cytokines are low molecular weight proteins, secreted by various cell types, that regulate the intensity and duration of immune responses and mediate cell-to-cell communication[36]. During AP IL-1, IL-6, and TNF-$\alpha$ are formed within the pancreas[32]. There is growing evidence that cytokines, mainly TNF-$\alpha$ and IL-1, could be the orchestrators of skeletal muscle dysfunction during sepsis. An experimental study showed that TNF-$\alpha$ at a lower concentration (50-400 ng/mL) causes no diaphragmatic fatigue in rats[37]. However at a higher concentration (10 $\mu$g/mL) of the cytokine, fatigue occurs in hamsters[38]. It has been noticed that TNF-$\alpha$ and IL-1 have a synergistic negative inotropic effect on the contractility of the diaphragm[39]. According to Matuszczak *et al*[32], cytokine levels need to be at a very high concentration to have a physiologic effect. Since the concentrations of cytokines in muscle during sepsis have not yet been quantified, the higher concentration of cytokines used in the study may be achieved in the natural physiologic setting of sepsis[32]. According to Norman *et al*[35], the intrapancreatic concentration of cytokines achieved in AP is much

higher than the systemic level and this argues in favor of the observation by Matuszczak *et al*[32] who used higher concentrations of cytokines to achieve the end result of diaphragmatic fatigue. According to these studies, it is possible that if IL-1, IL-6, TNF-α are present at high enough concentrations in the pancreatic juice, they could cause diaphragmatic fatigue. Diaphragmatic impairment may be the pathophysiologic cause of atelectasis in the lower regions of the lung via decreased ventilation in these regions[32].

## STAGE 3: ARDS

The most dangerous complication of the pulmonary system is ARDS. ARDS, a syndrome first described in 1967[40], is observed in association with AP[16]. Of the patients who develop AP, 15% to 20% develop ARDS with an associated mortality of 56%[41]. ARDS usually manifests itself between two to seven days following the onset of AP, but may have a much more rapid course. Clinical features include severe dyspnea and extreme hypoxemia refractory to a high inspired oxygen concentration. Multilobar pulmonary infiltrates in patients with previous normal radiographs and a relatively normal pulmonary capillary wedge pressure (< 18 mmHg) are noted[42,43]. Autopsy studies in patients with AP have shown that morphological changes in the lungs are indistinguishable from those in patients with ARDS caused by other conditions, including shock, sepsis, and severe trauma[44]. The lungs are characterized by increased alveolar capillary permeability with interstitial edema. The complication of ARDS parallels the presence of other poor prognostic signs of AP[15]. Although less often than necrotizing pancreatitis, patients with interstitial edematous pancreatitis are also at risk (10%) of developing respiratory failure[45].

There are three clinical features that must be present in ARDS: widespread bilateral radiographic infiltrates, a ratio of the partial pressure of arterial oxygen to the fraction of inspired oxygen (PaO2/FiO2) less than or equal to 200 regardless of the positive end-expiratory pressure (PEEP), and no clinical evidence for an elevated left atrial pressure (less than or equal to 18 mmHg)[21,46]. Hypoxia is worse in ARDS. ARDS accounts for 50%-90% of all deaths from pancreatitis[47]. Improved ventilatory and supportive care, has improved the outcome in ARDS. However the underlying etiology remains unclear, keeping the mortality still unacceptably high.

### *Pathophysiology*

The pathophysiology of ARDS in AP is poorly understood. Actions of pancreatic enzymes as well as inflammatory mediators released as a result of pancreatic injury play a key role in the pulmonary complications. The pathophysiology of ARDS is described as increased pulmonary vasculature leaking protein- rich transudate into the alveolar space and decreased lung compliance manifested clinically as refractory hypoxemia, and radiologically as diffuse infiltration in the lungs.

In the pathogenesis of systemic complications of AP, the role of active enzymes in circulation, liberation of proinflammatory cytokines, leukoagglutination, migration of neutrophils, activation of leukocytes, complement-mediated injury, and platelet activating factor decreasing the normal defense mechanisms and increased production of Nitric oxide (NO) are studied. The agents which are suspected to play a role in the pathogenesis of AP are noted in Table 2. A number of experimental and clinical studies have helped to elucidate the mechanisms involved in the pathogenesis of lung injury secondary to AP.

**Table 2 Pathogenic players in respiratory insufficiency**

| | |
|---|---|
| 1 | Phospholipase A2 |
| 2 | Trypsin inhibitor |
| 3 | FFA/Lipoprotein lipase |
| 4 | Complement activation (C5a) |
| 5 | MIF |
| 6 | IL-6, IL-8, IL-1β |
| 7 | NO |
| 8 | TNF-α |
| 9 | fMet-Leu-Phe (a bacterial wall product) |
| 10 | ICAM-1 |
| 11 | β-2-integrin (CD11b/CD18) |
| 12 | Trypsin |
| 13 | NF-κB |
| 14 | Substance P |

**Activated trypsin:** It is a possible source for the cause of pulmonary insufficiency. Trypsin causes damage to the pulmonary vasculature and increases endothelial permeability[48]. In laboratory experiments trypsin has been shown to cause leukostasis in the pulmonary vasculature and thus activated trypsin could intensify intravascular coagulation in the pulmonary microcirculation[49]. In post mortem studies, patients with acute pancreatitis have been shown to have intravascular fibrin thrombi in different tissues, including the lungs[16]. Trypsin is capable of activating different complement factors directly, which can stimulate cytolysis and chemotactic leukocytes. Complement has been shown to produce ARDS raising the possibility of developing respiratory insufficiency.

**Phospholipase A:** As an incriminating factor in pulmonary complications of AP phospholipase $A_2$ ($PLA_2$) has been investigated by many authors. $PLA_2$, which is activated by trypsin in the duodenum, is known for its ability to remove fatty acids from phospholipids. Pulmonary surfactant lines the surface of the alveoli and prevents the alveoli from collapsing by maintaining the surface tension. One of the main components of surfactant is phospholipid dipalmitoy lphosphatidylcholine, which is a perfect substrate for $PLA_2$. The basic reason for pulmonary insufficiency and ARDS in AP is due to the destruction of the surfactant. Büchler *et al*[45,50] showed that there is a strong correlation between serum-activated $PLA_2$ and pulmonary insufficiency. In their prospective study, patients with pulmonary failure demonstrated a notably higher catalytic $PLA_2$ activity during the first week of AP than patients without arterial hypoxemia. Büchler *et al*[45] and Kortesuo *et al*[51] observed that there are many isoenzymes of $PLA_2$. The source of IR-$PLA_2$ and group I $PLA_2$ is the pancreas, but has no correlation with the severity of pancreatitis or likelihood of development of pulmonary insufficiency. Group II $PLA_2$, which has an extra-pancreatic source, correlates

well with the severity of AP and is probably responsible in part for the pulmonary insufficiency occurring as an extra-pancreatic complication of AP[45,51,52]. The role of other isoenzymes of $PLA_2$ in AP has yet to be elucidated. Inflammatory cells do not seem to be the source of Group II $PLA_2$, but they cause the pulmonary insufficiency and ARDS in AP[53]. The low molecular weight $PLA_2$ inhibitor has been shown to decrease the level of group II $PLA_2$ activity and has no effect on group I $PLA_2$ activity. The inhibitor also decreases tissue destruction and protects pancreatic acinar cells[54]. This is an area which needs further investigation to improve the outcome of AP.

**Platelet activating factor:** PAF is a potent biological mediator whose effect is manifested throughout the body. PAF stimulates PMN white cells and regulates the interaction between PMN cells and endothelial cells, facilitating migration of activated white cells into tissue spaces. PAF is a structural component of membrane lipids and is released upon the action of PLA2[13,55]. In induced experimental pancreatitis (whether induced by immune complex, caerulein, or taurocholate) PAF is released into the pancreas, ascitic fluid, lung and blood[56]. PAF can be controlled via the PAF receptor and PAF antagonists decrease the severity of experimental AP by reducing oxidative injury, morphological changes, white cell infiltration, vascular permeability in pancreas and lungs, pulmonary damage, blood, and peritoneal PAF exudative concentrations[56]. Lexipafant, a computer image analysis generated imidazolyl derivative of $Sp^2$ nitrogen compounds, is a powerful PAF receptor antagonist having an affinity for the receptor seven times more avid than PAF itself[57]. Lexipafant has undergone a randomized double blind phase II clinical trial in human pancreatitis and is very successful in reducing organ failure[58]. However, during phase III clinical trial, Lexipafant is unsuccessful in reducing new organ failure or mortality. In the largest systematic prospective study of severe AP ever undertaken, Johnson *et al*[59] found that 58% of patients in the placebo group and 57% of patients in the Lexipafant group develop one or more organ failures, indicating that there is no difference between these groups. Systemic sepsis affects fewer patients in the Lexipafant group, the development of pseudocysts is 14% in the placebo group and 5% in the Lexipafant group. IL-8, a marker for neutrophil activation and E-selectin, a marker for endothelial damage, decrease more rapidly in the Lexipafant group than in the placebo group. This adequately powered study showed that antagonism of PAF activity on its own is not sufficient to ameliorate SIRS in severe AP or change mortality rates[59].

**Free fatty acid:** Another possible cause of pulmonary insufficiency is the release of free fatty acid (FFA). During the inflammatory process, pulmonary lipoprotein lipase is activated and releases FFA from albumin, to which they are physiologically bound. The free fatty acids released from triglycerides (TG), have been shown to damage capillary alveolar wall membranes[60].

**Digestive enzymes:** In a normal pancreas, potentially harmful digestive enzymes (i.e. protease and phospholipase) are synthesized as inactive proenzyme forms which are only activated after pancreatic secretions reach the duodenum. Enterokinase, a brush border enzyme, cleaves trypsinogen into 2 products, namely N-terminal portion termed trypsinogen-activation peptide (TAP) and active trypsin. Active trypsin then catalyzes the conversion of other proenzymes secreted by the pancreas into their active forms[61]. Gukovskaya *et al*[62] demonstrated that neutrophils infiltrate into the pancreas facilitating intrapancreatic trypsin activation during cerulean (cholecystokinin analogue)-induced experimental pancreatitis. The immunocytochemical localization experiments using specific antibodies to TAP indicate that the trypsin activation mediated by neutrophils occurs within the pancreatic acinar cells[62]. Steer[63], in an excellent hypothesis, suggested that intrapancreatic (i.e., intra-acinar cell) activation of trypsinogen during pancreatitis is itself a 2-phase event. According to this interpretation, the initial phase of trypsinogen activation would be neutrophil-independent and solely dependent on acinar cell events. This initial phase of trypsinogen activation can lead to early acinar cell injury that triggers the pancreatic inflammatory response[63].

Activated neutrophils throughout the body, are attracted to the pulmonary microvascular network by various factors such as complement activation, cytokine production, alveolar macrophages and the upregulation of adhesion molecules[64]. Chemoattractants such as TNF-α, IL-1, IL-6, IL-8, fMet-Leu-Phe (a bacterial wall product) and complement factor C5a upregulate the beta-2-integrin expression on neutrophils and the ICAM-1 receptor on endothelial cells[65,66]. The binding of ICAM-1 to beta-2-integrin (CD11b/CD18) can increase the permeability of the pulmonary vasculature, allowing neutrophils to enter the lung parenchyma. Upon entry into the parenchyma, neutrophils release compounds which have been implicated in the damage caused by ARDS. This theory is supported by studies demonstrating that mice deficient in ICAM-1, develop a much less severe form of pancreatitis, and that ICAM-1 expression is increased in mice with pancreatitis[67,68]. Several compounds released by neutrophils once within the lung parenchyma are discussed in the following paragraphs.

**TNF-α:** High concentrations of TNF-α are found in a variety of locations throughout the body during an episode of AP, and pancreatic parenchyma, ascitic fluid, lymphatic drainage and serum levels can predict disease severity and possible mortality[35,69-72]. TNF-α activates neutrophils and increases lung damage levels at higher concentration in the lung[73,74]. A tetravalent guanylhydrazone compound CNI-1493 has been developed in a research program to prevent the production of macrophage-derived NO. It was then discovered that CNI-1493 blocks the production of inflammatory mediators such as TNF-α, IL-1, IL-6, macroinflammatory peptide (MIP)-1, by preventing the phosphorylation of p38 mitogen- activated (MAP) kinase[75]. If p38 MAP kinase is prevented from being phosphorylated, then TNF-α is not produced or is produced at a significantly lower level[76]. Denham *et al*[77] reported that CNI-1493 significantly attenuates the increase in pulmonary TNF-α resulting in decreased lung injury, confirming that CNI-1493 inhibits its pancreatic and pulmonary TNF-α gene induction in two models

of murine pancreatitis. These results also support that pulmonary congestion is decreased when anti-TNF-α antibody is administered after pancreatitis is induced and overall morbidity and mortality are decreased[78,79]. Several recent studies have suggested that TNF-α is one of the agents orchestrating the early stages of AP, especially in recruitment of inflammatory cells, regulation of cytokine production, and promotion of pancreatic acinar cell death by apoptosis[61,69,79]. TNF-α also plays a protective role in preventing the release of proinflammatory cytokines[76,80]. TNF-α induces concomitantly proapoptotic and antiapoptotic mechanisms[81]. Antiapoptotic mechanisms are controlled by NF-B and MAP kinases. Pancreatitis-associated protein (PAP) prevents apoptosis[81,82]. Apoptosis is possibly a more favorable demise for acinar cells than necrosis because it results in a quick removal of damaged cells without loss of plasma membrane integrity as well as decreased recruitment of leukocytes[81]. According to Kaiser *et al*[81] severe forms of AP demonstrate little apoptosis while edematous pancreatitis shows a higher number of apoptotic cells. There is an interaction between apoptosis and necrosis, with inflammatory cells infiltrating the pancreas and causing necrosis of the apoptotic cells[81,83]. Apoptosis appears to be advantageous over necrosis, but needs to be controlled to prevent excessive tissue loss[81].

**Nitric oxide:** The role of nitric oxide (NO) in vascular collapse during AP is highly controversial[77,84]. CNI-1493 blocks the production of NO by macrophages, subsequently the level of NO decreases lessening the damage to the lungs[84]. However, Tsukahara *et al*[85] demonstrated that alveolar macrophages during experimental pancreatitis mediate endothelial injury as well as increase microvascular permeability resulting in lung injury. In contrast, O'Donovan *et al*[86] showed that administration of sodium nitroprusside, a NO donor, to rats with caerulein-induced pancreatitis decreases lung myeloperoxidase activity, bronchoalveolar lavage protein concentration, and wet-to-dry lung weight, suggesting that NO may be protective against lung injury during pancreatitis. A possible explanation for the contradictory findings between Tsukahara *et al*[85] and O'Donovan *et al*[86] is the different experimental models or is possibly attributable to the paradoxic nature of NO[77].

**Substance P:** Substance P is a neuropeptide which is located in the nerve endings throughout the body and is released into the gap where it acts via the NK1 receptor to mediate pain[12]. It has also been implicated in many inflammatory states, such as immune complex- mediated lung injury, asthma and inflammatory bowel disease[87,88]. Once pancreatitis occurs, there is an increase in substance P levels as well as NK1 receptors on pancreatic acinar cells[12]. Interestingly, mice which have been genetically engineered to be deficient in NK1 receptors are protected from AP[89]. This gives clinical medicine another possibility for preventing AP and thus also for protecting patients against pulmonary complications of AP.

**Chemokines:** Chemokines are a family of cytokines which are distinguished by the fact that they act on the superfamily of G-protein–coupled serpentine receptors[12]. There are two subsets of chemokines based on the orientation of the first two cysteines: CC chemokines and C-x-C chemokines. The CC chemokines stimulate monocytes and C-x-C chemokines stimulate neutrophils. IL-8 is a C-x-C chemokine[90]. There is no direct homologue of IL-8, but there is a homologue of growth-related oncogene-alpha (GRO-α) which is cytokine-induced neutrophil chemoattractant (CINC)[90]. CINC (also a C-x-C chemokine) is a specific neutrophil attractant. A recent study reported that anti-CINC antibody reduces lung damage. During the study, anti-CINC was administered to Caerulein- induced pancreatitis mice prophylactically and during Caerulein administration. The results demonstrate that both the prophylactic group and the therapeutic group have a reduction in lung injury, but not a reduction in AP[90].

**IL-8:** IL-8 is a strong attractant of neutrophils in the lungs and its high concentrations have been observed in the lungs of patients with ARDS[91,92]. Donnelly *et al*[93] showed that bronchoalveolar lavage (BAL) levels of IL-8 are significantly higher in patients who develop ARDS than in those who do not develop ARDS. However they have not found a relationship between blood levels of IL-8 and patients with or without ARDS, but found that IL-8 may be a prognostic factor for ARDS. Kurdowska *et al*[94] took the IL-8 issue one step further to determine if the anti-interleukin 8 and interleukin-8 complex is a marker of patients who are at risk of developing ARDS, and found that anti-interleukin 8 and interleukin-8 complexes may be a marker of patients who may progress to ARDS.

**Macrophage migration inhibitory factor (MIF):** MIF is a cytokine discovered in 1966 and produced by T lymphocytes and plays a role in preventing random migration by macrophages[95,96]. It is now known that MIF is released not only by T lymphocytes but also by monocytes, macrophages, pituitary corticotrophic cells, and epithelial cells. MIF is currently viewed as an important proinflammatory cytokine[97,98]. MIF has a critical role in regulating the immune system by overriding the anti-inflammatory and immunosuppressive effect of glucocorticoids on macrophages and T cells[98,99]. MIF has been shown to be elevated in several inflammatory diseases such as sepsis, rheumatoid arthritis, bronchial asthma and also in the bronchoalveolar lavage samples of adults with ARDS[100-103]. It was reported that MIF levels in the lungs of taurocholate (TCA) pancreatitis rats are significantly higher than those in the lungs of normal rats, whereas MIF levels in the pancreas and liver have no differences between the normal and TCA pancreatitis rats[103,104]. There is a possible connection between TNF-α and MIF[103,105]. Pulmonary TNF-α levels are increased significantly in TCA- induced pancreatitis and the increase can be attenuated by treatment with anti-MIF antibody, but not with the control antibody[103]. Anti-MIF antibody also improves the survival rate in both TCA pancreatitis rats and choline deficient, ethionine-supplemented (CDE) pancreatitis mice[103]. Serum MIF levels are also found to be significantly higher in severe AP than in mild AP or in healthy controls[103]. Combining the increased survivability with anti-MIF antibody, induction of IL-8, stimulus of TNF-α production, ability to override the effects of glucocorticoids and the increased levels of MIF found

**Table 3 Pro-inflammatory cytokines**

| |
|---|
| IL-1β -major inflammatory mediator, major activator of macrophages and enhances B and T cell activation |
| IL-6 -acute phase reactant, stimulates B cell differentiation |
| IL-8 -stimulates the upregulation of adhesion molecules, chemotactic factor for neutrophils and lymphocytes |
| TNF-α -recruitment of inflammatory cells, regulation of cytokine production, and promotion of pancreatic acinar cell death by apoptosis |

IL-10 is an anti-inflammatory cytokine by inhibiting macrophages releasing inflammator mediators.

in severe AP, suggests that MIF plays strongly a vital role in APand lung injury[103]. It also raises the possibility of treatment with anti-MIF antibody to prevent lung injury in AP in the future.

**IL-10:** IL-10 is one of the few known anti-inflammatory cytokines in human body which prevents the production of pro-inflammatory mediators particularly TNF-α by macrophages and T cells[106-109]. IL-10 also inhibits the release of IL-6, and IL-1-beta[12,108,110-112] as well as stimulates the synthesis of IL-1 receptor antagonist (IL-1ra) and the release of soluble p75 TNF receptor which inhibits the action of pro-inflammatory cytokines[113]. (Table 3) In particular IL-10 has been shown to inhibit the release of TNF-α from alveolar macrophages, thus implicating that it can decrease the likelihood of developing ARDS[114]. In another study, IL-10 was administered to one group of mice prior to the development of AP. IL-10 was administered to another group of mice 33 hafter the induction of AP. The study showed that mortality is decreased due to AP in both groups[115]. Two other studies showed that both IL-10 and IL-1ra significantly decrease the severity of AP when given after the induction of AP[113,115]. Several studies have shown that IL-10 when given after AP can decrease the severity of AP, which raises the possibility of IL-10 used in clinical treatment of AP[35,115].

## CONCLUSION

Mortality in AP occurs in two peaks and is usually associated with MODS. Pulmonary dysfunction is perhaps the most critical for all extra-pancreatic manifestations of AP[3,4]. The pathogenesis of pulmonary complications in AP has been the topic of intense research in the past two decades. In this review, we have evaluated the different types of respiratory complications, with their clinical significance assessed and their pathogenesis discussed. Although the data are predominantly from experimental studies, their clinical relevance is of increasing importance. Hypoxemia occurs in nearly 50%-60% of patients with AP[6,18,19]. The major cause of hypoxemia is ventilation/perfusion (V/Q) mismatch which results in right to left intrapulmonary shunting. Failure to promptly recognize hypoxemia and hypovolemia may precipitate MODS. Pleural effusion on the left side, once considered just a marker of AP, is indeed a sign of poor prognosis[24]. Atelectasis is noted to be secondary to a decrease in pulmonary surfactant[31]. The role of cytokines has become quite evident in a number of experimental studies of atelectasis[33-36]. The most dangerous pulmonary complication is ARDS[33-35]. Of the patients who develop ARDS nearly 50% would die[16]. The incidence is much less in edematous pancreatitis. The clinical features of ARDS include widespread bilateral infiltrates in CXR and a ratio of partial pressure of arterial O2 to fraction of inspired oxygen (PaO2/FiO2) $\leqslant$ 200 regardless of PEEP with no evidence of elevated left atrial pressure[21,46]. The pathophysiology of ARDS is complex. The action of enzymes in circulation (trypsin, and phospholipase A2, proinflammatory cytokines, leukoagglutination, migration of neutrophils, activation of leukocytes, complement mediated injury, PAF, NO) is noted to play a different role in the pathogenesis.

The future management of AP that currently carries an overall mortality of 5% and 15%-20% in necrotizing pancreatitis includes measures to counteract the proinflammatory agents[1,2]. With the exception of platelet activating inhibitor agents, such as Lexipafant, nothing seems to be clinically relevant at this time[57]. However, a better understanding of the pathogenesis of lung injury and other organ dysfunction is expected to revolutionize the treatment of AP.

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*REVIEW*

# Associations between NOD2/CARD15 genotype and phenotype in Crohn's disease-Are we there yet?

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## Abstract

There have been multiple NOD2/CARD15 genotype-phenotype analyses undertaken in patients with Crohn's disease since the gene's discovery in 2001. This review focuses on the major published series based upon their size and on the presence of specific clinical and genetic information provided in the published material from 2001 to 2005. Twelve studies provided raw data to carry out comparisons of disease location while ten studies included analysis of NOD2/CARD15 genotypes. NOD2/CARD15 variant frequency in ileal disease did not differ significantly among studies, whereas a comparison of disease location demonstrated highly significant differences among studies. Meta-analysis confirmed significant associations between NOD2/CARD15 variants and both ileal and ileocolonic disease locations, and with both stricturing and penetrating forms of disease behavior. This review underlines the significant phenotypic differences that exist among populations, including similar ethnic groups, and has demonstrated the need for further studies of patients with long-term "inflammatory" Crohn's disease.



**Key words**: Crohn's disease; Phenotypic heterogeneity; Genotype; Inter-observer agreement; Disease location; Disease behavior

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## INTRODUCTION

Chronic idiopathic inflammatory bowel diseases (IBD), made up predominantly of Crohn's disease (CD) and ulcerative colitis, remain fertile ground for both clinical and basic science research. These conditions have been held up as classic examples of complex polygenic disorders with the working hypothesis of a genetically susceptible host manifesting the disease phenotype after exposure to a single or a series of environmental triggers. Study of the genetics of IBD has expanded rapidly over the past 10 years since publication of the first genome-wide scan in 1996[1]. Technical advances have aided this advance with the culmination being the discovery of the NOD2/CARD15 gene within IBD1 on chromosome 16[2,3]. The case for further identification of IBD susceptibility genes has been put forward, specifically regarding the organic cationic transporters, OCTN1 and OCTN2, on chromosome 5 (IBD5) and DLG5 on chromosome 10[4,5]. These genes have not received universal support from independent studies and remain topics for discussion and research, specifically including further functional studies.

These rapid advances in IBD genetics, fuelled by the interest and enthusiasm of both clinicians and geneticists, has set the pace of IBD research over the past 8-10 years, which has succeeded the "IBD immunology" era of the 1990's. While the latter gave us a plethora of cytokine data, including the important role of TNF-α leading to anti-TNF strategies in CD, the discovery of NOD2 highlighted the significance of the gut's innate immune system including pathogen-associated molecular patterns (PAMPs)[6]. This progress has been particularly challenging for clinician researchers as they are expected to translate these critical discoveries into meaningful changes and advances in clinical practice, including assessment and treatment of IBD patients. In this review, we will assess the clinical interpretations of major breakthroughs in CD genetics focusing on the NOD2/CARD15 gene, including both the strengths and limitations of such studies and an analysis of significant differences in phenotypes and genotypes, and we will offer suggestions for future studies[7,8].

## CLASSIFICATION OF PHENOTYPE

Clinical classifications of CD seem to be used synonymously with terms for phenotypes, and a number of papers have described "genotype-phenotype

correlations". However, genotypes and phenotypes are derived in fundamentally different ways. While genotype is based upon a limited number of options that can be determined mechanically and thus objectively, derivation of phenotype is subjective and influenced by potentially multiple variables, with many yet to be identified. A number of variables may not be included in multivariate analysis when genotype-phenotype analyses are performed.

The clinical classification systems that have been put forward have been purposefully kept simple. This may have been done to encourage individuals and groups to adopt them and hence maximize opportunities to compare and combine datasets[9-11]. However, given this intention, it does not appear to have been successful for genotype-phenotype studies in CD. The updated Vienna classification for Crohn's disease, now known as the Montreal classification, has maintained a simple approach including age at diagnosis, location, and behavior as the major parameters, with perianal disease as a subgroup of behavior[11]. It has appropriately split age at diagnosis and perianal penetrating from internal penetrating, but has not clarified the issue of stricturing disease working independently of penetrating disease[12]. There is no mention of the need to demonstrate the presence of transmural inflammation, assuming that this is a prerequisite for the diagnosis of CD. This is relevant to subsequent study of those patients with inflammatory CD who do not evolve into a stricturing and/or penetrating pattern. Do these patients fit into the definition of CD, given that they do not appear to show any association with NOD2/CARD15 after correction for disease duration?[13] Other variables that may well influence natural history, including smoking, prior appendectomy, and body mass index, have thus far not been included in this classification[14-16]. The latter may influence development of penetrating disease, the level of inflammation and hence CRP and the risk of metabolic bone disease[17,18].

Clinical classification systems for ulcerative colitis are few. There was no such system included in the Vienna initiative. The Montreal paper presented a simple classification based upon distribution and activity, but both of these parameters are subjective, particularly when the former is based upon macroscopic and not microscopic observations. There is again no inclusion of smoking or prior appendectomy despite strong evidence supporting a role for appendectomy in (re)shaping the natural history of ulcerative colitis.

No classification system or phenotypic approach has attempted to embrace the microscopic pattern of inflammation, damage and repair seen in both CD and ulcerative colitis. Limited work has been done on granulomata and their association with both clinical outcomes and with some genetic polymorphisms[19-21]. This is in contrast to several other fields of research, including liver disease, where histological appearances are used as important aids in determining the risk of fibrosis, and colorectal cancer, in which significant heterogeneity has been identified among cancers based both upon histological appearances and molecular markers[22,23].

## STRENGTHS AND LIMITATIONS OF STUDIES TO DATE

Disease susceptibility and phenotype-genotype analyses have arrived in two "waves" since the seminal publications on NOD2/CARD15 and CD in 2001. The first wave of papers identified the key CD-causing variants in this gene and went on to establish an association with ileal location[12,24-29]. Some studies in this group supported a stronger association with stricturing disease behavior and not ileal location, after multivariate analysis[12,24,29]. Two studies carried out haplotype analysis[27,28]. The second wave included studies from smaller centers, predominantly across Europe, focusing on phenotype-genotype correlations[30,40]. There have been a limited number of studies that have investigated response to therapy[41,42].

The limitations of these studies center around two points: case-control ascertainment and phenotypic classification. Thus far, all of these studies have been sourced from specialist-based cohorts and often from large, tertiary referral centers[12,24-29]. Ascertainment of cases has also been influenced by earlier genetic studies where the primary aim has been "gene discovery" and hence there have been an exaggerated number of CD multiplex families[12,24]. Many of the studies combine the resources of multiple sites, which have contributed to collection of these multiplex families[12,24,25]. The method of ascertainment of controls is not always clear, and in the majority of cases they do not represent true population controls. Specifically, some studies have excluded controls with a family history of IBD thus biasing the population even further.

Since the majority of studies lack power to carry out detailed phenotype-genotype analyses, it is tempting to combine datasets in a meta-analysis. However, this has been hampered by a plethora of different approaches to clinical classification of the case population. Disease location is variously described as "any ileal" and "any colonic", or "ileal and right colon" with "left colon and rectum", without prior validation of these systems[24,26,28]. Similarly, disease behavior, which clearly evolves with time, was modified from the Vienna classification in several studies, again making it difficult to combine datasets. Disease duration has often been omitted. There have been inconsistencies in defining disease location, including the inclusion or exclusion of perianal abscess, combining ileal and ileocolonic locations without giving raw data on these locations individually and providing data on very specific subgroups, such as UC-like CD, where the number in this group ($n$ = 56) is greater than the "colon-only" location as a whole ($n$ = 29). More recent studies have concentrated on using the Vienna classification despite its known limitations, but have in some cases tried to address these limitations by carrying out supplemental analyses including investigation of an association between NOD2/CARD15 and stricturing disease independent of penetrating disease and internal penetrating disease independent of perianal disease.

In summary, studies thus far have provided us with

some important information on the strength of the NOD2/CARD15 association with CD as a susceptibility gene but these have not been based on population-based cohorts. Population-based studies are awaited and may be best coordinated through an international effort using agreed upon methods of case-control ascertainment, genotyping and clinical classification. Associations with ileal location have provided us with important clues to disease pathogenesis, including the role of Paneth cells and defensins. However, extensive further work is required using much larger datasets that include other key variables, such as treatment received, to determine the mechanisms by which NOD2/CARD15 variants may increase the risk of stricturing disease, and whether these variants are also implicated in the development of internal penetrating disease.

## RESULTS OF STUDIES ON NOD2/CARD15 TO DATE

Multiple studies have now investigated the contribution of variants in the LRR domain of the NOD2/CARD15 gene to development of CD. This has been summarized in a recent meta-analysis. Individuals carrying only one high-risk allele had 2.39-fold (95% CI: 2.00-2.86) increased risk of the disease, while those with 2 or more high risk alleles carried a 17.1-fold (10.7-27.2) increased risk of CD compared to individuals without any high-risk alleles. The greatest relative risk was identified for the SNP13 variant (OR 3.76, 95% CI: 3.22-4.38 for one variant) but significant heterogeneity existed among studies ($P = 0.01$)[40].

For the purposes of the rest of this paper, we have selected 15 studies that each provide both genotype and (some) phenotype data on a minimum dataset of 200 CD cases[12,24-36,38]. We will discuss the evidence for associations between NOD2/CARD15 and key clinical variables including age at diagnosis, disease location and behavior, need for surgery and the presence of granulomata. We have carried out further analyses on these studies as a combined dataset and as individual series, where sufficient clinical and genetic data are available in the original publication. We focus specifically on differences in phenotype and genotype among studies, and have carried out meta-analysis for NOD2 associations with disease location and behavior.

## DISEASE LOCATION

Of the 15 studies selected, 13 provide clearly interpretable data on disease location. Of these, 10/13 support a significant association with ileal disease (L1)[12,25-27,31-34,36,38], while some also demonstrate an association with absence of colonic location (L2)[24]. As indicated above, some studies have combined ileal (L1) with ileocolonic (L3) patients to investigate this association. This may be due to a lack of power in subgroup analysis. Clinically, this may be inappropriate. There are limited data on the natural history of ileocolonic disease compared with ileal disease but associations have been made between L3 and younger age at diagnosis and an increased risk of surgical recurrence compared to other disease locations[43,44]. In addition, some patients with L3 may have their major disease burden in the colon, thus weakening the association with NOD2/CARD15 compared to pure ileal disease or L1. Once again, the interpretation of results depends heavily on the clarity of describing phenotypes, and in this case how the presence or absence of ileal disease was determined, whether by endoscopy, histology, radiology or a combination of these.

## DISEASE BEHAVIOUR

Of 13 studies that provided data for the variable disease behaviour, 7 showed a significant association with stricturing behaviour[12,24,29,30,34,36,38]. Two studies show significant positive associations with both stricturing and penetrating disease behaviour[12,34], but one of these excluded perianal penetrating disease from the analysis. If included, this association with NOD2 variants is lost[12]. One study described a negative association with penetrating behaviour[26]. Penetrating disease is defined according to the Vienna classification in 8/13 studies and in another study it is defined as "internal penetrating" behaviour, separating it from perianal penetrating disease[12]. The rest of the studies describe their own "in house" definition of penetrating disease. Given the overlap between these forms of disease behaviour and the length of time some patients may take to develop "complex" CD, supplementary analyses seem necessary to clarify these observations. Specifically, stricturing CD will often occur in combination with penetrating disease, both being hallmarks of transmural inflammation. One may therefore predict that NOD2/CARD15 should be associated with both complications. If this is not the case, it may relate to a lack of association of NOD2/CARD15 with perianal penetrating disease that influences the relationship with penetrating disease overall, as suggested by Brant *et al*[12]. Alternatively, NOD2/CARD15 variants may promote the development of fibrosis over fissuring ulceration. These questions clearly need to be addressed in future studies.

## AGE AT DIAGNOSIS

Five of thirteen studies that looked for an association between NOD2/CARD15 and age of diagnosis found a significant association with early onset of the disease[12,24,26,32,38]. Of these, 4 found association only for NOD2/CARD15 homozygotes and compound heterozygotes and 1 found association with these groups as well as with the frameshift variant alone. Importantly, these findings remained significant after multivariate analysis when important confounders such as disease location and stricturing behaviour were included. These data support the results of previous linkage studies that demonstrated an association between IBD1 and earlier age at diagnosis[45]. They also support the concept of pediatric CD being a "more genetic" disease, consistent with other polygenic disease models.

## OTHER CLINICAL ASSOCIATIONS

Eleven studies provided data on surgery for CD and 8 of

these provided NOD2/CARD15 genotype data on the "surgical" cohort. Two groups have identified an association between NOD2/CARD15 and increased risk of surgery. Lakatos *et al* demonstrated this to have an independent effect on need for surgery after logistic regression (OR 1.71, 95% CI: 1.13-2.62, $P = 0.01$) together with presence of stricturing disease behaviour. This cohort had the largest complete dataset of 527 patients and provided the most comprehensive analysis of NOD2/CARD15 genotype-phenotype relation[38]. The second study showing this association also carried out similar statistical analysis, finding a higher risk for surgery with those carrying two variant alleles (OR 17.8, 95% CI: 4.9-64.3)[36].

## FURTHER ANALYSIS OF CLINICAL CHARACTERISTICS AND GENETIC ASSOCIATIONS

### *Statistical methods*

Differences in proportions among different studies were tested using simple $\chi^2$ distribution obtained as the sum of [(Observed – Expected)$^2$/Expected] with (R-1)*(C-1) degrees of freedom where R is the number of rows (studies) and C is the number of columns (locations, genotypes). The higher contribution to the $\chi^2$ from different studies suggested a higher degree of variation in the distribution of reported location.

Meta-analysis was performed by calculating the odds ratio and confidence interval using the frequency distribution of NOD2 variants and the variable of interest presented by studies involved in the analysis. Fixed analysis used an inverse variance method to calculate weight, and weighted average was calculated as a pooled estimate. Heterogeneity among studies was calculated using Cochran's Q (sum of squared difference between individual study effect and pooled effect across studies). Analysis was performed using Meta command in STATA 9[46].

### *Differences among studies*

Ten studies gave clear data on numbers of patients with pure ileal CD and their NOD2 variant frequencies. The majority of studies had small numbers in this important subgroup of CD (mean, 85 patients, range 23-136) with an overall NOD2 variant frequency of 41.1% (23.4%-53.6%). Comparisons between studies just failed to show a significant difference ($P = 0.06$). However, exclusion of one study from Finland, which is known to have lower NOD2 variant frequencies compared to the majority of other European populations, demonstrated similar proportions in the majority of these studies ($P = 0.53$).

In contrast, a comparison of disease location among 12 studies that provided data on this variable as per the Vienna and Montreal systems, demonstrated highly significant differences ($P < 0.0001$). Unlike the genotype data for NOD2, exclusion of any one study from this analysis did not influence heterogeneity. Proportions among studies did not appear to correlate with geographical region, but two groups emerged from the data; i.e., those with high rates of ileal CD (40%-50%), and those with lower rates of ileal disease (20%-30%). Does disease location for CD vary substantially among Caucasian populations? There are no population-based data to answer this question. Clearly, other factors may play a role, including rate of familial disease, smoking, type of center (medical or surgical bias), and methods of ascertainment. Interestingly, of the four studies with high rates of ileal disease, two had a high rate of familial CD (0.47, 0.86), one had a relatively low rate (0.11), and the fourth did not include data on familiality. Similarly, figures for surgery did not show consistency within this subgroup, ranging from 0.33 to 0.67, and rate of stricturing disease was surprisingly low (0.17-0.23). Alternatively, are there major differences in clinical assessment of patients and a corresponding reduction in interobserver agreement among centers? Studies have recently indicated that this may be so for both CD behavior and location[47,48]. The NOD2 analysis given above indicates that, at least for NOD2 variant ileal CD, there are limited differences among populations. As indicated above at the outset of this review, clinical characterization of CD, and IBD in general, remains a significant challenge.

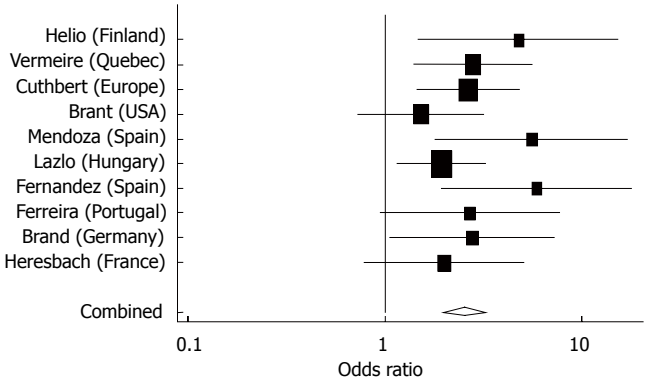


**Figure 1** Meta-analysis comparing odds of having a NOD2 variant (SNPs 8,12 and/or 13) among Ileal versus Colonic CD patients in 10 studies. Pooled estimate = 2.50 (95% CI 1.97-3.25), $P < 0.0001$), test of heterogeneity among studies, $P = 0.49$.

## META-ANALYSIS OF NOD2 ASSOCIATIONS WITH DISEASE LOCATION AND BEHAVIOUR

### *Location*

These analyses confirmed the strong association between NOD2 variants and pure ileal CD compared to pure colonic disease, which was used as the reference group (Figure 1). Similar results were obtained in comparing patients with ileocolonic disease and the reference group (Figure 2). Both meta analyses achieved significance ($P < 0.0001$), while there was no significant heterogeneity demonstrated between studies.

### *Behaviour*

Nine studies provided adequate data for inclusion in these meta analyses, of which four provided data on disease duration (range, 8.2-17.6 years). A strong association was confirmed between NOD2 variants and stricturing CD

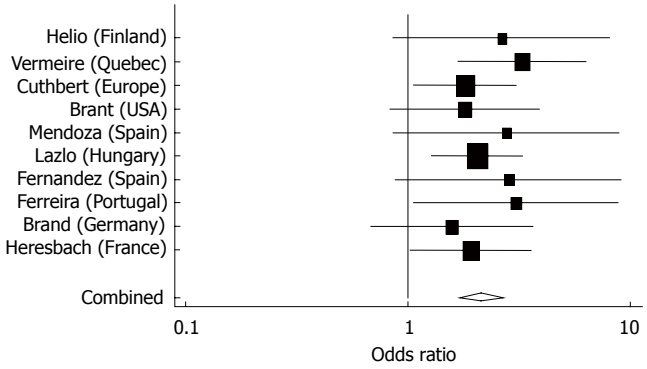


**Figure 2** Meta-analysis comparing odds of having a NOD2 variant among Ileocolonic *versus* Colonic CD patients in 10 studies. Pooled estimate = 2.13 (95% CI 1.7-2.7), $P < 0.0001$, test of heterogeneity among studies, $P = 0.927$.

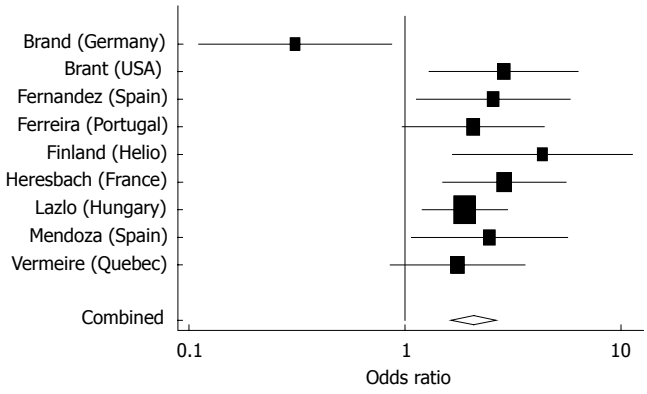


**Figure 3** Meta-analysis comparing odds of having a NOD2 variant among Stricturing *versus* Inflammatory CD patients in nine studies. Pooled estimate = 2.06 (95% CI 1.42-2.98), $P < 0.0001$, test of heterogeneity among studies, $P = 0.02$.

(Figure 3) with inflammatory disease (non-stricturing, non-penetrating) as the reference group. Significant heterogeneity was detected among studies ($P = 0.02$) related to one "outlier". Importantly, and not surprisingly, the other form of complex disease behavior also showed significant association with NOD2 variants (Figure 4). This association is likely to be strengthened if data were available for internal penetrating disease independent of perianal penetrating disease for all studies.

## CONCLUSION

The discovery of NOD2 as the first susceptibility gene for CD has resulted in a leap forward in our understanding of disease pathogenesis. It has also highlighted the heterogeneity of the disease. NOD2 represents the "low-lying fruit" of CD genetics-other susceptibility genes for this disease and for ulcerative colitis may not carry the same relative risk. The phenotype-genotype studies carried out to date have provided us with the critical observations that link NOD2 variants to ileal location, and this has highlighted the role of Paneth cells and antimicrobial peptides. However, there remain a number of questions. All these studies have been drawn from specialist cohorts leaving no accurate figure for NOD2 population attributable risk. There is significant heterogeneity in

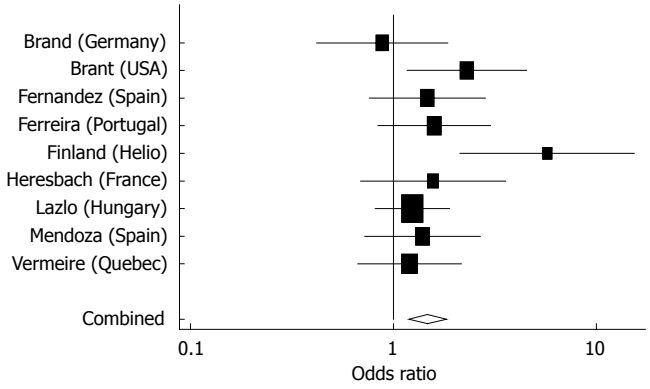


**Figure 4** Meta-analysis comparing odds of having a NOD2 variant among Penetrating versus Inflammatory CD patients in nine studies. Pooled estimate = 1.47 (95% CI 1.19-1.82), $P < 0.0001$, test of heterogeneity among studies, $P = 0.15$.

phenotype among studies, raising concerns with respect to clinical characterization of patients at different centers and thus the utility of current classification systems and interobserver agreement in this field. Our meta-analysis has demonstrated a highly significant association between penetrating CD and NOD2 variants, and confirms the associations with stricturing behaviour and with both pure ileal and ileocolonic locations. These observations highlight the need for further clinical and genetic characterization of phenotypes not associated with NOD2, including inflammatory CD, perianal (penetrating) disease, and pure colonic disease.

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*ESOPHAGEAL CANCER*

# Identification of squamous cell carcinoma associated proteins by proteomics and loss of beta tropomyosin expression in esophageal cancer

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## Abstract

**AIM:** To assess the proteome of normal versus tumor tissue in squamous cell carcinoma of the esophagus (SCCE) in Iranian patients and compare our results with former reports by using proteomics.

**METHODS:** Protein was extracted from normal and tumor tissues. Two dimensional electrophoresis was carried out and spots with differential expression were identified with mass spectrometry. RNA extraction and RT-PCR along with immunodetection were performed.

**RESULTS:** Fourteen proteins were found whose expression levels differed in tumor compared to normal tissues. Mass spectrometric analysis resulted in the identification of β-tropomyosin (TMβ), myosin light chain 2 (and its isoform), myosin regulatory light chain 2, peroxyredoxin 2, annexin I and an unknown polypeptide as the down regulated polypeptides in tumor tissue. Heat shock protein 70 (HSP70), TPM4-ALK fusion oncoprotein 2, myosin light polypeptide 6, keratin I , GH16431p and calreticulin were the up-regulated polypeptides found in tumor tissue. Several of these proteins, such as TMβ, HSP70, annexin I , calreticulin, TPM4-ALK and isoforms of myosins, have been well recognized in tumorigenesis of esophageal or other types of cancers.

**CONCLUSION:** Our study not only supports the involvement of some of the formerly reported proteins in SCCE but also introduces additional proteins found to be lost in SCCE, including TMβ.



**Key words:** Squamous Cell Carcinoma; Esophagus; Esophageal; Proteomics; Two dimensional electrophoresis; Polypeptide marker

Jazii FR, Najafi Z, Malekzadeh R, Conrads TP, Ziaee AA, Abnet C, Yazdznbod M, Karkhane AA, Salekdeh GH. Identification of squamous cell carcinoma associated proteins by proteomics and loss of beta tropomyosin expression in esophageal cancer. *World J Gastroenterol* 2006; 12(44): 7104-7112

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## INTRODUCTION

As a complex disease, cancer arises from a range of genetic alterations that result in both structural and functional cellular changes. Such alterations, including up and down regulation of certain genes, mutations, chromosomal alterations, and suppression or activation of specific genes, confer cells with growth advantage and clonal expansion[1,2]. As a result, cells may acquire the ability to produce new proteins and other proteins that normally are present may be modified, reduced, augmented or even eliminated[3,4]. Quantitative variations in protein abundance represent alteration in functional gene expression, which demands analysis. Proteomics has provided simultaneous analysis of a large number of cellular protein constituents and is the most powerful direct analytical method for protein detection and evaluation[5,6].

Squamous cell carcinoma of the esophagus (SCCE) is the sixth most common cancer in the world. However, in developing countries it ranks fourth and occurs with higher frequencies in certain regions of the world, such as Iran, China, South Africa and France. The last epidemiologic

report indicated the highest incidence rate of this cancer from Iran[8]. So far, many molecular studies have been carried out on this cancer and the role of oncogenes, tumor suppressors, chromosomal abnormalities and other molecular events have been investigated. Noteworthy among these molecular alterations is the down regulation of important tumor suppressor genes and consequently their protein products, such as P53, Retinoblastoma (RB), Mutated in Colon Carcinoma (MCC), and Deleted in Colon Carcinoma (DCC)[9], which further indicates the importance of Tumor Suppressors (TSs) in the carcinogenesis of this type of cancer.

The present report represents further extension of our earlier work on esophageal cancer[10]. Here we applied proteomics as a method of comprehensive analysis of differential protein abundances present in esophageal cancer. Among the fourteen differentially expressed proteins identified in this study, we observed loss of ß-tropomyosin (TMβ) in all tumor samples, a protein that has been suggested to function as a tumor suppressor. Furthermore, this study additionally supports the involvement of TSs in the etiology of SCCE.

## MATERIALS AND METHODS

### Chemicals

All chemicals were purchased from Sigma unless otherwise specified.

### Patients and tissue sampling

Cancerous and normal tissue specimens were collected from 45 patients with SCCE whom underwent surgery. Tissue samples were collected immediately after surgery, wrapped in aluminum foil, snap frozen in liquid nitrogen and maintained at -70℃. The age of the patients at the time of diagnosis ranged from 27 to 86 years (63% males, 37% females) with a mean of 55 years.

### Protein preparation

100-150 mg of tissue was sliced on ice and pulverized under liquid nitrogen using a microdismemberator (Braun, Germany). Subsequently 600 μL homogenization buffer (10 mmol/L Tris-HCL (BioRad), 5 mmol/L $MgCl_2$ PH 7.4) was added to the pulverized tissues, mixed and 10 μL of the following protease inhibitors were added: Pepestatin (1 mg/mL in isopropanol), benzamidine (16 mg/mL in $H_2O$), phenylmethylsulphonyl fluoride (PMSF at 25 mg/mL in isopropanol). To this homogenate, 10 μL of RNaseA (10 mg/mL in homogenization buffer) and DNase Ⅰ (1 mg/mL in homogenization buffer**)** were added and incubated on ice for 20 min. Subsequently, urea at 7 mol/L, thiourea at 2 mol/L, 5% ß-mercaptoethanol and 0.5% SDS were gradually added and the volume of solution was adjusted to 1.5 mL with the homogenization buffer. Samples were centrifuged at high speed for removal of insoluble particles and five μL of each was used for protein concentration assessment using the Bradford assay.

### Two-dimensional electrophoresis

Samples were subjected to isoelectrofocusing (IEF) following to adaptations and slight modification[11]. The first dimension gel was composed of 4.2% acrylamide, 0.22% N, N'methylenebisacrylamide, 8.5 mol/L urea, 0.27% (V/V) Nonidet P-40 (NP-40), 5% sucrose, and 6% ampholytes (pH 4-6, 5-7 and 6-8 at a ratio of 2:1:2, respectively) and 0.05% TEMED (N, N, N', N'-Tetamethylethylene diamine). The solution was degassed and 0.04% ammonium persulfate was added, mixed and poured to a height of 130 mm in cylindrical glass tubes with a 1.5 mm internal diameter. A volume of sample equal to 75 μg total protein was mixed with 0.33 volume of neutralizing buffer (9 mol/L urea, 8% NP-40 and 5% ampholytes pH 3.5-10), loaded on IEF gel, overlaid with 10 μL sample buffer (4 mol/L urea, 1% ampholytes pH 3.5-10) and filled with catholyte. The upper chamber buffer or catholyte was composed of extensively degassed 0.02 mol/L NaOH and the lower chamber buffer or anolyte; 0.01 mol/L phosphoric acid. Isoelectric focusing was applied, without prefocusing, at 300 V for 1 h, afterward at 600 V for 10 h and 800 V for 1 h in order to final focusing. Gels were removed and equilibrated for 20 min at room temperature in equilibration solution (60 mmol/L Tris-HCl, pH 6.8, 2% SDS, 5% β- mercaptoethanol (V/V), 10% glycerol (V/V), and 0.002% bromophenol blue). The second dimension gel consisted of 33.3 mL of 30% stock acrylamide solution and N, N'-methylene bisacrylamide (29.2% and 0.8% W/V respectively), 41.7 mL deionized water and 25 mL separation gel buffer (1.5 mol/L Tris-HCl, pH 8.8, 0.4% SDS [Sodium Dodecyl Sulfate]), 0.034% (W/V) ammonium persulfate and 0.05% TEMED. The equilibrated first dimension gel was layered on the second dimension gel and fixed in place with 1% agarose and electrophoresis was carried out at 30 mA/plate at 10℃ constant temperature by applying a cooling system.

### Protein detection

Proteins were detected using a slight modification of the previously reported method[11]. The gel was fixed (methanol, water, acetic acid and formaldehyde: 50/38/12/0.05 per volume) for at least 1h with constant shaking followed by 3 times 20 min washes with 50% ethanol, pretreated with sodium thiosulfate ($Na_2S_2O_3.5H_2O$; 0.2 g/L) for 1 min, and washed three times each for 20 s with $ddH_2O$. Impregnation of the gel with $AgNO_3$ (1.9 g/L and 0.075% (V/V) of 37% formaldehyde) was carried out and the residual $AgNO_3$ was removed by 3 × 20 s successive washes with de-ionized water. Successively the gel was developed by soaking in developing solution containing $Na_2CO_3$ (60 g/L), 0.05% (V/V) of 37% formaldehyde and 4 mg/L of $Na_2S_2O_3 \cdot 5H_2O$ for 10 min up to the appearance of yellowish brown spots. The gel was then rinsed twice, each for 2 min, with $ddH_2O$. Further development was stopped by immersing the gel in stop solution (50% methanol and 12% acetic acid) and stored in 30% ethanol at 4℃ until scanning.

### Isoelectric point determination

The isoelectric point (pI) of polypeptides was determined by either application of protein PI markers (Sigma) or by determining PI change along the entire length of the first dimension gel[12]. Gels were cut into 0.5 cm long pieces. Each piece was placed in a separate tube containing 0.5

mL of distilled $H_2O$, crushed and incubated at room temperature for 2 h. The pH of each tube was then measured.

### Image analysis

Silver-stained gels were scanned using a calibrated GS-800 densitometer (Bio-Rad) at resolution of 600 dots and 12-bits per inch. Spot detection and gel matching were done according to Melanie-4 default settings and spot pairs were investigated visually (GeneBio, Geneva, Switzerland). Pairs of Two dimensional electrophoresis (2DE) gels composed of tumor and normal gels from each patient were used and the percent volume of each spot was estimated and analyzed for image analysis. The polypeptide variants of each pair of gels (tumor versus normal) were recorded and the most common changes in the polypeptide expression pattern (70% or higher) in all 2DE gels were used as a reference for comparison between tumor and normal gels.

### Immunological detection and localization of actin

Following to the 2DE, gels were equilibrated for 30 min in transfer buffer (25 mmol/L Tris-base, 192 mmol/L glycine, 20% methanol and 0.1% SDS) and polypeptides were electrophoretically transferred to nitrocellulose membranes at 14 V overnight. Membranes were blocked for 2 h in blocking solution (TBST; 100 mmol/L Tris-base or HCl, pH7.5, 0.9% NaCl and 0.05% Tween 20) and exposed to biotinylated anti-actin antibody at 1/3000 dilution for 1 h with constant shaking. Membranes were subsequently washed 3X with TBST, incubated with strptavidin conjugated alkaline phosphatase in TBST at 1/4000 dilution and further incubated (1 h) with constant shaking. Membranes were washed two times with TBST, once with TBS and exposed to color solution (33 μL NBT from 50 mg/mL stock solution in 70% dimethylformamide and 17 μL BCIP from 50 mg/mL stock solution in 100% dimethylformamide). To stop excess color formation, membranes were washed with distilled water[13].

### RNA extraction and RT-PCR of TMβ and beta actin

Total RNA was extracted by acid phenol method and the first strand cDNA was synthesized applying oligo dT 18 (Roche). Subsequently amplification was carried out using the forward primer 5'-GGC TGA TGA GAG CGA GAG AG-3' and the reverse primer 5'-GCA CTG GCC AAG GTC TCT TC-3' for amplification of TMβ and 5'-TGA CGG GGT CAC CCA CAC TGT GCC CAT CTA-3' as forward and 5'-CTA GAA GCA TTT GCG GTG GAC GAT GGA GGG-3' as reverse primers for amplification of beta actin. The PCR condition was composed of primary denaturation at 95℃ 1 min followed by 30 cycles of amplification according to the subsequent scheme; denaturation 1 min at 95℃, annealing at 56℃ 1 min and extension at 72℃ 1 min and final extension at 72℃ for 10 min. Subsequently 4 μL of the PCR product was used for agarose gel electrophoresis.

### Mass spectrometry

Silver stained protein spots containing the proteins of interest were destained thoroughly with 1% $H_2O_2$ (typically 1 min) and lyophilized to dryness[14]. Silver stain removal using $H_2O_2$ was performed to enhance peptide adsorption by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI)[14]. The dehydrated gel bands were hydrated with 15 μg/L (Promega, Madison, WI) of porcine trypsin in 25 mmol/L $NH_4HCO_3$, pH8.2 on ice for 45 min. Excess trypsin was removed; gel bands were covered with 25 mmol/L $NH_4HCO_3$, pH8.2 and incubated at 37℃ overnight. Tryptic peptides were extracted from the gel bands with 70% acetonitrile and 0.1% trifluoroacetic acid. Samples were desalted with C18 Zip Tips (Millipore, Bedford, MA) as per manufacturer's protocols. 0.5 μL of sample was co-crystallized with 0.5 μL of α-cyano-4-hydroxycinnamic acid in 50% acetonitrile, 1% trifluoroacetic acid and spotted directly on a stainless steel MALDI target plate. Mass spectra were acquired using a MALDI-TOF/TOF mass spectrometer (Voyager 4700, Applied Biosystems, Foster City, CA). MALDI-TOF/TOF spectra were internally calibrated (< 20 ppm) using trypsin autolysis products. Post-acquisition baseline correction and smoothing was carried out using software provided with the TOF/TOF instrument. Spectra were submitted to Mascot (http://matrixscience.com) for peptide mass fingerprinting.

## RESULTS

### Protein extraction, 2DE and image analysis

Before doing 2DE, the concentrations of extracted proteins from tissue samples were determined. The protein extraction efficiencies were similar for tumor tissues and their adjacent normal nonmalignant tissues. The yields of extractions were approximately 80 μg/mg for both tumor and normal tissues. Subsequently seventy five micrograms of proteins from either tissue type were used in the first dimension. Because of the epithelial origin of squamous cell carcinoma, the mucosa of the normal non malignant tissues were separated from the rest of the esophageal tract for protein extraction and proteome comparison with corresponding tumors. For further verification that an equal amount of protein from either type of tissue was applied, immunological detection of actin was carried out following to 2DE. Following electrophoresis, the resolved proteins were recorded for each of the 2DE gel pairs. The densitometry map contained almost 800 features ranging in the molecular masses from 10 to 220 kDa and pIs from 4.8 to 8.0. For deciphering tumor-associated polypeptides, 2DE gels of tumor tissues were compared with their corresponding matched normal tissues. Compared with the matched normal tissues, 92 definite proteins (spots) in 2DE gels from 45 tumor tissues belonging to 45 patients were found indicating lower levels of expression. In contrast, 88 spots were identified in tumors that indicated higher levels of expression with respect to the 2DE gels of their corresponding matched normal tissues. To eliminate the possibility of artifacts, pairs of 2DE gels (tumor versus normal for each patient) were matched and compared and spot variants that occurred most often, or at least in 70% or higher of all 2DE patterns, were considered to be significant and were used for further comparisons. There were 7 out of 92 spots that indicated lower levels

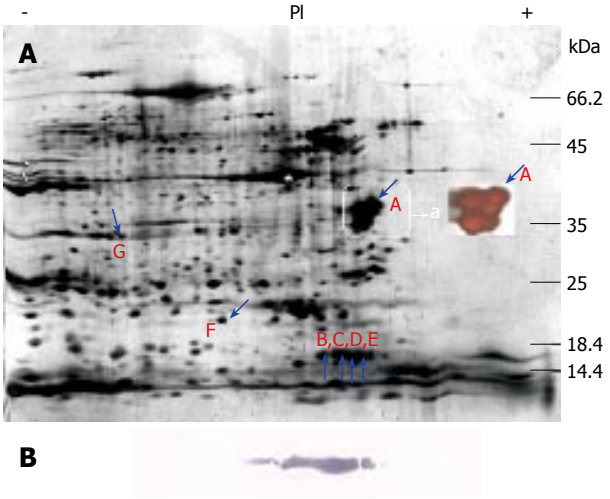


**Figure 1** **A**: A representative 2DE gel of a normal tissue. Proteins that become down-regulated in corresponding tumor (Figure 2A) are shown with arrows and capital letters. For a better visualization of spots within the box, silver stained image of another gel is shown; **B**: Immunodection of actin as an internal control of protein loading, the * represents the location of actin.

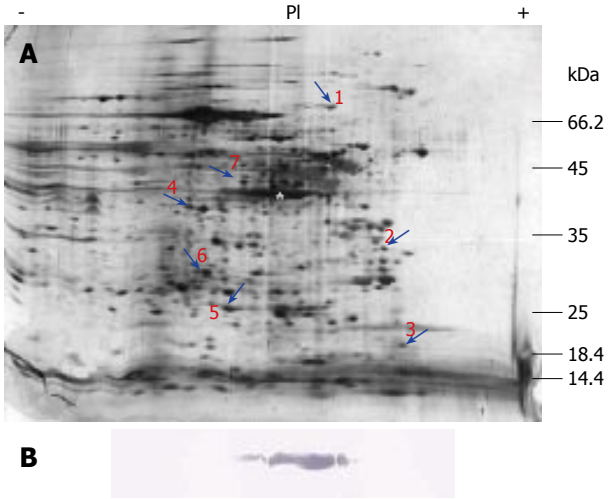


**Figure 2** **A**: A representative 2DE gel of tumor tissue. Arrows and numbers indicate up-regulated proteins in comparison with their matched normal tissue (Figure 1A); **B**: Immunodetection of actin and the * represents the location of actin.

**Table 1** Mass spectrometric identification and characteristics of the 14 proteins whose expression were subjected to change in SCCE

| Polypeptide | Tissue specification | Protein name | Accession number based on NCBI | Chromosome | PI/MW | Expression profiling or level |
|---|---|---|---|---|---|---|
| A | Normal | Beta tropomyosin | NM-003289 | 9p13.2-p13.1 | 5.5/40.3 | Disappearance |
| B | Normal | Myosin regulatory light chain 2 | XM-027060 | 18p11.31 | 5.45/17.6 | Down Regulation |
| C | Normal | Myosin light chain 2 | NM-000432 | 16p11.2 | 5.6/17.6 | Down Regulation |
| D | Normal | Unknown | NC-000963 | Map No | 5.8/17.6 | Down Regulation |
| E | Normal | Myosin light chain 2 | NM-000432 | 16p11.2 | 6/17.6 | Down Regulation |
| F | Normal | Peroxiredoxin 2 | NM-005809 | 19p13/2 | 6.6/26 | Down Regulation |
| G | Normal | Annexin 1 | NM-000700 | 9 | 7.3/35.5 | Down Regulation |
| 1 | Tumor | Heat shock protein 70 kD | XM-044201 | Unpublish | 6/64 | Up Regulation |
| 2 | Tumor | TPM4-ALK fusion oncoprotein 2 | A186109 | 19p13/1 | 5.5/32.5 | Up Regulation |
| 3 | Tumor | Myosin light polypeptide 6 | NM-0211019 | 12p13/3 | 5.4/20.1 | Up Regulation |
| 4 | Tumor | Keratin 1 | AF304164 | 12q13 | 6.9/42.5 | Up Regulation |
| 5 | Tumor | DNA directed RNA polymerase B (ropB) | NC-000915 | | 6.1/27.2 | Up Regulation |
| 6 | Tumor | GH16431P | Ay051511 | | 6.6/30.1 | Up Regulation |
| 7 | Tumor | Calreticulin | NM-004343 | 19p13/3 | 5.2/50 | Up Regulation |

of expression and 7 out of 88 spots that indicated higher levels of expression respectively. Figures 1A and 2A represent the resulting 2DE gels of the normal and the corresponding tumor tissues of one patient.

To identify whether an equal amount of protein was applied in 2DE, immunodetection of actin was carried out as an internal control of loading (Figures 1B and 2B). As Figures 1B and 2B show, equal amount of proteins were applied in the first dimension. The 14 observed differentially abundant proteins are indicated by arrows (Figures 1A and 2A). The proteins that were observed with decreased abundance in tumors, compared with their corresponding normal tissues, are labeled alphabetically as A, B, C, D, E, F and G. Spots B, C and E are observed in a chain of spots and likely indicate that they are isoforms or modified forms of a specific protein. On the other hand, those proteins that were observed to have an increased abundance in tumors were numbered as 1, 2, 3, 4, 5, 6 and 7. The apparent molecular weight of polypeptides, approximate isoelectric points and changes in expression level, is presented in Table 1. It was found that spot A was absent in tumor samples whereas, spots B, C, D and E were considerably down regulated and spots F and G were shown to be more than two-fold down regulated. It suggests that down regulated proteins were involved in the maintenance of normal phenotype whereas the other up-regulated polypeptides were involved in the development of malignancy.

### *Mass spectrometric analysis, identification of polypeptides and RT-PCR*

Following to 2DE, spots of interest were excised, in-gel

**Table 2 An example of MALDI/TOF/TOF mass spectrometry. The identified protein was β tropomyosin, one of the fourteen identified proteins as designated by letter A in Figure 1A**

| Calculated mass | Observed mass | Start Seq. | End Seq. | Sequence | Modification |
|---|---|---|---|---|---|
| 846.4679 | 846.433 | 232 | 238 | LKEAETR | |
| 894.4679 | 894.4463 | 162 | 168 | YEEVARK | |
| 916.4734 | 916.4508 | 192 | 198 | QLEEELR | |
| 1107.5792 | 1107.5211 | 239 | 248 | AEFAERSVAK | |
| 1143.6116 | 1143.5856 | 190 | 198 | ARQLEEELR | |
| 1170.6727 | 1170.6464 | 169 | 178 | LVILEGELER | |
| 1243.6528 | 1243.6317 | 92 | 101 | IQLVEEELDR | |
| 1262.597 | 1262.5764 | 179 | 189 | SEERAEVAESR | |
| 1298.7677 | 1298.7423 | 168 | 178 | KLVILEGELER | |
| 1332.6389 | 1332.6127 | 78 | 90 | ATDAEADVASLNR | |
| 1343.6801 | 1343.6615 | 38 | 48 | QLEEEQQALQK | |
| 1399.7539 | 1399.7324 | 91 | 101 | RIQLVEEELDR | |
| 1443.8053 | 1443.7762 | 106 | 118 | LATALQKLEEAEK | |
| 1460.7339 | 1460.7119 | 77 | 90 | KATDAEADVASLNR | |
| 1471.775 | 1471.7477 | 38 | 49 | QLEEEQQALQKK | |
| 1488.74 | 1488.715 | 78 | 91 | ATDAEADVASLNRR | |
| 1493.7338 | 1493.7158 | 141 | 152 | MELQEMQLKEAK | Oxidation (M) |
| 1616.835 | 1616.8109 | 77 | 91 | KATDAEADVASLNRR | |
| 1671.8911 | 1671.8682 | 169 | 182 | LVILEGELERSEER | |
| 1702.8792 | 1702.8088 | 36 | 49 | CKQLEEEQQALQKK | |
| 1719.8582 | 1719.834 | 192 | 205 | QLEEELRTMDQAL | Oxidation (M) |
| 1727.8922 | 1727.8687 | 92 | 105 | IQLVEEELDRAQER | |
| 1799.9861 | 1799.9597 | 168 | 182 | KLVILEGELERSEER | |
| 1817.8776 | 1817.8594 | 153 | 167 | HIAEDSDRKYEEVAR | |
| 1883.9933 | 1883.9731 | 91 | 105 | RIQLVEEELDRAQER | |
| 1946.9963 | 1946.9734 | 190 | 205 | ARQLEEELRTMDQALK | Oxidation (M) |
| 2202.1248 | 2202.1091 | 106 | 125 | LATALQKLEEAEKAADESER | |
| 2414.252 | 2414.2375 | 169 | 189 | LVILEGELERSEERAEVAESR | |
| 2534.1636 | 2534.271 | 199 | 220 | TMDQALKSLMASEEEYSTKEDK | |
| 3751.876 | 3751.9631 | 252 | 284 | TIDDLEETLASAKEENVEIHQTLDQTLLELNNL | |

digested and analyzed with a MALDI/TOF/TOF (Matrix-assisted laser desorption/Ionization/ Time of Flight/ Time of Flight) mass spectrometer. The observed peptide mass spectra were analyzed for protein identification by peptide mass fingerprinting that resulted in the identification of the 14 proteins that are listed in Table 1. The identified protein corresponding to spot A was found to be β-tropomyosin as shown in Table 2. As mentioned above, this protein was found to be lost in tumors thus, for further validation of results achieved regarding with TMβ, RT-PCR was conducted by applying TMβ specific primers. As Figure 3A indicates, the 431 bp amplification product is only limited to the cDNA synthesized from RNA extracted from normal tissue. Applying beta actin as an internal control of RT-PCR, which gives rise to a 670 bp amplification product (Figure 3B), and running the extracted RNA (Figure 3C) indicate that lack of amplification in tumor tissues should be due to the loss of expression of TMβ. These observations further point out that down regulation of TMβ occurs at transcriptional level in SCCE. As discussed later in the Discussion section, TMβ is a down stream target for several oncogenes and functions as a tumor suppressor gene.

## DISCUSSION

Former studies on SCCE have led to the identification of Annexin I [15,16], tumor rejection antigen (gp96)[16], clustrin[17], tropomyosin 3, retinoblastoma binding like protein and K506 binding protein[18] as potential markers of esophageal and other types of cancers, some of which were also identified in the present study for instance loss of TMβ in breast cancer[19], myosin light chain 2 isoforms in transformed osteosarcoma[20], peroxiredoxin Ⅱ in pancreatic adenocarcinoma[21], calreticulin in bladder[22] and colon cancers[23], HSP70 in hepatocellular[4] and keratin 1 in gastric carcinoma cell lines[24]. In addition to those proteins previously introduced for esophageal cancer, we identified new proteins, among which TMβ was found to be completely absent in all tumor samples. Therefore we suggest this protein as a potential marker for SCCE. Loss or down regulation of TMβ in cancer is not restricted to our study. Studies on other types of cancers such as oral tongue squamous carcinoma[25] and carcinomatous breast lesions[26] have also led to this observation.

Different studies have indicated that TMβ functions as a tumor suppressor. For example, reversion of neoplastic phenotype, anti-angiogenic activity[27,28], induction of slow growth rate, anchorage, cytoskeleton organization in breast cancer MCF7 cell lines[29] and anoikis[19] are all the consequence of TMβ expression. TMβ is required for cytoskeleton establishment and mediation of TGF-β mediated stress fiber formation. Alternatively, the Ras-ERK pathway antagonizes with TGF-β induced stress fiber formation by suppressing TMβ expression[30]. Indeed TMβ is a down stream target for the ras oncogene[31]. To the best of our knowledge, no strong evidence has been documented regarding with the involvement of the

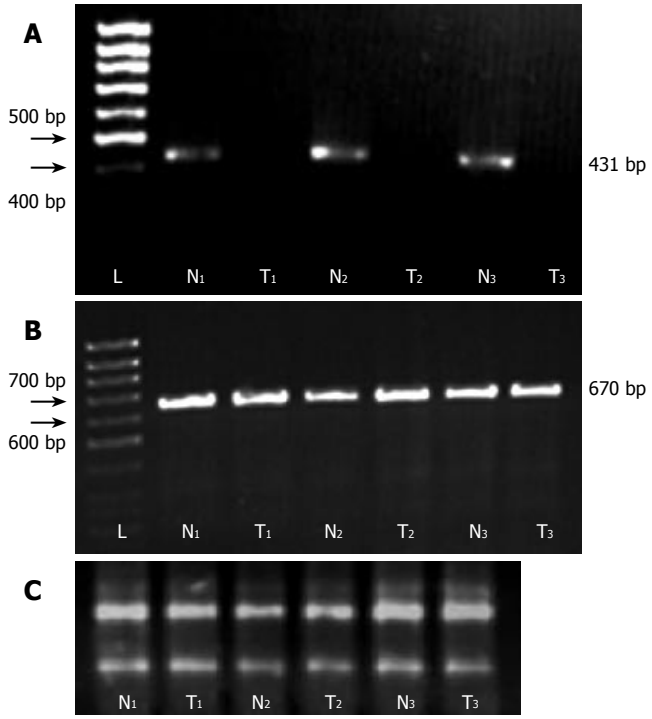


**Figure 3** **A**: Verifying differential expression pattern of ß tropomyosin by RT-PCR in three separate experiments as indicated by numbers (1 to 3), normal versus tumor tissues. The amplification product (431bp) is limited to normal tissue which indicates loss or strong down regulation of this protein as observed by 2DE; **B**: RT-PCR amplification of ß-actin as an internal control of RT-PCR; **C**: Electrophoresis of the total RNA from normal and tumor tissues used for cDNA synthesis and RT-PCR. L: DNA marker; N: normal tissue; T: tumor tissue.

ras family of oncogenes in esophageal carcinogenesis, therefore it is likely that other oncogenes may be involved in the down regulation of TMβ, such as TGF-α and activation of EGFR, both of which participate in the down regulation of TMα and β[32]. Interestingly high levels of TGF-α were reported in all gastrointestinal cancers, including the late stages of esophageal cancer[33], along with amplification of EGFR in esophageal cancer[34]. Loss of TMβ in tumors suggests that this protein may play an important role in tumor suppression of esophageal carcinogenesis.

In addition to TMβ, down regulation of several other proteins was also observed in this study, including annexin Ⅰ. As we mentioned above, reports on the loss of annexins Ⅰ or its isoforms was not limited to our study but other research groups[15,16,37] have also observed this event. It was suggested that such a loss of expression in esophageal and prostate tumors is an early event[15] of carcinogenesis. Hypermethylation of annexin Ⅰ was suggested as another mechanism leading to inactivation and loss of expression in cell lines such as B cell non-Hodgkin's lymphoma[35]. Annexin Ⅰ induces both spontaneous and caspase-3 activated apoptosis[36]. Furthermore, mutations in p53 were also found to result to the down regulation of annexin 10, another member of the annexins family in hepatocellular carcinoma, along with vascular invasion, early recurrence and poor prognosis[38]. We formerly showed mutations in the p53 tumor suppressor gene in SCCE[10], thus it is possible that such mutations correlate with down regulation of annexin 10 as well as annexin Ⅰ. It is well-demonstrated that EGFR amplification occurs in SCCE[34]. On the other hand, annexin Ⅰ undergoes phosphorylation and inactivation by EGFR[39]. Thus our result additionally supports former report[16] regarding with loss of annexin Ⅰ in SCCE.

To our knowledge this is the first report introducing down regulation of peroxyredoxin Ⅱ in SCCE. Peroxiredoxins are members of the phase Ⅱ detoxifying enzymes involved in cytoprotection and cellular defense against oxidants by detoxifying xenobiotics and oxidants, especially $H_2O_2$. By reducing $H_2O_2$, peroxyredoxins protect cells from oxidative stress induced apoptosis and proapoptotic signals. Peroxyredoxins inhibit $H_2O_2$ induced signal cascade in response to PDGF and EGF. They also inhibit NFκB and tumor necrosis factor α in response to external $H_2O_2$ and $H_2O_2$ induced expression of proapoptotic protein; BAX[40,41]. Thus, it is possible that loss or down regulation of this protein or other members of detoxifying enzymes in esophageal epithelium, which normally could be exposed to stresses, contribute to the carcinogenesis by cellular failure in detoxification of oxidative agents affecting this tissue.

Loss of muscle forming proteins is an important cause of body weight loss or cachexia in cancers primarily due to loss of the myosin heavy chain[42]. Myosin light chain 2 is an indispensable component of cytoskeleton, migration and apoptosis[43]. Down regulation of myosin light chain 1 and myosin regulatory light chain 2 proteins was shown in oral tongue squamous cell carcinoma[25]. Due to a common embryonic origin of oral and esophagus tissues, strong down regulation of myosin regulatory chain 2 indicates an important role for this protein in both organs. Formerly Kumar and Chang have shown that transformation of osteosarcoma cells is associated with complete loss of myosin light chain 2 isoforms, which indicates myosin light chain 2 as a target for cellular transformation and malignancy[20]. Our report is the first demonstrating down regulation of myosin light chain isoforms in esophageal cancer. Alternatively, up regulation of myosin light polypeptide 6 in tumors could possibly be due to the selective down regulation of myosin heavy chain and other isoforms of myosin light chain. Supporting our results is the report of Samoszuk *et al* that have shown up regulation of this protein in clonogenic human breast cancer[44].

We also found several up regulated proteins in tumors, among which is heat shock protein 70. Investigations on gastric, hepatocellular and colonic adenocarcinoma cancers have indicated higher levels of expression of HSP70 along with other stress associated proteins[4,45]. HSP70 exerts antiapoptotic activity by suppressing tumor necrosis factor induced apoptosis and caspase 3 down stream events[46], interfering with SAPK/JNK and ceramide induced apoptosis and cleavage of caspase 3 substrate enzyme PARP, which is involved in DNA repair[47]. Theses findings have raised the idea that HSP70 not only should be considered as a tumor marker for HCV-caused hepatocellular carcinoma[48] but also as a target for cancer treatment[49]. Thus, as an organ of the digestive system, esophagus carcinogenesis might follow the same molecular path as other organs of this system.

Calreticulin and keratin 1 were among other up

regulated proteins in tumors. The involvement of calreticulin in tumors has been reported by several groups. Rendon Huerta *et al*[50] have shown that protein kinase C isoforms bind and phosphorylate calreticulin. This would suggest a possible role for calreticulin in signal transduction and involvement in cell division. It was also found that calreticulin regulates p53 function by affecting its rate of degradation and nuclear localization[51]. Thus, association of p53 mutations with esophageal carcinogenesis[10] as well as p53 degradation could explain the role of calreticulin in the development of esophageal tumor. In addition to calreticulin we also found overexpression of Keratin 1, a member of keratin family in SCCE. Formerly Trask and colleagues have shown different expression patterns of keratin isoforms in normal versus tumor tissues[52]. They proposed the expression pattern of keratin isoforms as biomarkers for differentiating normal from malignant cells. Furthermore, Kinjo and coworkers have shown overexpression of keratin 1 in colonic adenocarcinoma cells[53] and Nishikawa *et al*[24] have found up to 17 folds of truncated keratin 1 in Epstein-Barr virus transfected gastric carcinoma cell lines. In esophageal cancer, several groups have reported an increased level of cytokeratin 19 (keratin 19) in serum and secretion by cell lines[54]. The later groups have proposed cytokeratin 19 to have the best specificity and sensitivity as a prognostic marker for evaluating esophageal cancer. Thus involvement of another member of the keratin family in SCCE not only supports former results but also introduces a new marker from this family.

Activation of anaplastic leukemia kinase (ALK) is an example of chromosomal abnormalities, such as translocations, that lead to fusion proteins. ALK was first identified in anaplastic large cell lymphomas (ALCL) and subsequently in inflammatory myofibroblastic tumors (ITM). The aberrantly expressed protein activates several protein kinases, such as IP3 kinase, STAT5 and transcription factors, which end up to mitogenic effects[55]. The fusion of ALK with nucleoplasmin is frequently observed in ALCL. Walking on chromosome 2, Lamant *et al* have shown the fusion of tropomyosin 3 and ALK as a result of t (1; 2) (q25; p23)[56]. They suggested that such a fusion provides an active promoter for ALK, because nucleoplasmin is a housekeeping gene and TM3 is constitutively expressed. Further studies led to the identification of not only TM3-ALK but also TM4-AKL fusion proteins in patients with inflammatory myofibroblastic tumors[57] and as a result perturbation of normal tumor suppressor activity of TM isoforms. It was also suggested that the extent of such rearrangements and fusions could possibly engage other proteins. Here we demonstrated that expression of ALK is not restricted only to the neural but also to other tumors. TPM4-ALK fusion protein is also among proteins being introduced for the first time in SCCE.

There were also three other proteins found in SCCE as homologues of formerly identified proteins in other organisms. Thus, we interpret them to be newly found proteins in SCCE. Among these proteins, two were up regulated in tumors that were identified to be DNA-directed RNA polymerase Beta subunit (ropβ) with almost 100% (99.998) homology with that of *H pylori*. The other protein, spot 6 had a mass spectrum that matched with a high score (99.925) with GH16431P of Drosophila melanogaster (NCBI ACCESSION; AAK92935). Looking into patients pathology files indicated that almost all were *H Pylori* positive, thus we think that up regulation of this protein in tumors, to some extent, may be due to contamination with this bacterium. However, the question that remains unanswered is why such contamination was not observed in normal tissues. A possible explanation could be a preferentially higher growth of bacteria in tumors than in normal tissues. The other protein was the spot D in normal tissue whose mass spectrum matched with high score (99.963) to an unknown protein (NCBI Accession; NC 000963). This protein is highly expressed in normal tissue while loss of it was observed in tumor specimens. Being an unknown protein, further studies are required to reveal its true biological activity in tumor suppression.

In conclusion, this is the first report on the proteomics of SCCE from Iran, a country with the highest incidence rate of SCCE[8]. We were able to identify fourteen differentially expressed proteins in esophageal tumors. Our study further showed that several proteins, which are commonly affected in other digestive organs such as liver, are also affected in SCCE, which may indicate a common molecular mechanism for digestive system tumorigenesis and putative candidate biomarkers. It should also be mentioned that our study brings further evidence for the importance and requirement of a deeper investigation of the molecular events down stream to the EGFR activation in SCCE. The expression levels of several proteins identified in our study is affected by the activation of this receptor. To our knowledge a general molecular marker has not been introduced for cancers, however, finding the disappearance of TMβ in SCCE could introduce a useful tumor marker for SCCE diagnosis, evaluation and follow up.

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# Hypermethylation and expression regulation of secreted frizzled-related protein genes in colorectal tumor

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## Abstract

**AIM:** To investigate the functions of promoter hypermethylation of secreted frizzled-related proteins (sFRPs) genes in colorectal tumorigenesis and progression.

**METHODS:** The promoter hypermethylation and expression of *sFRP* genes in 72 sporadic colorectal carcinomas, 33 adenomas, 18 aberrant crypt foci (ACF) and colorectal cancer cell lines RKO, HCT116 and SW480 were detected by methylation-specific PCR and reverse transcription PCR, respectively.

**RESULTS:** None of the normal colorectal mucosa tissues showed methylated bands of any of four *sFRP* genes. *sFRP1*, *2*, *4* and *5* were frequently methylated in colorectal carcinoma, adenoma and ACF (*sFRP1* > 85%, *sFRP2* >75%, *sFRP5* > 50%), and the differences between three colorectal tissues were not significant ($P > 0.05$). Methylation in colorectal tumors was more frequent than in normal mucosa and adjacent normal mucosa. The mRNA of *sFRP1-5* genes was expressed in all normal colorectal mucosa samples. Expression of *sFRP1, 2, 4* and *5* and *sFRP1*, *2* and *5* was downregulated in carcinoma and adenoma, respectively. The downregulation of sFRP2, 4 and 5 was more frequent in carcinoma than in adenoma. Expression of *sFRP3* which promoter has no CpG island was downregulated in only a few of colorectal tumor samples (7/105). The downregulation of *sFRP1*, *2*, *4* and *5* expression was significantly associated with promoter hypermethylation in colorectal tumor. After cells were treated by DAC/TSA combination, the silenced *sFRP* mRNA expression could be effectively re-expressed in colorectal cancer cell lines.

**CONCLUSION:** Hypermethylation of *sFRP* genes is a common early event in the evolution of colorectal tumor, occurring frequently in ACF, which is regarded as the earliest lesion of multistage colorectal carcinogenesis. It appears to functionally silence *sFRP* genes expression. Methylation of *sFRP1*, *2* and *5* genes might serve as indicators for colorectal tumor.



**Key words:** Colorectal tumor; *Secreted frizzled-related protein* genes; Methylation; Indicator; Re-expression

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## INTRODUCTION

Secreted frizzled-related proteins (sFRPs) comprise a family of five secreted glycoproteins that antagonize Wnt canonical and noncanonical signaling by different mechanisms directly or indirectly[1]. Wnt signaling regulates cell growth, motility and differentiation in animal development and has increasingly been implicated in tissue homeostasis in adult organisms. Aberrant Wnt signaling pathway is an early progression event in 90% of colorectal cancers, contributing to the growth, proliferation and loss of apoptosis of tumor cells[2]. Thus, role of sFRP as a negative regulator of Wnt signaling may have important implications in tumorigenesis, and its downregulation has been correlated with human cancers. To date, no mutation in *sFRP* genes has been associated with their dysregulation in tumors. Aberrant hypermethylation of CpG islands in gene promoter has been found to be a primary mechanism in the inactivation of several tumor suppressor genes. As we know, colorectal tumor is one of the tumors with high frequencies of gene methylation[3,4]. Epigenetic gene silencing plays an extremely important role in the early stage of colorectal tumorigenesis. In this study, we examined hypermethylation and expression of *sFRP* genes in different stages of colorectal tumor and colorectal cancer cell lines in order to investigate the functions of promoter hypermethylation of *sFRP* genes in colorectal tumorigenesis and progression, and whether it can serve as an indicator for colorectal tumor.

## MATERIALS AND METHODS

### Materials

**Tissue samples:** Tissue samples, including 72 sporadic colorectal carcinomas, 33 sporadic colorectal adenomas (male 56, female 49, age 27-76 years), 38 adjacent normal mucosa from 28 colorectal carcinomas and 15 colorectal adenomas, and 20 normal colorectal mucosa tissues (male 11, female 9, age 20-54 years) were obtained from surgical resection of patients receiving endoscopy examination at the clinic of Zhongnan Hospital of Wuhan University and The Tumor Hospital of Hubei Province between October 2003 and April 2006. Eighteen ACFs from 15 colorectal carcinomas were also collected[5]. Samples were stored at -70℃ until processing. None of the patients had received chemotherapy or radiation therapy prior to surgery. All patients gave informed consent for their participation in the study which had been approved by the Ethical Committee of our university.

**Cell culture and treatment:** Three colorectal carcinoma cell lines, including RKO, HCT116, and SW480 (cell lines were purchased from China Center for Type Culture Collection) were used in this study. Cell lines were cultured in RPMI 1640 medium (GIBCO BRL) supplemented with 10% fetal bovine serum and treated with 5-aza-2' -deoxycytidine (DAC), a DNA methyltransferase (DNMT) inhibitor, and trichostatin A (TSA), a histone deacetylase (HDAC) inhibitor. Three cell lines all received four different treatments: low-dose DAC (200 nmol/L, 48 h), high-dose DAC (5 μmol/L, 72 h), TSA (300 nmol/L from a 1.5 mmol/L ethanol-dissolved stock, 24 h), and DAC (200 nmol/L, 48 h) followed by addition of TSA (300 nmol/L, 24 h), the drugs and medium being replaced every 24 h.

### Methods

**Methylation analysis:** Methylation-specific PCR (MSP) was used to examine the promoter hypermethylation of *sFRP1, 2, 4* and *5* genes. Genomic DNA was extracted by the phenol-chloroform standard methods. Bisulfite genomic DNA modification and purification was performed as previously reported[6]. The modified DNA was amplified by methylated and unmethylated primers (exact primers are available upon request). Products were visualized by 12% polyacrylamide gel electrophoresis. MSP was not performed for *sFRP3*, which has no CpG island in promoter region.

**Reverse transcription PCR (RT-PCR) analysis:** RT-PCR was employed to detect *sFRP1-5* genes mRNA expression. TRIZOL Reagent (Invitrogen) was used to extract total RNA from tissues and cells. The integrity of RNA was identified by methanal denatured agarose electrophoresis. Ultraviolet-visible light spectrophotometer (Beckman Coulter DU530) was used to determine $A_{260/280}$ of total RNA. Reverse transcription was performed on 1 μg of total RNA in a reaction volume of 20 μL. PCR assays were performed in 50 μL volume using 100 ng cDNA. Glyceraldehyde phosphate dehydrogenase (*GAPDH*) was employed as an internal reference gene to ensure cDNA quality and loading accuracy. Products were visualized by 2.5% agarose electrophoresis, and then analyzed by gel imaging system (SynGene GGM) and GeneTools software. RT-PCR was performed three times for the tumor samples with detectable mRNA, and then the gene expression index (density Lum of samples mRNA/density Lum of *GAPDH* mRNA) of these samples was compared with the normal samples (*t* test). Those with reduced mRNA expression and $P < 0.01$ were considered as significantly downregulated.

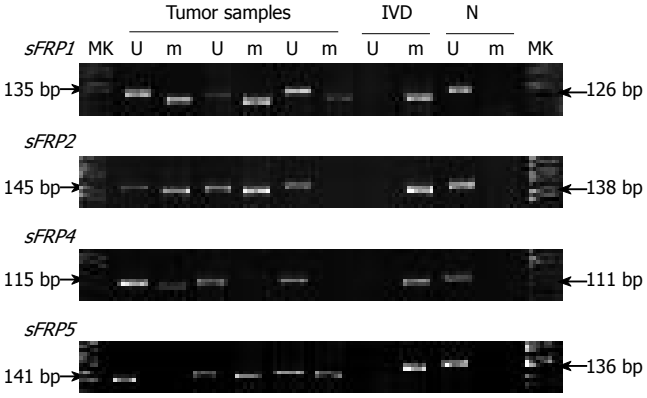


**Figure 1** Hypermethylation of *sFRP* genes in colorectal tissues. Tumor samples showed unmethylated (U) and methylted (m) bands, but normal tissues (N) showed only unmethylated bands. The *in vitro* methylated DNA (IVD) serves as a positive control of methylated *sFRP* genes.

### Statistical analysis

Statistical analysis was performed using SPSS 11.5 software. Associations between the discrete variables were assessed using the two-sided Fisher's exact test or Pearson's chi square tests. *P* value less than 0.05 was regarded as statistically significant.

## RESULTS

### Methylation of sFRP genes

None of normal mucosa showed methylated bands. *SFRP1, 2, 4* and *5* were methylated in 93.1% (67/72), 83.3% (60/72), 36.1% (26/72) and 52.8% (38/72) of colorectal carcinoma; 87.9% (29/33), 81.8% (27/33), 24.2% (8/33) and 57.6% (19/33) of colorectal adenoma (Figure 1); 52.6% (20/38), 28.9% (11/38), 2.6% (1/38) and 18.4% (7/38) of adjacent normal mucosa to colorectal carcinomas and adenomas, respectively. Most of *sFRP* genes were methylated in tumor samples when methylation was presented in the adjacent normal mucosa from the same sample, *sFRP1* (20/20), *sFRP2* (9/11), *sFRP4* (1/1) and *sFRP5* (6/7). The tumors showed stronger methylation signals than the adjacent normal tissues (Figure 2).

The methylation of each of the *sFRP1, 2, 4* and *5* genes was significantly different between carcinoma and normal mucosa ($P < 0.001$ for *sFRP1, 2* and *5*, $P = 0.002$ for *sFRP4*), between carcinoma and adjacent normal mucosa ($P < 0.001$ for *sFRP1, 2, 4* and *5*), between adenoma and normal mucosa ($P < 0.001$ for *sFRP1, 2* and *5*, $P = 0.0462$ for *sFRP4*), and between adenoma and adjacent normal mucosa ($P = 0.001$ for *sFRP1*, $P < 0.001$ for *sFRP2* and *5*, $P = 0.018$ for *sFRP4*). While no significant difference was found between carcinoma and adenoma ($P = 0.614$ for

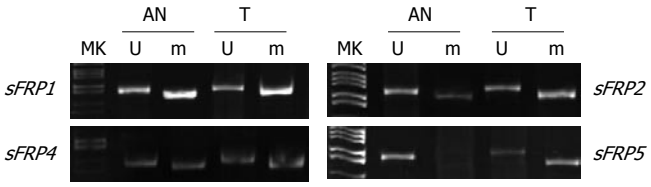


**Figure 2** Hypermethylation of *sFRP* genes in colorectal tumor samples (T) and the corresponding normal tissues adjacent to them (AN). The tumors showed stronger methylation signals than the adjacent normal tissues.

**Table 1 Correlation of sFRP expression with methylation status**

| Gene | | Colorectal tumor | | |
|---|---|---|---|---|
| | | Unmethylated | Methylated | $\chi^2$, *P* |
| *sFRP1* | + | 6 | 9 | $\chi^2 = 17.627$ |
| | - | 3 | 87 | $P < 0.001$ |
| *sFRP2* | + | 11 | 28 | $\chi^2 = 5.345$ |
| | - | 7 | 59 | $P = 0.021$ |
| *sFRP4* | + | 68 | 15 | $\chi^2 = 37.041$ |
| | - | 3 | 19 | $P < 0.001$ |
| *sFRP5* | + | 38 | 10 | $\chi^2 = 39.872$ |
| | - | 10 | 47 | $P < 0.001$ |

Expression (+) and reduced expression (-).

*sFRP1*, $P = 0.848$ for *sFRP2*, $P = 0.228$ for *sFRP4* and $P = 0.647$ for *sFRP5*).

*SFRP1, 2, 4* and *5* were methylated in 94.4% (17/18), 77.8% (14/18), 27.8% (5/18) and 55.6% (10/18) of 18 ACF samples. *SFRP1, 2, 4* and *5* methylation differed significantly between ACF and normal mucosa ($P < 0.001$ for *sFRP1*, *2* and *5* and $P = 0.017$ for *sFRP4, Fisher's* exact test). While no significant difference was found between ACF and carcinoma ($P = 1.000$ for *sFRP1*, $P = 0.836$ for *sFRP2*, $P = 0.506$ for *sFRP4* and $P = 0.833$ for *sFRP5*), and between ACF and adenoma ($P = 0.794$ for *sFRP1*, $P = 1.000$ for *sFRP2*, $P = 1.000$ for *sFRP4* and $P = 0.059$ for *sFRP5*). At least one of the four *sFRP* genes was methylated in 18 ACF samples.

### Expression of sFRP genes

The 20 normal colorectal mucosa specimens expressed considerable levels of sFRP1-5. Compared with the normal mucosa, *sFRP1, 2, 4* and *5* expression was silenced or significantly reduced in 90.3% (65/72), 70.8% (51/72), 26.4% (19/72) and 61.1% (44/72) of colorectal carcinomas, respectively ($P < 0.001$ for *sFRP1*, *2* and *5* and $P = 0.023$ for *sFRP4*); *sFRP1, 2,* and *5* were markedly downregulated or silenced in 75.8% (25/33), 45.5% (15/33) and 39.4% (13/33) of colorectal adenomas ($P < 0.001$ for *sFRP1, 2* and *5*). *SFRP4* was downregulated or silenced in 9.1% (3/33) of colorectal adenomas relative to the normal mucosa ($P = 0.438$). The downregulation of *sFRP 2, 4* and *5* expression was more frequent in carcinoma than in adenoma ($P = 0.012$, $P = 0.043$ and $P = 0.038$). *SFRP1* expression had no significant difference between carcinoma and adenoma ($P = 0.094$). A minority of adjacent normal mucosa samples had reduced expression

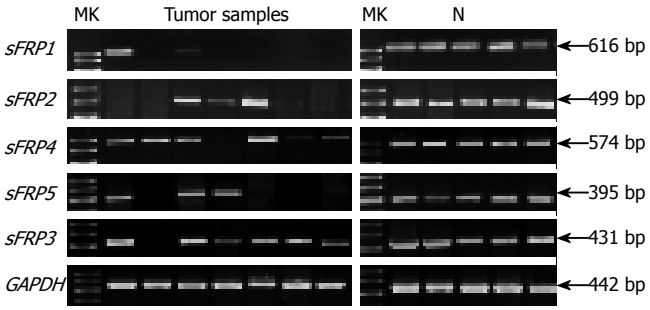


**Figure 3** Expression of *sFRP* gene family in colorectal tumor and normal tissues (N). The gene *GAPDH* serves as a positive indicator for RNA quality and loading.

of *sFRP1-5* genes, and the ratio was 13.2% (5/38), 5.3% (2/38), 0% (0/38), 7.9% (3/38) and 7.9% (3/38), respectively. *SFRP3* that has no CpG island in promoter region was downregulated in only 7 of 105 colorectal tumor samples (Figure 3). Reduced expression of *sFRP1, 2, 4* and *5* genes was significantly associated with aberrant hypermethylation of these genes (Table 1).

### Re-expression of silenced sFRP genes in colorectal carcinoma cell lines

*SFRP1*, *2*, *4* and *5* genes were methylated in RKO and HCT116 cell lines. *SFRP1* and *2* but not *sFRP 4* and *5* were also methylated in SW480 cell line (Figure 4A). When *sFRP* genes were methylated, their corresponding mRNA expression was absent in three colorectal carcinoma cell lines. SW480 cell line expressed *sFRP 4* and *5* mRNA, in which methylation was not detected in *sFRP 4* and *5* promoter. *SFRP3* was expressed in three colorectal carcinoma cell lines.

However, after cells were treated by low-dose DAC, the silenced *sFRP1, 2* and *5* mRNA expression could be re-expressed in RKO and HCT116 cell lines; and *sFRP1* expression could be re-expressed in SW480 cell line. High-dose DAC treatment could strengthen the effects of low-dose DAC treatment, and result in reexpression of *sFRP 4* (RKO and HCT116) and *2* (SW480) mRNA, which could not be induced by low-dose DAC. The addition of TSA to DAC rendered the silenced *sFRP* genes mRNA to be re-expressed effectively, and markedly elevated the mRNA expression level that had been re-expressed or increased by high-dose DAC. A single application of TSA could not induce re-expression of *sFRP* genes mRNA. The influence of demethylation treatment on *sFRP3* expression was minimal (Figure 4B and 4C).

## DISCUSSION

Aberrant activation of the Wnt canonical signaling pathway is associated with a variety of human cancers, such as head and neck carcinoma, lung cancer, melanoma, and mesothelioma, and particularly colorectal cancer, as well as the growth, proliferation and loss of apoptosis of tumor cells. The tumor suppressor adenomatous polyposis coli (APC) mutates in a high proportion of colorectal carcinomas; meanwhile it is an important member of Wnt canonical pathway involved in the degradation of

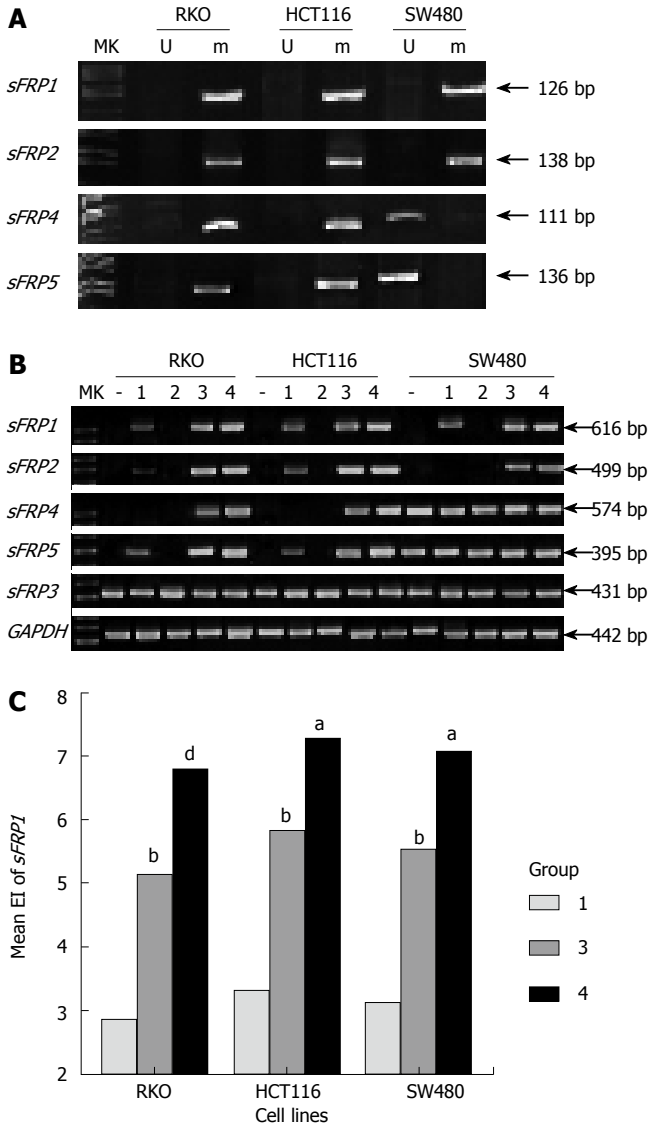


**Figure 4** **A**: Hypermethylation of *sFRP* genes in colorectal cancer cell lines; **B**: The mRNA expression level of *sFRP* genes in colorectal cancer cell lines before and after demethylation treatment. Three cell lines all received 4 different treatments: never treated (-), low-dose DAC (1), TSA (2), high-dose DAC (3), DAC + TSA (4); **C**: The *sFRP1* gene expression index (EI) in three cell lines after demethylation treatment, low-dose DAC (1), high-dose DAC (3), DAC+TSA (4). $^{b}P < 0.01$ *vs* 1, $^{d}P < 0.01$ *vs* 3, $^{a}P < 0.05$ *vs* 3.

β-catenin. Wnt signaling has been identified as one of the key signaling pathways in cancer, regulating cell growth, motility and differentiation. It has been reported that the noncanonical pathway is also involved in tumorigenesis, being associated with inhibition of apoptosis[7].

SFRPs contain a characteristic cysteine-rich domain (CRD) that shares homology with the CRD of frizzled (Fz) receptor of Wnt in the N-terminal half of the proteins. Thus, sFRPs may block Wnt signaling either by interacting with Wnt proteins to prevent them from binding to Fz proteins or by forming nonfunctional complexes with Fz[1]. It has been reported that sFRPs can potentially inhibit the entire canonical Wnt pathway even in the presence of activating mutations of *APC* or β-*catenin* downstream of the Fz receptor in colon cancer cell[8]. Therefore, silence of *sFRP* genes may be essential for the aberrant activation of the Wnt canonical pathway in colorectal tumorigenesis.

To date, no mutation in *sFRP* genes has been associated with their dysregulation in tumors. Aberrant hypermethylation of CpG islands in gene promoters has been found to be a primary mechanism in the inactivation of several tumor suppressor genes. However, the presence of gene silencing by hypermethylation is an important character of colorectal tumor[3,4]. This study demonstrated that genes of the sFRP family were methylated in most cases of colorectal carcinoma, adenoma and ACF, especially *sFRP1* and *2*. The proportion of *sFRP1* and *2* methylation was more than 85% and 75%, respectively. However, *sFRP* genes methylation was much less common in normal-appearing epithelia adjacent to colorectal tumor and never detected in normal mucosa. These data suggest that methylation of *sFRP* genes occurs as an early event in the evolution of ACF-adenoma-carcinoma sequence and is increased through carcinogenic transformation. Notably, 80.6% of colorectal carcinomas showed methylation of both *sFRP1* and *2*, and at least one of the four *sFRP* genes with CpG islands was methylated in 97.2% of the colorectal carcinomas and all ACF samples. *SFRP1*, *2 and 5* appeared to be more specific for colorectal carcinoma, adenoma and ACF, and may be more suitable candidate markers for these lesions.

Based on our study, methylation represents a likely mechanism of *sFRP* gene silencing in colorectal tumor: (1) *sFRP* expression is absent or markedly decreased in colorectal carcinoma and adenoma but generally high in normal mucosa, which is associated with *sFRP* gene methylation; (2) mRNA expression of *sFRP* genes is absent in cancer cell lines with corresponding *sFRP* gene methylation but high in cell lines with unmethylated *sFRP* genes; and (3) *sFRP* gene expression is reactivated in methylated cancer cell lines treated by demethylation; (4) s*FRP3* that has no CpG island in promoter region is expressed in cancer cell lines and a majority of tumor and normal samples. These data suggest *sFRP* gene silencing induced by promoter hypermethylation plays a key role in colorectal tumorigenesis by permitting aberrant activation of Wnt canonical signaling. Furthermore, we propose that the inhibiting effect of s*FRP3* on Wnt signaling is weak. Although sFRP3 is still expressed in tumor cells, it is not sufficient to block Wnt signaling. To confirm that, further studies are needed on the action of Wnt signaling in colorectal tumor cells.

We also found promoter hypermethylation of *sFRP* genes was present at equal frequency in colorectal carcinoma and adenoma, but the downregulation of *sFRP 2*, *4* and *5* expression was more frequent in carcinoma than in adenoma. These suggest that *sFRP* genes are not completely silenced by promoter hypermethylation in the stage of adenoma, although methylation has been presented in adenoma and even early stage ACF. The silence of *sFRP* genes tends to increase with the colorectal tumor progression. Thus, the downregulation of *sFRP* genes may be associated with the progression and malignant potential of colorectal tumor. Our data showed *sFRP1* methylation and downregulation were both extremely frequent in colorectal carcinomas and adenomas. It suggests the methylation and downregulation of *sFRP1* occur more frequently and earlier than other *sFRP* genes

in colorectal tumor, and perhaps play more important role in the progression of colorectal tumorigenesis. However, the methylation and downregulation of *sFRP4* were less common than *sFRP1, 2* and *5* genes in colorectal tumor, though they were both high in mesothelioma and esophageal adenocarcinoma as previously reported[9,10]. It has been reported *sFRP4* has the least homology with other family members[11]. These suggest the Wnt signaling is regulated by different sFRP molecules in different histiocytes. In colorectal tumor cells, the role of sFRP1, 2 and 5, especially the sFRP1, is more important than sFRP3 and 4.

Two key epigenetic pathways are involved in gene transcription regulation: DNA methylation and histone acetylation. DNA methylation is carried out by three DNA methyltransferases (DNMT), DNMT1, DNMT3A, and DNMT3B. DNA methylation represses transcription directly, by inhibiting the binding of specific transcription factors, and indirectly, by recruiting methyl-CpG-binding proteins and their associated repressive chromatin remodeling activities[12]. Deacetylation catalyzed by histone deacetylases is important for turning off genes and maintaining some genes in a repressed state[13]. DAC is a DNMT inhibitor that induces DNA demethylation by metabolic incorporation into genomic DNA, thereby covalently arresting DNMT. TSA is an HDAC inhibitor that causes hyperacetylation on histones H3 and H4. Our data showed *sFRP* genes were effectively demethylated by the high-dose DAC or combination of DAC and TSA treatment and re-expressed in colorectal cancer cell lines. The effect of combination of DAC and TSA treatment was better than high-dose DAC, even though the DAC concentration was low in the combination. A single application of TSA could not induce re-expression of *sFRP* genes mRNA, although some genes were upregulated in expression by TSA alone in the previous report[14]. These data indicate DNA methylation and histone deacetylation are synergetic in the inhibition of gene transcription, and in terms of *sFRP* genes, DNA methylation plays the dominant role.

Our study shows that hypermethylation of *sFRP* genes is a common early event in the evolution of colorectal tumor, and hypermethylation patterns of *sFRP* genes may provide a potentially useful marker system for predicting the risk of colonic neoplasia.

Silence of *sFRP* genes induced by promoter hypermethylation plays a key role in colorectal tumorigenesis. As DNA sequence is not altered by epigenetic modification, and gene silenced by promoter hypermethylation could normally transcript after demethylation, modulation of Wnt protein-driven cell growth, through reversal of *sFRP* genes silencing, may represent potential targets for colorectal cancer prevention and treatment.

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*VIRAL HEPATITIS*

# Genetic vaccination with Flt3-L and GM-CSF as adjuvants: Enhancement of cellular and humoral immune responses that results in protective immunity in a murine model of hepatitis C virus infection

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## Abstract

**AIM:** To investigate whether transfection of plasmid DNA encoding these cytokines enhances both humoral and cellular immune responses to hepatitis C virus (HCV) in a murine model.

**METHODS:** We established a tumor model of HCV infection using syngenic mouse myeloma cells stably transfected with NS5. Co-vaccination of DNA encoding granulocyte macrophage colony-stimulating factor (GM-CSF) and Flt-3 ligand together with a plasmid encoding for the HCV NS5 protein was carried out. Mice were sacrificed 14 d after the last immunization event with collection of spleen cells and serum to determine humoral and cellular immune responses.

**RESULTS:** Co-vaccination of DNA encoding GM-CSF and Flt-3 ligand together with a plasmid encoding for the HCV NS5 protein induced increased antibody responses and CD4+ T cell proliferation to this protein. Vaccination with DNA encoding GM-CSF and Flt-3L promoted protection against tumor formation and/or reduction in mice co-immunized with cytokine-encoding DNA constructs. This suggests this strategy is capable of generating cytotoxic T lymphocyte activity *in vivo*. Following inoculation with plasmid DNA encoding Flt-3L, no increase in spleen size or in dendritic cell (DC) and natural killer cell numbers was observed. This was in contrast to a dramatic increase of both cell types after administration of recombinant Flt3-L *in vivo*. This suggests that vaccination with plasmid DNA encoding cytokines that regulate DC generation and mobilization may not promote unwanted side effects, such as autoimmunity, splenic fibrosis or hematopoietic malignancies that may occur with administration of recombinant forms of these proteins.

**CONCLUSION:** Our data support the view that plasmid DNA vaccination is a promising approach for HCV immunization, and may provide a general adjuvant vaccination strategy against malignancies and other pathogens.



**Key words:** DNA-vaccination; Dendritic cells; Flt3-L; granulocyte macrophage colony-stimulating factor; Hepatitis C virus

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## INTRODUCTION

Dendritic cells (DCs) play a pivotal role in the initiation of immune responses and are considered as important targets for effective immunotherapeutic strategies against cancer and infectious diseases[1,2]. DCs acquire antigen in peripheral tissues, migrate to peripheral lymphoid tissue and subsequently present MHC-peptide complexes to naive T cells for the induction of immune responses. In this scheme, DCs are also believed to control the type of immune response that is generated through the secretion of cytokines such IFN-γ or IL-4 which regulate Th1 or Th2 responses, respectively. The development of DC

and their activation state is dependent upon growth factors, such as fms-like tyrosine kinase 3 ligand (Flt-3L) and granulocyte macrophage colony-stimulating factor (GM-CSF). When administered *in vivo* in the form of recombinant proteins, these cytokines can dramatically alter the absolute number of DC and the subset composition of lymphoid tissue and blood-borne DC[3,4]. This has important implications for the use of these cytokines as adjuvants, since recent evidence suggests that distinct DC subsets are responsible for the generation of different classes of immune responses. Monocyte-derived CD11c+ DCs induce T cells to produce Th1 cytokines *in vitro*, whereas the CD11c- plasmacytoid T cell-derived DCs elicit the production of Th2 cytokines. Flt-3L can increase the number of cells in both subsets of DC, but GM-CSF only increases the CD11c- precursors[3,4]. A number of studies have shown that recombinant Flt-3L protein can augment *in vivo* immune responses and that this cytokine has immunotherapeutic potential[5-7]. It is important to establish if the adjuvant effects of Flt-3L can be achieved by plasmid DNA immunization, which would provide a more versatile delivery system for antigen delivery in vaccination protocols that involve this cytokine[8].

Direct intramuscular or intradermal injection of plasmid DNA generates potent cell-mediated and humoral immune responses against a variety of pathogens, such as human immunodeficiency virus (HIV), hepatitis B virus (HBV), ebola virus, influenza virus, mycobacteria and malaria in animal models of these diseases[9-11]. Genetic vaccines induce antigenic expression that resembles a response to native viral epitopes more closely than do other conventional vaccines. Live attenuated or killed vaccines are often altered in protein structure and antigenicity and may be rendered less efficient at stimulating protective immunity. An important feature of DNA-based immunization is the induction of cytotoxic T cells (CTL) that recognize and lyse virus-infected cells, thereby limiting viral spread. Recent DNA-based immunization studies have reported the induction of anti-HIV CTL activity and anti-plasmodium T cell responses in chronically infected individuals who were immunological naïve for these pathogens[12,13]. Several lines of evidence implicate DC as the principal antigen-presenting cell (APC) in mediating effective immune responses that involve plasmid DNA vaccination. Firstly, bone marrow-chimeric mice have shown that bone marrow-derived APCs are responsible for the induction of the immune response after DNA vaccination[14,15]. Secondly, DCs but not B cells or keratinocytes, isolated from DNA-vaccinated mice were able to present the immunizing antigen to T cells *in vitro*[17]. Thirdly, injection of DNA leads to the direct transfection of DCs *in vivo*[1]. Finally, direct *in vivo* visualization of antigen-expressing DCs from draining lymph nodes after gene-gun vaccination has been demonstrated[18]. Collectively, these reports indicate plasmid DNA immunization with a DC-specific adjuvant is likely to lead to a potent immune response.

Hepatitis C virus (HCV) infection is the major causative agent of transfusion-associated and community-acquired non-A or non-B hepatitis. An estimated 170 million individuals are infected with HCV worldwide and this represents a viral pandemic[19]. Infection with HCV is associated with high morbidity and mortality, since 75%-85% of individuals infected with HCV develop chronic infection and hepatitis whilst approximately one third progress to cirrhosis and eventual liver failure[20]. Moreover, HCV-infected individuals are at increased risk for hepatocellular carcinoma (HCC) within 10-20 years of infection[21]. An effective therapeutic and/or prophylactic anti-HCV vaccine is, therefore, of considerable clinical importance[22]. We have previously identified various HCV structural and non-structural proteins that may be used for vaccination studies designed to limit HCV pathogenesis[23-25]. Here we have shown that co-immunization of DNA encoding GM-CSF and Flt-3L, together with DNA encoding HCV viral antigen leads to enhanced humoral and cellular immune responses that collectively provide protective immunity against HCV-induced pathogenesis in mice.

## MATERIALS AND METHODS

### Construction of DNA expression vectors

A plasmid coding for HCV NS5 was cloned as described in detail previously[25]. pcDNA3-Flt3-L was cloned from a plasmid PCRblunt-mFlt3-L encoding the leader sequence and the extracellular domain of mouse Flt3-L. PCR amplification was performed with the following primer pair: in sense direction 5´-CGG*GGTACC***ATG**ACAGTGCTGGCGCCAGCCTGG-3´ (*Kpn* I *site* and **engineered start codon** ) and in antisense direction 5´-GC*TCTAGAAAGCTT***TTATTA**ATGGTGATGATGGTGATGCTGCCTGGGCCGAGGCTC-3´ (*Xba* Ⅰ *and Hind* Ⅲ *site*, **engineered stop codons** and 6xHis Tag) with a proof reading high fidelity system (Roche, Germany) and a standard PCR protocol as follows: Pre-denaturation at 95℃ for 2 min; 30 amplification cycles, each cycle consisting of denaturation at 95℃ for 1 min, primers annealing at 57℃ for i min and extension at 72℃ for 1 min; and finally an extra incubation at 72℃ for 7 min to ensure full extension of the products. After restriction enzyme digest with *Kpn* Ⅰ and *Xba* Ⅰ for 20 h, the insert was cloned into a standard mammalian transfection vector with a CMV promoter and a given number of CpG motifs (pcDNA3; Invitrogen, Groningen, Netherlands)). Standard sequencing was performed to verify correct nucleotide sequence (Sequence Laboratories, Göttingen, Germany). Cloning and characterization of pGM-CSF was described elsewhere[25]. Plasmids were grown in DH5α cells, and plasmid DNA was subsequently purified by Qiagen Giga Kit using the endofree buffer system (Hilden, Germany).

### HCV infection model

In the absence of a small animal model for HCV infection, we used a tumor model expressing a target HCV antigen (HCV-NS5). A stable HCV NS5-expressing mouse myeloma cell line SP2-NS5-21 (H-$2^d$ background) syngenic to BALB/c mice was established by electroporation and limiting dilution, followed by Western blot analysis for HCV NS5 expression. BALB/c mice inoculated with $2 \times 10^6$ syngenic myeloma cells (SP2-NS5-21) into the flank

develop visible tumors within 7 d and succumb to terminal tumor burden within 15-25 d. The tumors constitutively express HCV NS5 protein and are an established model of HCV viral challenge that has been characterized previously[25].

### *In vitro expression*

A human hepatoma cell line (HuH-7) was transiently transfected with the various plasmids pcDNA-Flt3-L, pcDNA3-NS5 and pcGM-CSF to verify protein and antigen expression as determined by Western blot and ELISA. Detail protocols for immunoblot for NS5 and for Western blot and ELISA for GM-CSF are described previously[23,25]. Flt3-L expression was confirmed by Western blot technique with an anti-His-Tag antibody and a mouse Flt3-L-specific ELISA (R&D, Minneapolis, MN). Cell supernatants were collected 48 h after standard calcium phosphate transfection and cell lysates were prepared in a RIPA buffer (50 mmol/L Tris, 150 mmol/L NaCl, 10 mL/L Nonidet P-40, 5 g/L sodium deoxycholate, 10 g/L SDS). Proteins were lysed and supernatants were loaded onto a 150 g/L-SDS-PAGE gel and blotted onto nitrocellulose membranes. After blocking with 30 g/L BSA in TBS for 2 h, membranes were incubated for 1 h with an anti-His-Tag antibody (Quiagen, Hilden, Germany) in TBS at room temperature (dilution 1:1000), followed by an incubation with sheep-anti-mouse Ig horseradish peroxidase antibody (Amersham Pharmacia, Freiburg, Germany) at 1:1000 dilution. Chemiluminescence detection was performed with the ECL system following the manufacturer's protocol (Amersham Pharmacia). Flt3-L ELISA was performed with a commercially available ELISA kit (R&D, Minneapolis, MN). Lysates and supernatants were used undiluted. To analyze Flt3-L serum levels of immunized mice, a 1:50 dilution of serum collected through tail vein bleeding was used as first antibody in this assay.

### *Plasmid DNA Immunization*

BALB/c (H-$2^d$) and C57/BL6 (H-$2^b$) mice were purchased from Charles River Laboratories and maintained under standard pathogen-free conditions in the animal facility (Zentrales Tierlabor) at our institution (University of Heidelberg). Five days prior to plasmid immunization, 100 μL of 50 μg/mL Flt3-L or mock plasmid DNA was injected into the quadriceps muscle over five different sites. Plasmid immunization was performed three times thereafter (100 μg plasmid DNA in 100 μL 9 g/L NaCl) at bi-weekly intervals (administration into the opposite leg). Empty plasmid vector (mock) immunization was employed as a negative control. Animals ($n$ = 5) were injected with pcDNA3-NS5 (50 μg) alone or in combination with an Flt3-L (50 μg) or GM-CSF (50 μg) plasmid. Furthermore, one group was immunized with 50 μg of HCV NS5 and Flt3-L combined with GM-CSF (both 25 μg) to study the combined effect of both cytokines. One group was immunized subcutaneously into the footpad. Mice were sacrificed 14 d after the last immunization event with collection of spleen cells and serum to determine humoral and cellular immune responses. The immunization experiments were once repeated; each immunization group contained at least 5 animals.

### *Measurement of humoral immune responses*

Anti-HCV NS5 antibody level in the serum of each immunized animal was determined by ELISA. In brief, microtiter plates (Nunc Maxisorp) were coated with 0.5 μg/well recombinant HCV NS5-4 protein (Mikrogen, Munich, Germany), incubated overnight at 4℃ and blocked with foetal bovine serum (FBS) for 2 h at 20℃. A 1:50 dilution of mouse serum was added to the plates, incubated for an additional 1 h at 20℃, and washed 4 times with phosphate buffered saline (PBS) containing 5 mL/L Tween-20. A peroxidase-conjugated AffinePure goat anti-mouse IgG (Dianova, Hamburg, Germany) was applied to the plates at a 1:2000 dilution, incubated for 1 h, washed, and substrate (OPD) was added for color development (Abbott).

### *Lymphoproliferation and cytokine release assays*

Mice were anesthetized with diethylether for harvesting of spleen cells. Erythrocytes were removed by incubation in 8.3 g/L $NH_4Cl$ / 0.17 mol/L Tris (pH 7.4) for 5 min at 25℃. Spleen cells were washed 2 times and cultured in triplicate using 96-well round bottom plates at $5 \times 10^5$ cells/well in 200 μL DMEM (Cellgro Mediatech, Washington, DC) containing 100 mL/L FBS and 2-mercaptoethanol ($5 \times 10^{-5}$ mol/L). Cells were stimulated for 3 d with recombinant HCV NS5-4 protein (aa 2622–2868) (Mikrogen, Munich, Germany) at various concentrations (0, 0.01, 0.1 and 1 μg/mL). Following the addition of radioactive $^3$H-thymidine (1 μCi/well), cells were incubated for an additional 18 h, and $^3$H-thymidine uptake into DNA was measured after harvesting; incorporation of radioactivity was corrected for background activity (Δ cpm). To determine cytokine secretion levels of lymphoproliferative cells stimulated with recombinant HCV NS5-4 protein, cells were cultured as described, and mIFN-γ and mIL-4 levels were measured by commercial kits according to manufacturer's instructions (Pharmingen, San Diego, CA).

### *ELISPOT assay*

To assess the number of IFN-γ-secreting cells at the individual cell level, single cell suspensions from spleens harvested from immunized mice mouse were analyzed in an IFN-γ ELISPOT assay. Cells were directly assessed in this assay without prior *in vitro* expansion in the presence of 1 μg/mL recombinant NS5-4 protein at 37℃ (50 mL/L $CO_2$) in IFN-γ-bound microtiter plates to measure IFN-γ cytokine secretion as means of CD8+-T-cell function (AID, Straßberg, Germany). After washing with PBS-Tween buffer, cells were incubated with a secondary antibody suspended in DMEM supplemented with 100 mL/l FCS and 2-mercaptoethanol.

### *Assessment of CTL activity in vivo*

BALB/c mice were immunized im three times with either mock DNA or NS5 vector and co-immunized with Flt3-L or Flt3-L and GM-CSF. One week after the last immunization event with $2 \times 10^6$ syngenic SP2/0-derived cells stably expressing NS5 were washed (Sp2-NS5-21), resuspended in 200 μL of PBS, and inoculated sc into

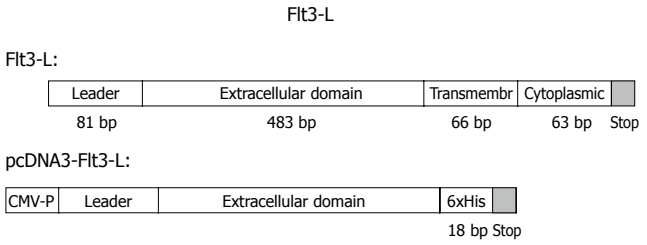


**Figure 1** Cloning of the soluble form of mouse Flt3-L containing the leader and the extracellular domain into a mammalian expression vector with CMV-promoter and RSV-enhancer. The C-terminus was tagged with a 6xHis. The soluble form of mFlt3-L is fully active.

the right flank. SP2/0 cells that stably expressed HCV core protein (SP2/19) were used as a control in selected animals. Tumor formation, tumor size, mouse weight and mouse survival was assessed at distinct intervals post inoculation.

## RESULTS

### *In vitro expression of Flt-3L from Flt-3L-pcDNA3*

Flt-3L is type I transmembrane, non-disulphide linked, homodimer glycoprotein of approximately 30 ku. This cytokine may also be found as a soluble form in serum that comprises only the extracellular domain of approximately 160 amino acids that retains full biological activity[26,27]. We, using PCR, have cloned Flt-3L DNA encoding the leader sequence and extracellular domain, together with a hexahistidine C-terminal tag, into the eukaryotic expression vector pcDNA-3 for use as a Flt-3L delivery system. Figure 1 shows a schematic representation of the major domains of full-length and truncated forms of Flt-3L. DNA encoding the leader and extracellular domains of Flt-3L was generated by PCR and ligated into the eukaryotic expression vector pcDNA3 to generate Flt-3L-pcDNA3. Restriction digest and DNA sequence analysis confirmed the presence of the predicted insert size and its correct orientation (data not shown).

Figure 2 shows that Flt-3L-pcDNA3 was authentically expressed after transient transfection into HuH-7 cells. Figure 2A shows a Western blot of Flt-3L-pcDNA3-transfected HuH-7 cell lysate and culture supernatant probed with an anti-His monoclonal antibody. Anti-His reactive protein bands of 20 – 30 ku were present in both cell lysate and supernatant of cells transfected with Flt-3L-pcDNA3. This protein band pattern most likely represents the different glycosylation forms of Flt-3L which have been described previously[27]. Additional bands of 40 – 42 ku were detected in the cell culture supernatants that may represent the dimeric form of Flt-3L. No reactivity with the anti-His monoclonal antibody was seen in either cell lysate or culture supernatants from HuH-7 cells transfected with control vector. Further confirmation that Flt-3L was expressed from Flt-3L-pcDNA3 was provided by ELISA using an anti-Flt-3L specific monoclonal antibody with samples from Flt-3L-pcDNA3-transfected HuH-7 cells as substrate. Figure 2B shows that Flt-3L could be detected in cell lysate and culture supernatant from Flt-3L-pcDNA3-transfected cells, whereas no reactivity was detected in equivalent samples from control transfected cells. This

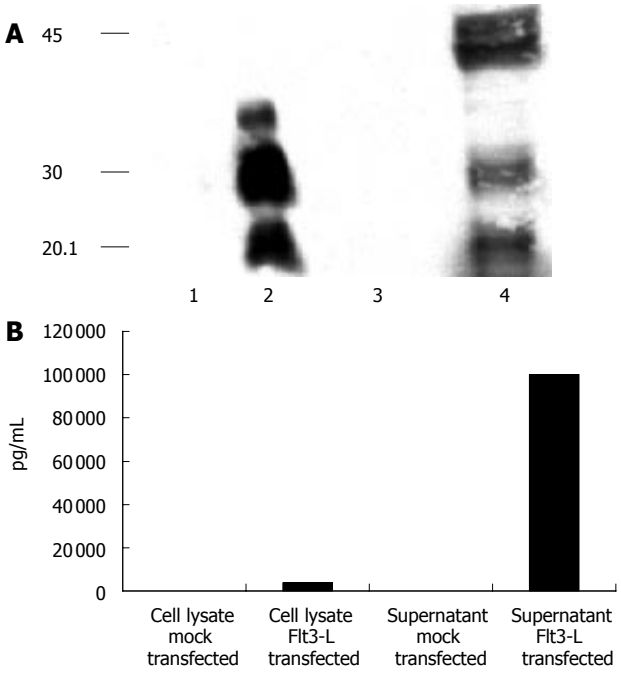


**Figure 2** Expression of Flt3-L in transiently transfected HuH-7 cells: (**A**) Detection of mouse Flt3-L on a 150 g/L SDS-PAGE using an anti-His-Tag antibody. Different glycosylation forms of Flt3-L in cell were detected in cell lysates and supernatants. Lane 1 and 3: mock-transfected cells; lane 2 and 4: Flt3-L-transfected cells; lane 1 and 2: supernatants after transient transfection; lane 3 and 4: cell lysates. (**B**) To confirm these results, supernatants and cell lysates were incubated by an Flt3-L-specific ELISA. Again, secretion levels into the supernatant were more than 20-fold higher compared to levels seen in the cell compartment.

data demonstrates that Flt-3L protein could be generated from Flt-3L-pcDNA3.

### *Flt-3L- or GM-CSF-pcDNA3 enhances anti-HCV immune responses*

Immune responses in mice to the HVC structural protein NS5 in the presence or absence cytokines that regulate dendritic cells were examined. Mice were immunized intramuscularly three times, at bi-weekly intervals, with pcDNA3-NS5 in combination with either Flt-3L-pcDNA3 or GM-CSF-pcDNA3. Serum samples from vaccinated mice were investigated for the presence of anti-HCV antibodies by ELISA using as substrate, the peptide NS5-4 which comprises amino acids 2622-2868 of the protein NS5. The data in Figure 3 shows that co-immunization of pcDNA3-NS5 with Flt-3L-pcDNA3 led to elevated levels of anti-HCV antibody compared to that found when pcDNA3-NS5 was administered alone. The level of anti-HCV antibody was further increased when pcDNA3-NS5 was co-immunized with GM-CSF-pcDNA3. Similar results were obtained using either BALB/c or C57/BL6 mice (data not shown). These experiments showed that administration of plasmid DNA encoding cytokines that regulate the development and maturation of dendritic cells, at the same time as inoculation with DNA encoding HCV protein, can produce elevated anti-HCV humoral immune responses. The adjuvant effect of Flt-3L or GM-CSF did not appear to affect the level of anti-HCV-specific T cell proliferation not readily evident at the level of T cell activation and proliferation. Spleen T cells from NS5 plasmid DNA-immunized mice were stimulated *in vitro* with recombinant

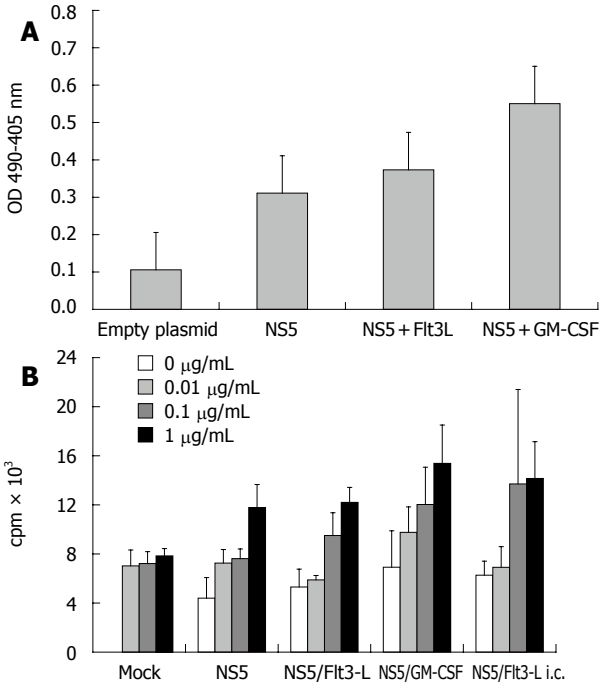


**Figure 3** **A:** Anti-HCV NS5 ELISA showing mean levels of specific antibodies after genetic immunization with HCV-NS5-expressing plasmid. Controls included wells coated with BSA and sera derived from mock-immunized mice. Each group comprised 10 BALB/c mice and mice sera were pooled before the assay. ELISA plates were coated with HCV-NS5-4, therefore, antibody levels may be underestimated; **B:** T-cell proliferation assay ($n$ = 10 mice/group) with different amounts of stimulating recombinant HCV NS5-4 protein (0.01-1 μg/mL). An increase of thymidine incorporation was seen after stimulation with 0.1 and 1 μg/mL recombinant protein. However, levels of T-cell proliferation after co-immunization with Flt3-L were only slightly higher than in NS5-immunized mice. Note that stimulation with a non-relevant protein (HBsAg) induced only background activity, demonstrating the antigen specificity (data not shown).

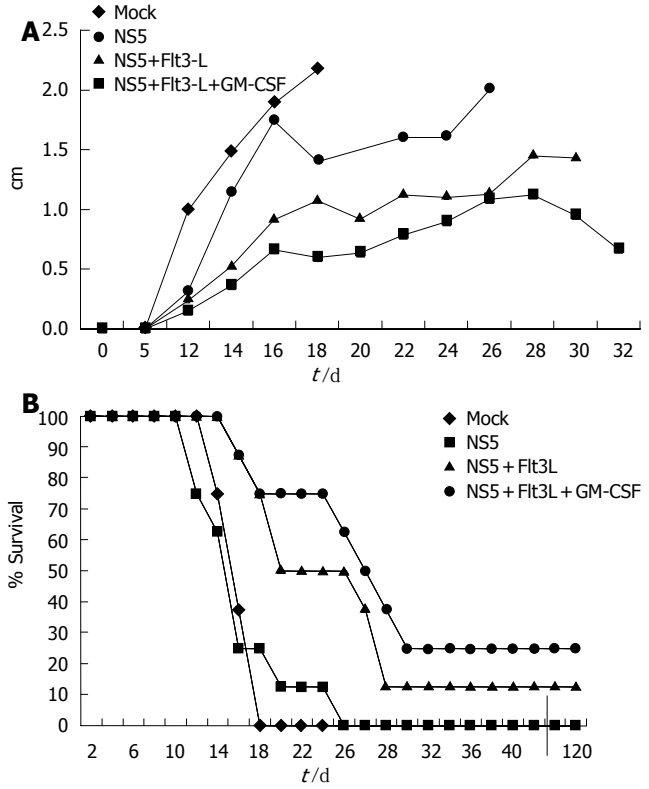


**Figure 4** Tumor model to assess CTL activity generated *in vivo* after genetic vaccination. Mice were immunized three times with a total of 100 μg of plasmid DNA. Each vaccination group ($n$ = 8) was challenged with 2 × $10^6$ syngenic mouse myeloma cells stably expressing HCV-NS5 protein in the right flank. Mice developed notable tumors around d 8-10. (**A**) Tumor size in different immunization groups. Note that mice had to be sacrificed at maximum tumor diameter size of 2 cm. Sacrificed mice were then taken out from the measurement. (**B**) Mouse survival after genetic vaccination in each group. Three mice were long-term survivor and never developed significant tumor burden.

HCV NS5-4 peptide and the subsequent proliferative response measured by 3-H-thymidine uptake. The data in Figure 3 shows that there was no significant difference in anti-NS5 HCV T lymphocyte proliferation between cells isolated from mice immunized with NS5-pcDNA3 alone or in combination with either Flt-3L pcDN3 or GM-CSF-pcDNA3. In addition, when IFN-γ and IL-4 levels in the supernatant of stimulated T cells were measured by ELISA, there were no significant differences between any of the immunization groups (data not shown).

### *Protective immunity generated by Flt-3L and GM-CSF*

*In vivo* CTL activity was assessed by a tumor model. Ten days after the last immunization event, animals were challenged in the right flank with a HCV-NS5 mouse myeloma cell line. Eighteen days after challenge, all mock plasmid-immunized animals were dead. Animals immunized with 50 μg of HCV-NS5 plasmid lived slightly longer, but all animals died by d 26. Co-vaccination with Flt3-L or Flt3-L and GM-CSF lead to survival and complete protection in some animals: 25% and 10%, respectively. Tumor size was also significantly reduced in co-immunized animals (Flt3-L and combination of Flt3-L and GM-CSF). Mice immunized with the same syngenic SP2/0 cell line expressing a different HCV structural protein (HCV core) as a control died as well by d 17, like mock-immunized animals. Indeed, 100% of mice immunized with mock DNA or challenged with SP2/19 cells demonstrated tumor formation, confirming the specificity of the cytotoxic activity in this tumor model (Figure 4).

### *In vitro and in vivo CTL activity: ELISPOT and tumor model*

We used IFN-γ-bound microtiter plates to measure IFN-γ cytokine secretion as means of CD8+-T cell function in an ELISPOT on an individual cell level. Spleen cells were harvested and cultured overnight in the presence or absence of 1 mg/L recombinant NS5-4 protein. Flt3-L, GM-CSF, the combination of both cytokines injected each with smaller amounts of DNA (25 μg per vaccination) and the subcutaneous injection with Flt3-L resulted in comparable levels of NS5-4-specific IFN-γ-producing T cells. The number of cells was significantly higher compared to NS5-immunized animals alone and the mock-injected animals (Figure 5).

### *In vivo expression of Flt3-L from Flt3-L- pcDNA3*

In studies by He *et al* where Flt-3L DNA has been administered by hypodynamic needle, levels of serum Flt-3L have been raised 10-fold compared to control values. To determine whether Flt3-L-pcDNA3 administration *in vivo* also led to elevated levels of serum Flt-3L, mice were inoculated with this vector by intramuscular injection and serum samples harvested at 3, 6, 12, 24 h and at 3 d and 7

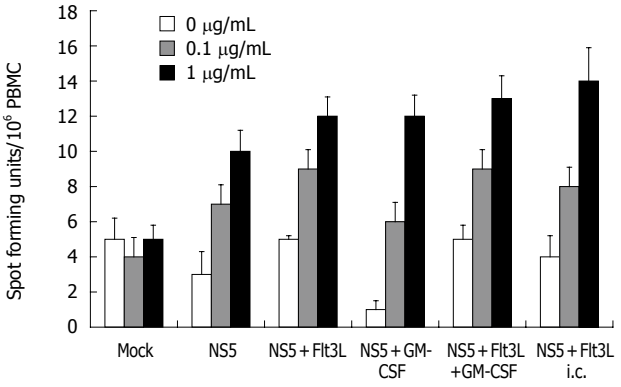


**Figure 5** ELISPOT assay: Assessment at the individual single cell level of the number of mIFN-γ-secreting cells as means of CD8 T cell function. Spleen cells were stimulated with recombinant HCV-NS5 protein at 0.1 and 1 μg/mL, and the number of spot forming units/$10^6$ PBMC was counted after 24 h incubation period ($n$ = 10 mice/group). Note that lymphocytes were not restimulated in NS5-expressing cells.

d post-inoculation. Serum samples were assessed for Flt-3 L by Flt-3L-specific ELISA. On no occasion, using either BALB/c or C57/BL6 mice, were the levels of serum Flt-3L significantly increased compared to control mice (data not shown). In addition, there was no increase in the size of lymphoid tissue, such as the spleen (data not shown), as has been reported following administration of recombinant Flt-3L protein or Flt-3L DNA by hypodynamic needle.

## DISCUSSION

DNA vaccination is an efficient and versatile mechanism for the delivery of antigen to the immune system. The mechanism of antigen delivery is believed to operate by direct transfection of dendritic cells with the inoculated DNA construct; plasmid expression within these cells and the subsequent processing and presentation of antigen to T cells[17]. Dendritic cells are potent antigen-presenting cells for naïve antigen-specific T cells and, therefore, have the capacity to initiate humoral and cellular immune responses, which is an essential requisite of any vaccination strategy[28,29]. A feature of plasmid DNA vaccination has been the reported longevity of expression of the DNA construct which may lead to evidence of immunity for periods greater than 12 mo[14]. This may represent continuous expression of the plasmid DNA construct within lymphoid tissue where antigen-presenting cells, such as dendritic cells and antigen-specific T cells, interact in the appropriate environment to allow the necessary cellular interactions that lead to an effective immune response[30]. Because of these features, plasmid DNA vaccination has been considered as an effective means to provide prophylactic or therapeutic immune responses against HCV infection[31]. However, several studies to date that have investigated plasmid DNA vaccination in animal models of human HCV infection have so far only demonstrated low antibody titers against the immunizing HCV proteins[23-25]. It is clear if plasmid DNA vaccination is to provide effective immunization against HCV infection that enhanced immune responses will need to be generated. In order to address this issue, we considered it appropriate to target dendritic cells in an attempt to promote enhanced plasmid DNA-induced anti-HCV immunity in mice.

In this study, we have successfully generated an enhanced immune response against a structural HCV protein by co-immunization with plasmid DNA-encoding cytokines that regulate development and maturation of dendritic cells. When HCV plasmid and Flt-3L DNA were co-injected, the resultant anti-viral antibody titer was significantly increased, and was further enhanced by co-immunization with GM-CSF plasmid DNA. These phenomena parallel what has been seen upon administration of recombinant Flt-3L and GM-CSF protein during immunization with protein antigens. Importantly, we have demonstrated that this enhanced plasmid DNA-based immunization is effective at providing protective immunity against HCV-based tumor formation. We used an *in vivo* tumor model that expressed a HCV non-structural protein. Control mice died after 18 d, whereas co-immunized mice survived to some extend, especially after co-administration of an Flt3-L and a GM-CSF plasmid underlining synergistic effects of both cytokines seen in *in vitro* studies[8]. This underlines the local and antigen-specific immune induction, because models of recombinant administration (up to 500 μg/kg per day) or virally transduction with Flt3-L DNA have also demonstrated inhibition of the growth of B16 melanoma, EL-4 lymphoma, murine leukemia, and C3L5 breast tumors in mice[32-35]. However, these models used large amounts of recombinant FLT3-L and repeated injections are required to induce these effects, unlike the relatively small amounts of plasmid DNA injection used in this study.

A feature of our study is that protective immunity against HCV-induced tumor formation was achieved in the apparent absence of any detectable increase in serum cytokine level from the inoculated plasmids. In humans, serum levels of Flt3-L are relatively low (< 100 pg/mL). In mice we measured serum levels around 300 pg/mL and did not find an increase after genetic vaccination with Flt3-L plasmid. These results are in contrast to data that have reported an initial increase in serum Flt3-L levels after a single injection of hydrodynamic human Flt3-L gene delivery when serum levels up to 40 μg/mL were observed and remained above 1 μg/mL for 6 d in mice[36]. In addition, elevated levels of serum Flt-3L, such as those following *in vivo* administration, resulted in an increase in the size of peripheral lymphoid tissues like spleen and lymph nodes. Furthermore, these elevated levels of Flt3-L were associated with significant increases in the number of DCs and NK-cells in various tissues, such as spleen and liver[36]. Our observations on the absence of any change in serum Flt3-L following plasmid DNA vaccination with this cytokine are in agreement with reports by others using other cytokines, such as IL-2, GM-CSF or IL-12, for plasmid DNA co-vaccination studies[23,37,38]. High levels of DC in a number of different organs may, under certain conditions, be associated with adverse side effects, including induction of haematopoietic malignant disease and the induction of autoimmunity[39]. Interestingly, a very recent study demonstrated the successful local recruitment of DC to the side of injection and the induction of CD4+ proliferative

responses in a large animal model of a veterinary disease (calves) after co-vaccination with Flt3-L and GM-CSF plasmids, which has important implications of translating results from the small animal model mouse to men[40]. In this regard, a local increase in DC numbers with an accompanied enhancement of the immune response would be distinct clinical advantage and is demonstrated against HCV in our study. This approach of co-immunization with Flt3-L and GM-CSF has, therefore, important implications for the development of an effective antiviral HCV vaccine.

## ACKNOWLEDGMENTS

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# Elicitation of strong immune responses by a DNA vaccine expressing a secreted form of hepatitis C virus envelope protein E2 in murine and porcine animal models

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## Abstract

**AIM:** To characterize the immunogenicity of a hepatitis C virus (HCV) E2 DNA vaccine alone or with a protein vaccine boost in murine and porcine animal models.

**METHODS:** A DNA vaccine expressing a secreted form of HCV E2 protein was constructed and used to vaccinate mice and piglets with or without boosting with a recombinant E2 protein vaccine formulated with CpG ODN and 10% Emulsigen. The immunogenicity of HCV E2 vaccines was analyzed by ELISA for antibody responses, MTT assay for lymphocyte proliferation, ELISPOT for the number of interferon-γ secreting cells, and cytotoxic T lymphocyte assays.

**RESULTS:** Intradermal injection of E2 DNA vaccine induced strong Th1-like immune responses in mice. In piglets, E2 DNA vaccine elicited moderate and more balanced immune responses. A DNA vaccine prime and protein boost vaccination strategy induced significantly higher E2-specific antibody levels and shifted the immune response towards Th2-like ones in piglets.

**CONCLUSION:** A DNA vaccine expressing a secreted form of HCV E2 protein elicited E2-specific immune responses in mice and piglets. Recombinant E2 protein vaccination following DNA immunization significantly increased the antibody response in piglets. These HCV E2 vaccines may represent promising hepatitis C vaccine candidates for further investigations.



**Key words:** Hepatitis C virus E2; DNA vaccine; DNA vaccine prime-protein boost

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## INTRODUCTION

Hepatitis C continues to be a severe health threat to a large population with about 123 million people being affected globally[1]. The etiologic agent, hepatitis C virus (HCV)[2] is able to establish persistent infections in up to 85% of infected individuals with severe clinical consequences[3]. Current therapy with pegylated interferon and ribavirin is only effective in about 50% of the patients[4]. Although vaccines represent one of the most effective means to combat infectious diseases, there is no vaccine available for hepatitis C[5]. Hence, evaluating different vaccination strategies that can induce HCV-specific immunity is critical for the development of effective vaccines to reduce HCV-related mortality and morbidity.

HCV is the only member of the *Hepacivirus* genus in the *Flaviviridae* family[6]. The positive-sense, single-stranded RNA genome encodes a polyprotein of about 3100 amino acids in length[7]. Processing of the polyprotein by cellular or viral proteases generates up to 11 viral proteins, including three structural proteins (core, envelope proteins E1 and E2) and six non-structural proteins (NS-2, -3, -4A, -4B, -5A, and -5B). As the major envelope protein in HCV particles[8,9], the E2 protein is likely to be critical for inducing antibody responses against HCV infections. In line with this notion, anti-E2 antibodies have been consistently detected in hepatitis C patients[10-13]. Furthermore, there is evidence to suggest that anti-E2 antibodies can inhibit HCV infections in both *in vivo* and *in vitro* settings[14-17]. However, generation of E2-specific antibodies in hepatitis C patients is usually delayed and of low magnitude, which may be one of the reasons for such a high rate of persistent HCV infections[12,13]. These findings indicate that E2-specific antibodies are beneficial and induction of these antibodies should be taken into

consideration when designing a vaccine against hepatitis C.

Although the correlations for a successful immune response that can resolve HCV infections have not been well characterized, previous studies suggest a rapid, vigorous, and broadly targeted cell-mediated immune response tends to be associated with HCV clearance[18-22]. In line with this notion, E2-specific cellular immune responses have been detected in hepatitis C patients as demonstrated by E2-specific lymphocyte proliferation, cytotoxic T lymphocyte, and ELISPOT assays[23-25]. More importantly, it has been demonstrated that a stronger E2-specific cell-mediated response is associated with better response to interferon therapy and viral clearance[25]. These findings indicate that it is desirable for a candidate HCV E2 vaccine to induce cell-mediated immune responses.

Delivery of transgenes by plasmid DNA is a novel platform technology for vaccine development. DNA vaccines tend to induce a Th1-biased response in the host[26-30]. Manipulation of protein subcellular localization may enhance antibody responses to the antigen. For instance, directing antigenic expression to secretion pathways by adding a signal peptide sequence may increase B-cell mediated responses[31]. In addition, boosting with a protein subunit vaccine following DNA vaccination is another feasible means for inducing strong antigen-specific humoral responses[32-36].

In this study, a DNA vaccine was designed to induce expression of a secreted form of HCV E2 protein. Immunogenicity studies using inbred mice showed that this HCV E2 DNA vaccine elicited strong Th1-like immune responses. In piglets, the E2 DNA vaccine elicited a moderate and more Th1-Th2 balanced response. E2 protein vaccination after DNA immunization had a more pronounced boosting effect in piglets than in mice by significantly increasing E2-specific antibody response, causing a shift of the immune response towards a Th2-type.

## MATERIALS AND METHODS

### *HCV E2 DNA vaccine construction*

To generate a DNA vaccine encoding a secreted form of HCV E2 protein, a portion of the E2 coding sequence (amino acid residues 412-661 of HCV polyprotein) without the hypervariable region 1 (HVR1) and the hydrophobic region was amplified by polymerase chain reaction (PCR). The template was plasmid pDM22, a cDNA clone of the BK isolate (genotype 1b; kindly provided by Dr. A. Takamizawa)[37]. The primers were E2-412-Nhe-Sense (5'-AATTGCTAGCCAGCTTATAAACACCAATGGG-3', *Nhe*I site is underlined) and E2-661-Bgl-AS (5'-AATTAGATCTTCACTCCGGCCTATCCCTGTC-3', a stop codon TGA was added followed by a *Bgl*Ⅱ site which is underlined). The PCR product was cloned into a plasmid vector pSLIA-tPAs[38] with *Nhe*I and *Bgl*Ⅱ (New England Biolabs), allowing the addition of the signal peptide sequence of the tissue plasminogen activator (tPA) to the amino-terminus of the truncated E2 protein. The identity of PCR amplified E2 gene was confirmed by DNA sequencing experiments. Subsequently, the *Hin*dⅢ-*Bgl*Ⅱ fragment containing the tPAs-tE2 fusion gene was cloned into a CpG enriched DNA vaccine vector pBISIA24[39], which contains 24 copies of a Th1-promoting CpG motif (GTCGTT). An unexpected extension of the open reading frame resulted from the *Nde*I recognition sequence in the multiple cloning site of plasmid pBISIA24 was removed by deleting the small fragment after *Pst*I digestion and subsequent ligation of the large fragment. The final DNA vaccine construct was designated pBISIA24-tPAs-tE2 (Figure 1). Plasmid prepared by endotoxin-free plasmid purification reagents (Qiagen) was used for immunization.

### *Transfection and immunoblotting analysis*

To determine whether the constructed DNA vaccine plasmid expressed the E2 protein, TE671 cells, a human rhabdomyosarcoma cell line, were transfected with plasmids pBISIA24-tPAs-tE2 and pBISIA24 using the calcium phosphate method[40]. At 24 h after transfection, culture medium was collected and cells were lyzed in a lysis buffer (1% SDS, 10 mmol/L Tris-HCl, pH8.0). The protein concentration was determined by the Bradford assay (Bio-Rad) using bovine serum albumin (BSA) (Sigma) as a standard. Twenty-five micrograms of protein were loaded on a 10% SDS protein gel and transferred to a PVDF membrane (GE Healthcare). The membrane was incubated with a monoclonal antibody against HCV E2 protein (H52, kindly provided by Dr. J. Dubuisson). After incubation with an HRP-conjugated secondary antibody, immuno-reactive protein bands were visualized by the enhanced chemiluminescence system (ECL$^{Plus}$, GE Healthcare).

### *Recombinant E2 protein expression and purification*

To generate a recombinant E2 protein vaccine, HCV E2 protein was expressed and purified as a fusion protein with a poly-histidine tag. For this purpose, the coding sequence of HCV E2 was amplified by PCR using plasmid pDM22 as the template. The primers for the PCR reaction were E2-Bam-Nhe-Sense (5'-GGGGGGGGATCCGCTAGCGATACCCACGTGACAGGGG-3', *Bam*HI and *Nhe*I sites are underlined) and E2-746-Bgl-AS (5'-GGGGGGAGATCTCAGGCCTCGGCCTGGGCTA-3', a stop codon TGA was added followed by a *Bgl*Ⅱ site which is underlined). The PCR fragment was cloned into an expression vector pRSETA (Invitrogen) using restriction enzymes *Bam*HI and *Bgl*Ⅱ, allowing the addition of a poly-histidine tag at the amino-terminus of the E2 protein. The resultant plasmid, designated pRSET-E2, was confirmed by restriction analysis and DNA sequencing. The *Not*I-*Bgl*Ⅱ fragment of plasmid pRSET-E2 was substituted by the *Not*I-*Bgl*Ⅱ fragment of pBISIA24-tPAs-tE2, generating plasmid pRSET-tE2 encoding a poly-histidine-tagged E2 protein without the carboxyl hydrophobic region. Plasmid pRSET-tE2 was then transformed into *E. coli* BL21 (pLysS) and expression of E2 protein was induced by isopropylthio-β-galactoside (IPTG, Invitrogen). The induced poly-histidine-tagged tE2 protein was purified under denaturing conditions in the presence of 8 mol/L urea using Ni-NTA agarose (Qiagen) *as per* manufacturer's instructions. The urea was removed from the protein preparation after dialysis against phosphate buffered saline (PBS). The level of endotoxin was determined by the

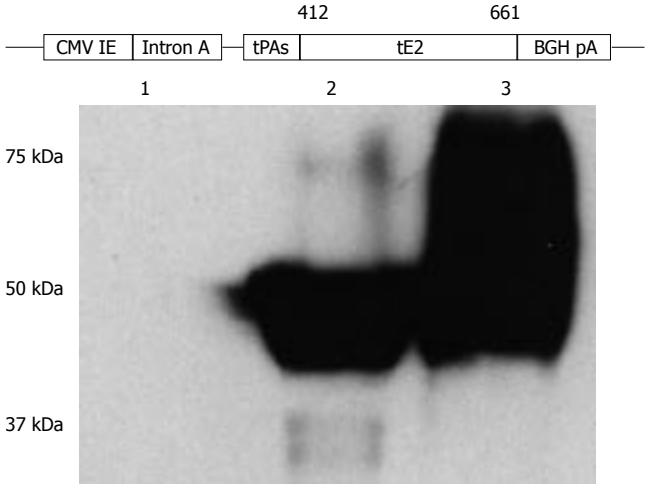


**Figure 1** Construction and characterization of HCV E2 DNA vaccine. A secreted form of HCV E2 protein generated by removing the carboxyl-terminal hydrophobic region and replacing the hypervariable region 1 with the signal peptide sequence of the tissue plasminogen activator (tPAs) was cloned into a DNA vaccine vector pBISIA24. The construction of E2 DNA vaccine, pBISIA24-tPAs-tE2, is schematically presented. TE 671 cells were transfected with pBISIA24 (lane 1) or pBISIA24-tPAs-tE2 (lanes 2 and 3). Cell lysates (lanes 1 and 2) and culture medium (lane 3) were analyzed in immunoblotting using an E2-specific antibody.

*Limulus* amoebocyte test (QCL-1000 Chromogenic *Limulus* amebocyte lysate kit, Cambrex).

### Mouse and piglet immunization

The experimental protocols were approved by the Committee of Animal Care and Supply, University of Saskatchewan. In the mouse trial, 24 six-week old B6C3F1 (H-2$^d$) female mice were randomly divided into three groups. Groups of mice were immunized either three times with 40 μL saline subcutaneously (s.c.), three times with 50 μg of DNA vaccine pBISIA24-tPAs-tE2 intradermally (i.d.), or twice with 50 μg of DNA vaccine pBISIA24-tPAs-tE2 i.d. followed by one subcutaneous vaccination with 5 μg of recombinant tE2 protein. The recombinant truncated E2 protein vaccine was formulated with 10 μg of CpG oligonucleotide (ODN) 1826 (5' -TCCATGACGTTCCTGACGTT-3', CpG motifs are underlined; kindly provided by Merial Limited) and 10% Emulsigen (MVP Laboratories) per dose. This formulation has been shown to elicit strong immune responses with a superior safety profile[41]. The vaccines were given three weeks apart. Blood was sampled two weeks after each immunization and spleens were collected two weeks after final immunization for analyzing immune responses.

In the piglet trial, 24 out-bred piglets (Landrace cross, 4-5 wk old, Prairie Swine Center) were randomly allocated into three groups with eight piglets in each group. The vaccination schedule was the same as that in the mouse trial but the doses were 10-fold of those used for mice. In addition, CpG ODN 2007 (5'-TCGTCGTTGTCGTTTTGGTCGTT-3', kindly provided by Merial Limited), which can stimulate porcine peripheral blood mononuclear cells (PBMCs)[42], was used for protein vaccine formulation.

### Enzyme-linked immunosorbent assay (ELISA)

To analyze antibody levels in murine or porcine sera after immunization, 96-well polystyrene plates (Immulon 2, Dynatech Laboratories) were coated with purified E2 protein at 100 ng/well in a carbonate buffer (pH9.6) overnight at 4℃. Serially diluted murine or porcine sera were added to each well and incubated for 2 h at room temperature (RT). To determine the total IgG levels, biotinylated goat anti-mouse IgG antibody (Caltag Laboratories) at a dilution of 1:10000 or alkaline phosphatase labeled goat anti-porcine IgG (KPL) at a dilution of 1:2500 was applied to detect bound IgG. To determine E2-specific IgG1 and IgG2a antibody levels in murine sera, bound antibodies were incubated with biotinylated goat anti-mouse IgG1 or IgG2a antibodies (Caltag Laboratories) at a dilution of 1:10000, respectively, followed by streptavidin-AP (Jackson ImmunoResearch Laboratories) at a dilution of 1:5000. To determine E2-specific IgG1 and IgG2 antibody levels in porcine sera, bound antibodies were incubated with mouse anti-porcine IgG1 or IgG2 antibodies (Serotec) at a dilution of 1:100, respectively, followed by biotinylated goat anti-mouse IgG antibody (Caltag Laboratories) at a dilution of 1:10000. The reactions were developed by adding *p*-nitrophenyl phosphate (PNPP) (Sigma) at 100 ng/well and the optical density was recorded at 405 nm.

### Enzyme-linked immunospot (ELISPOT) assay

ELISPOT assay was performed to determine the frequency of interferon-γ (IFN-γ) secreting cells in mouse splenocytes or porcine PBMCs after vaccination. Microplate Devices Unifilter 96-well plates (Waterman) were coated with anti-mouse IFN-γ antibody (125 ng/well) (BioSource International) or anti-porcine IFN-γ monoclonal antibody (500 ng/well) (Endogen) overnight at 4℃. After washing and blocking, $1 \times 10^6$ murine splenocytes or porcine PBMCs were added along with 200 ng of purified E2 protein into each well. The plates were incubated at 37℃ and 5% $CO_2$ for 40 h. For mouse sera, biotinylated anti-mouse IFN-γ antibody (125 ng/well) was added and incubated at 37℃ and 5% $CO_2$ for 3 h. For porcine sera, rabbit anti-porcine IFN-γ antibody (200 ng/well) (Endogen) was added and incubated at RT for 4 h followed by the addition of biotinylated goat rabbit IgG (Zymed) at a dilution of 1:5000 for 2 h at RT. All the plates were then incubated for 1.5 h at RT with streptavidin-AP (Jackson ImmunoResearch Laboratories) at a dilution of 1:500 and developed with 5-bromo-4-chloro-3-indolylphospohate and nitroblue tetrazolium substrate tablets (Sigma). The plates were dried and the spots were recorded.

### Lymphocyte proliferation-MTT assay

Splenocytes ($3 \times 10^5$ cells) isolated from each group of mice were seeded into each well of round bottom tissue culture 96-well plates (Nunc). Purified E2 protein (100 ng) was added to each well and incubated at 37℃ and 5% $CO_2$ for 72 h. Eighty ng of 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT, Sigma) was added to each well and incubated at 37℃ and 5% $CO_2$ for 3 h. The plates were centrifuged at 1000 × g for 10 min. One hundred μL of the supernatant was mixed with 100 μL of acidified isopropanol (0.375% HCl in isopropanol) and the optical density was recorded at 590 nm.

### *Cytotoxic T lymphocyte (CTL) assay*

**Effector cell preparation and stimulation:** To prepare effector cells, splenocytes were isolated from each group of mice and pooled 14 d after vaccination. To generate stimulating cells, splenocytes isolated from naïve, syngenic mice were infected with a recombinant vaccinia virus expressing HCV BK E2 protein (VP1478, kindly provided by Sanofi Pasteur MSD) at an m.o.i. of 10 for 1 h at 37℃ followed by an irradiation at 3000 rads. The splenocytes from experimental groups were cultured with irradiated stimulating cells for four days at 37℃ and 5% $CO_2$ before they were used as effector cells in CTL assays.

**Target cell preparation:** To generate target cells, a syngeneic mastocytoma cell line P1-HTR-TK$^+$[43] (H-2$^d$, a highly transfectable variant of P815 cells, kindly provided by Dr. T. Boon) was transfected with a plasmid expressing HCV BK E2. Stable transfectants, designated P1-E2 cells, were selected with 800 μg/mL of Geneticin (Invitrogen). The expression of HCV E2 protein was confirmed by immunohistochemical staining using an E2-specific polyclonal antibody as previously described[44]. In brief, after fixation and blocking, P1-E2 cells or plasmid vector-transfected P1 cells were incubated with an anti-E2 antibody at a dilution of 1:1000 in PBS for 30 min. Cells were then washed and incubated with a biotinylated secondary antibody for 30 min. The reaction was developed with 3, 3'-diaminobenzidine tetrahydrochloride (DAB) (Vector Laboratories).

**CTL assay:** CTL assays were performed based on the evaluation of the cytoplasmic lactate dehydrogenase (LDH) after cell lysis as described previously[45]. In brief, effector cells were harvested by centrifugation at 800 × g for 10 min and washed twice with medium. The cells were then adjusted to $2.0 \times 10^7$ cells/mL in AIM-V medium and incubated with target cells at standard effector:target (E:T) ratios in triplicates in a 96-well round bottom plate. After 4 h incubation, the plate was centrifuged at 250 × g for 4 min. Fifty μL of the supernatant was transferred to a 96-well flat bottom plate and mixed with the Substrate mix provided in the Cytotox 96 Non-radioactive Cytotoxicity Assay kit (Promega). After 30 min, the reaction was terminated by adding 50 μL of the Stop Solution into each well and the optical density was determined at 490 nm. Spontaneous and maximal LDH release by target cells was determined by incubating the target cells with medium alone or with medium plus the lysis buffer containing 0.9% Triton X-100 (Promega). HCV E2-specific lysis was calculated as [(experimental release-spontaneous release)/(maximal release-spontaneous release)] × 100.

### *Statistical analysis*

The experimental data were analyzed by software programs Prism 4 (GraphPad) or Excel (Microsoft) and were expressed as mean ± SE. A $P$ value of ≤ 0.05 determined by Student's $t$ test was considered statistically significant.

## RESULTS

### *Construction and characterization of a DNA vaccine expressing a secreted form of HCV E2 protein*

We designed a DNA vaccine encoding a secreted form of HCV E2 protein by removing the transmembrane domain and replacing the hypervariable region 1 (HVR1) of the E2 protein with the tissue plasminogen activator signal peptide sequence (tPAs). The corresponding coding sequence was amplified by PCR and cloned into a DNA vaccine vector pBISIA24[39], resulting in the plasmid pBISIA24-tPAs-tE2 (Figure 1). To determine the expression of E2 protein, TE671 cells were transfected with pBISIA24 (vector control) and pBISIS24-tPAs-tE2. The presence of E2 protein in cell lysates and culture medium was analyzed by immunoblotting. As shown in Figure 1, an E2-specific antibody recognized proteins of about 50 kDa in cell lysates and of 50-75 kDa in culture medium after pBISIA24-tPAs-tE2 transfection, whereas these proteins were not detected after pBISIA24 vector transfection, indicating the expression and secretion of the E2 protein by pBISIA24-tPAs-tE2. The apparent molecular masses (50-75 kDa) of tE2 protein are larger than the calculated one (31 kDa) and the extracellular form contained larger protein species than the intracellular form, suggesting that E2 protein may have been modified post-translationally by glycosylation.

### *Recombinant tE2 protein expression and purification*

The E2 protein used in immunization was produced in *E. coli*. The expression of poly-histidine tagged tE2 was induced by IPTG and purified by affinity chromatography (not shown). The purified poly-histidine tagged tE2 protein was dialyzed into phosphate-buffered saline before it was used in mouse and piglet immunization. The protein vaccine contained endotoxin at a concentration of 80 ng/mg of protein as determined by the *Limulus* amoebocyte test.

### *Antibody responses to HCV E2 DNA vaccine in mice*

To determine whether the DNA vaccine expressing a secreted E2 protein elicited antibody responses in mice, mouse sera collected after vaccination were analyzed for E2-specific IgG titers by ELISA assays. As shown in Figure 2A, vaccinated-mice all developed E2-specific IgG antibody after intradermal DNA vaccination with the average IgG titer being $1.6 \times 10^5$. To test whether a protein vaccine could enhance the IgG response, mice were injected twice with the DNA vaccine followed by recombinant E2 protein boosting. Mice that received the protein boosting vaccination had about two-fold higher titers of E2-specific IgG ($3.0 \times 10^5$) (Figure 2A). These results indicate that the DNA vaccine expressing a secreted form of HCV E2 protein induced strong antibody responses in mice, which were further increased by a protein vaccine.

Since the relative levels of IgG subclasses are an indicator for evaluating the quality of the immune response[46,47], the titers of IgG1 and IgG2a were determined. E2 DNA vaccine induced higher IgG2a titers than IgG1 (Figure 2), indicating a Th1-biased response. Boosting with E2 protein formulated with CpG ODN and 10% Emulsigen did not change this pattern, although the IgG1 and IgG2a levels were elevated (Figure 2).

### *Cell-mediated responses to HCV E2 DNA vaccine in mice*

To determine whether the HCV E2 vaccine induced cell-mediated immune responses in mice, we analyzed the

responses of mouse splenocytes after vaccination by measuring cell proliferation and interferon-γ secretion upon antigen re-stimulation *in vitro*.

In lymphocyte proliferation assay, splenocytes from saline vaccine group showed detectable proliferation after antigen stimulation (Figure 3A). Splenocytes isolated from mice after DNA vaccination expanded to a significantly higher degree after E2 antigen re-stimulation than medium control ($P < 0.001$, Figure 3A). Protein boost immunization did not change E2-specific lymphocyte proliferation (DNA *vs* DNA/Protein, $P = 0.47$, Figure 3A). The results of IFN-γ ELISPOT assay demonstrated that DNA vaccination elicited a strong IFN-γ response (DNA *vs* saline, $P < 0.001$, Figure 3B), whereas a protein boost immunization induced fewer IFN-γ secreting spots (DNA *vs* DNA/Protein, $P = 0.03$, Figure 3B). These results indicate that stronger E2-specific Th1-type immune responses were induced in mice that received the DNA vaccine three times than in mice that received twice DNA vaccination followed by a protein boosting injection.

Since cytotoxic T lymphocytes are one of the major effectors in cell-mediated immune responses, we also determined the presence of E2-specific cytotoxic T lymphocytes after vaccination. For this purpose, a syngeneic cell line expressing HCV E2 protein was generated. Immunohistochemistry staining using an anti-E2 antibody detected specific signal in P1-HTR cells stably transfected by the E2-expressing plasmid, but not in vector-transfected cells, demonstrating the expression of the E2 protein by the stable transfectant (not shown). When the E2-expressing cell line was used as target cells in the CTL assay, effector cells from DNA vaccine- or DNA and protein vaccine-immunized mice showed 36% or 31% specific lysis at an effector to target ratio of 100:1, respectively (Figure 4). No CTL activity was detected after saline immunization (Figure 4). These results demonstrate the elicitation of E2-specific cytotoxic T lymphocytes in mice immunized with E2 DNA vaccine or DNA vaccine followed by a protein boost.

### *Immune responses to HCV E2 vaccines in piglets*

Since vaccine efficacy can be different in various test species[31] and pigs represent a promising model for human biology because of its body size and physiology[48], we evaluated our E2 vaccines in piglets. As shown in Figure 5A, all the piglets receiving the E2 DNA vaccine three times intradermally developed E2-specific IgG titers with the average titer being $1 \times 10^3$. Vaccination with the E2 protein after two DNA vaccine priming injections significantly boosted the E2-specific IgG titers (DNA vaccine *vs* DNA/Protein, $P = 0.0156$, Figure 5A). These results indicate that E2 DNA vaccine was also effective in inducing antigen-specific antibody response in piglets, which was boosted significantly by a protein vaccine.

To analyze the type of the immune responses, we determined the ratios of E2-specific IgG1 to IgG2 levels in the porcine serum samples after vaccination. As illustrated in Figure 5B, DNA vaccination induced a slightly Th1-biased immune response with the IgG2 to IgG1 ratio being 1.4, whereas protein boosting after DNA vaccination shifted the immune response towards Th2 direction as demonstrated by a reduced IgG2/IgG1 ratio (0.58).

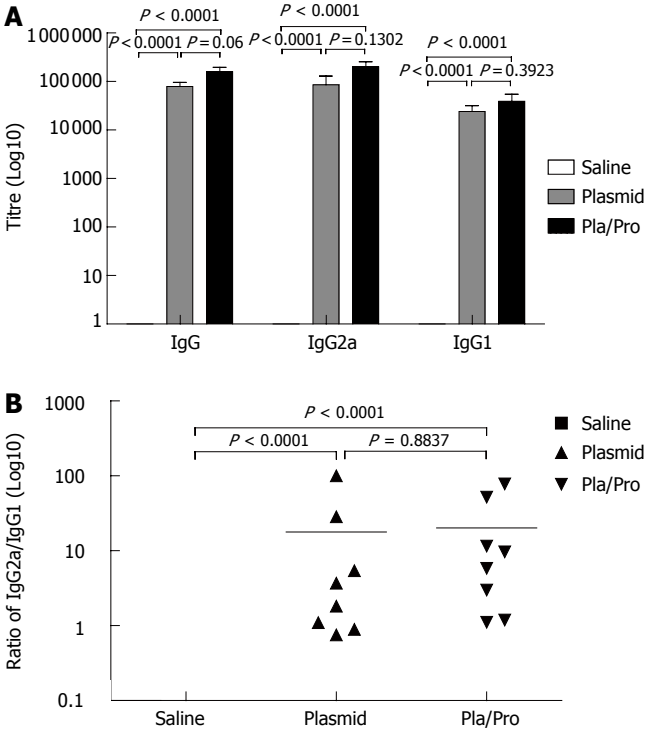


**Figure 2** Antibody responses to HCV E2 vaccines in mice. B6C3F1 mice were intradermally injected with pBISIA24-tPAs-tE2 three times or pBISIA24-tPAs-tE2 twice followed by subcutaneous vaccination with E2 protein formulated with CpG ODN 1826 and 10% Emulsigen at 3-wk intervals. E2-specific IgG, IgG1, and IgG2a levels were determined by ELISA assays (**A**). The ratios of IgG2a to IgG1 were determined (**B**) ($P < 0.0001$, *vs* saline).

To further characterize the immune responses of piglets to E2 vaccines, the number of IFN-γ secreting cells in porcine PBMCs was determined after vaccination. As shown in Figure 6, PBMCs from E2 vaccinated piglets contained significantly higher number of IFN-γ secreting cells than saline treated piglets after *in vitro* antigen re-stimulation in ELISPOT assay (DNA *vs* saline, $P < 0.0001$, DNA/Protein *vs* saline, $P < 0.0001$). No difference was detected between the DNA vaccine alone group and the DNA prime and protein boost group (Figure 6, DNA *vs* DNA/Protein, $P = 0.4531$).

## DISCUSSION

Numerous research groups have evaluated the potential of using E2 protein as a hepatitis C vaccine candidate through different strategies[5,36,49-74]. These studies have demonstrated E2-specific immune responses, mostly in mouse trials, when E2 alone or as part of the entire HCV structural region was delivered as a DNA vaccine, viral vectored vaccine, or subunit protein vaccine. The most important finding of these studies is that the vaccine-induced E2-specific immune responses were able to delay HCV infections in chimpanzees or humans[55,61,62,64]. While these studies have demonstrated the feasibility of inducing E2-specific immune responses through vaccination, the efficacy of E2 vaccines is less satisfactory in inducing sterilizing immunity. It is thus obvious that further

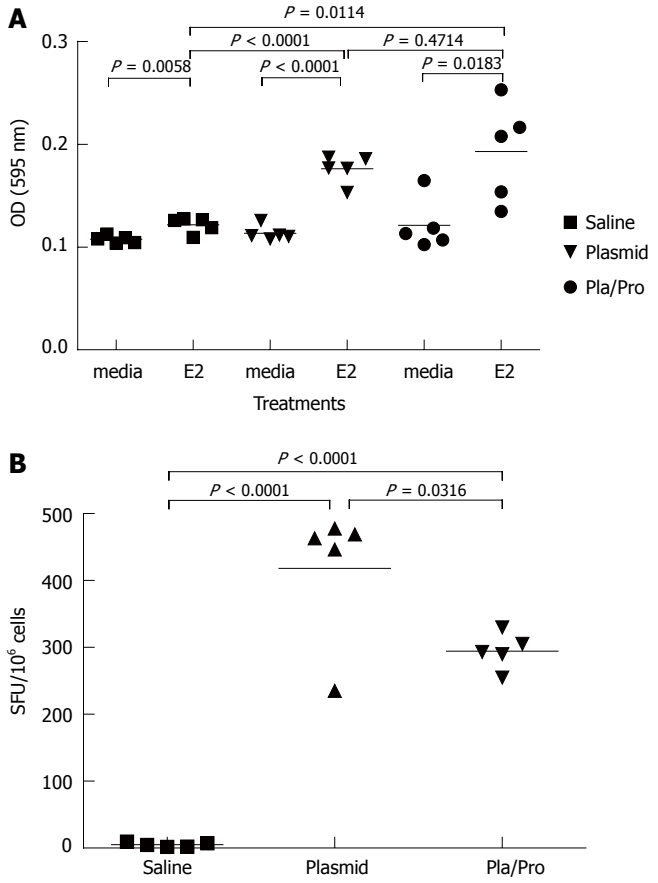


**Figure 3** Cell-mediated immune responses to HCV E2 vaccines in mice. Murine splenocytes were isolated 2 wk after last immunization and were re-stimulated with E2 protein. Cell proliferation was analyzed by MTT assay (**A**). ($P < 0.0001$ *vs* saline; $P < 0.05$, $P < 0.01$ *vs* media). The number of interferon-γ secreting cells was determined by ELISPOT assay (**B**). ($P < 0.05$ *vs* plasmid; $P < 0.0001$ *vs* saline).

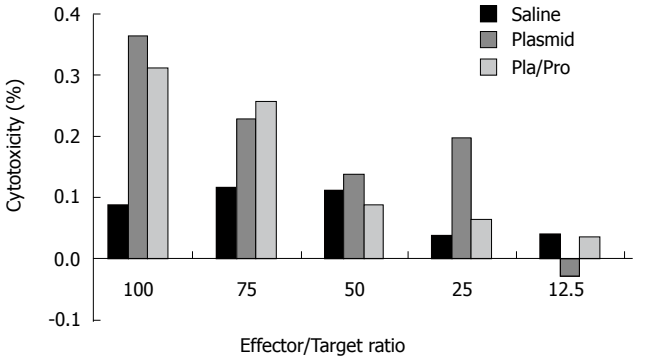


**Figure 4** Cytotoxic T lymphocyte (CTL) response to HCV E2 vaccines in mice. Murine splenocytes isolated from groups of mice two weeks after final immunization were pooled and stimulated with feeder cells infected with recombinant vaccinia virus expressing HCV E2 protein. The cytotoxic activity was determined by measuring the released lactate dehydrogenase (LDH) by the target cells after incubation with the effectors at different ratios.

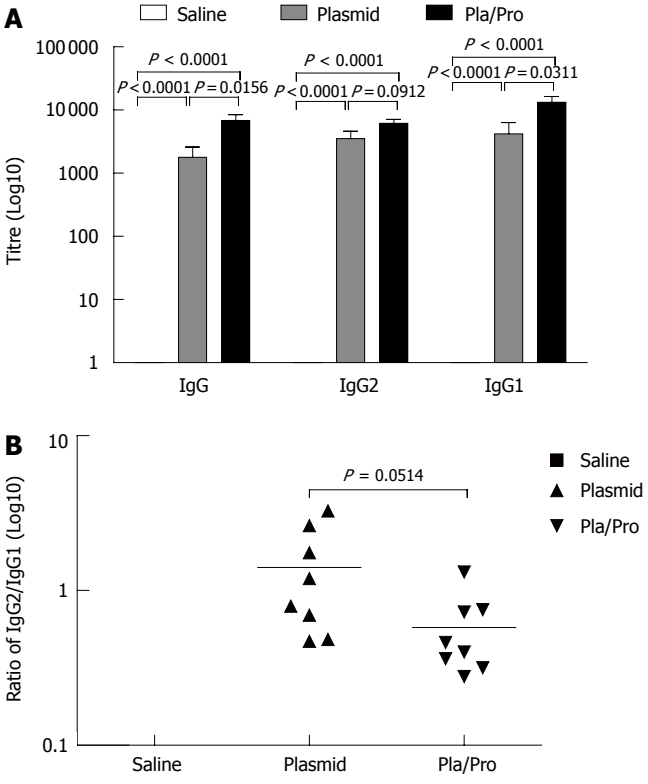


**Figure 5** Antibody responses of piglets to HCV E2 vaccines. Piglets were immunized by saline, E2 DNA vaccine three times, or E2 DNA vaccine twice followed by subcutaneous vaccination with E2 protein formulated with CpG ODN 2007 and 10% Emulsigen at three-week intervals. ELISA assays were performed to determine E2-specific IgG, IgG1, and IgG2 levels (**A**), as well as the ratios of IgG2 to IgG1 (**B**) ($P < 0.05$ *vs* plasmid; $P < 0.0001$ *vs* saline).

improvement of E2 vaccines is required by evaluating additional vaccination strategies.

Given the importance of E2 protein in inducing host immune responses, we have chosen E2 as a vaccine candidate against hepatitis C. It has been established that HCV E2 expressed by a number of systems is an intracellular protein modified by high-mannose type oligosaccharides[75-78]. However, the envelope proteins on the hepatitis C virions have been shown to be modified by complex type oligosaccharides[79], suggesting secretion of E2 protein has occurred in HCV life cycle. In agreement with this notion, the secreted, complex form E2 protein possesses greater immunoreactivity against hepatitis C patient sera than the intracellular E2 protein when it is expressed in cell culture[78]. The retention of the expressed E2 protein is due to the presence of a membrane anchor domain (amino acids 718-746) at its carboxyl-terminus[80-82] and deletion of the transmembrane domain leads to E2 protein secretion[83]. However, a comparative study of a series of carboxyl-terminally truncated E2 proteins has demonstrated that truncation to amino acid residue 661 is necessary to achieve proper folding of the secreted E2 protein[83]. Hepatitis C virus is known to be able to quickly develop multiple sequence variants after infection within the host due to the lack of proof-reading activity of RNA-dependent RNA polymerase[84]. A short stretch of coding sequence (amino acids 384-411, the hypervariable region 1) at the very amino-terminus of E2 has shown extremely high variability[85,86] and may contribute to the generation of immune escape HCV variants. Although E2 HVR-1 may contain antigenic epitopes[15,16], rapid appearance of variable mutants and their interference with the development of cellular immunity in the host[87] make it a less favorable component in an E2 DNA vaccine. Based on

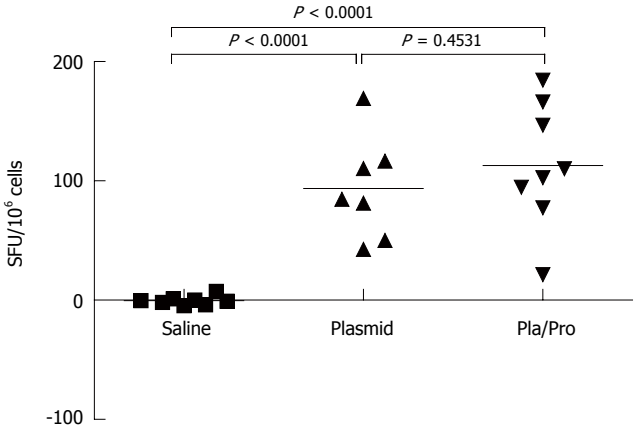


**Figure 6** Cell-mediated immune response of piglets to HCV E2 vaccines. Porcine peripheral blood mononuclear cells (PBMCs) were isolated three weeks after final immunization. After re-stimulation with E2 antigen, the number of interferon-γ secreting cells was determined by ELISPOT assay ($P < 0.0001$ *vs* saline).

the above findings and analysis, we designed an E2 DNA vaccine by deleting the carboxyl-terminal hydrophobic region after amino acid residue 661 and replacing HVR1 by a strong signal peptide sequence derived from tissue plasminogen activator[88]. Indeed, we showed that the engineered E2 protein was efficiently secreted into the culture medium (Figure 1). In addition, in agreement with previous studies[83], the apparent molecular masses of the intracellular as well as secreted tE2 proteins are larger than that calculated from the amino acid sequence and the secreted tE2 proteins contain species of even higher molecular mass than the intracellular tE2 protein, suggesting that intracellular and extracellular E2 proteins may have been modified by different glycosylation.

Intradermal injection of this E2 DNA vaccine elicited strong E2-specific antibody responses in mice as demonstrated by high IgG levels (Figure 2). IgG isotyping, IFN-γ ELISPOT, lymphocyte proliferation, and CTL assays (Figures 2-4) indicated that both Th1 lymphocyte and cytotoxic T lymphocyte responses were induced by E2 DNA vaccine. Interestingly, DNA vaccine alone or DNA vaccine followed by an E2 protein formulated with CpG ODN and 10% Emulsigen induced comparable E2-specific IgG2a to IgG1 ratios (Figure 2B) and CTL responses (Figure 4). This is in agreement with the proven roles of immuno-stimulatory CpG ODN in promoting Th1 immune responses[41,89-91]. However, although DNA prime and protein boost strategy induced a relatively strong IFN-γ response, the number of IFN-γ secreting cells in the splenocytes derived from mice immunized with DNA prime and protein boost was significantly lower than that of DNA vaccine group (Figure 3B).

DNA vaccines are often less effective in large model animals and in humans than in mice[31,92]. Therefore, it is critical to test the vaccine efficacy in an out-bred, large animal model. Our piglet trial indicated that E2 DNA vaccine induced E2-specific IgG titers (Figure 5A) that were about 100-fold lower than those detected in mice (Figure 2A). As for the type of vaccine-induced immune response, E2 DNA vaccine induced a more balanced response in piglets (Figure 5B), in contrast to a strongly Th1-biased response in mice (Figure 2B). The effect of protein boost injection in modulating the type of the immune response was also different: while protein boost had little effect in mice (Figure 2B), a shift towards Th2 direction was detected in piglets (Figure 5B). Taken together, while these results demonstrate that E2 vaccines were also effective in inducing antigen-specific immune responses in piglets, inter-species difference in immune responses to different vaccine strategies requires further investigation.

In conclusion, our results demonstrate that a DNA vaccine expressing the secreted form of HCV E2 protein induces E2-specific immune responses in mice and piglets. An E2 protein vaccine formulated with CpG ODN and 10% Emulsigen further increases the antibody responses. In addition, our results highlight the importance of testing the magnitude and type of vaccine-induced immune responses in multiple model species before primate or human trials are initiated.

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## COMMENTS

### Background
Hepatitis C is a devastating liver disease worldwide. Current interferon and ribavirin therapy is far from satisfactory. An effective vaccine for hepatitis C is urgently needed.

### Research frontiers
To develop an effective vaccine for hepatitis C, a rational design based on the understanding of the magnitude and the quality of the immune responses is critical for a success. The application of appropriate adjuvants and vaccine delivery means also play a major role.

### Innovations and breakthroughs
The novelty of this article lies in the design of the HCV E2 vaccine, namely the hypervariable region-1 was replaced by the signal peptide sequence of the tissue plasminogen activator and the hydrophobic region at the carboxyl-terminus was removed.

### Applications
The HCV E2 DNA vaccine developed in this study should be further tested in primate models with hepatitis C virus challenge to demonstrate protective activity of the vaccine.

### Peer review
HCV infection is a big burden worldwide. Numerous studies have evaluated the potential of using E2 protein as a hepatitis C vaccine candidate through different strategies, but the efficacy of E2 vaccines is less satisfactory in inducing sterilizing immunity. It is interesting to investigate further improvement of E2 vaccines by evaluating additional vaccination strategies. The authors characterized the immunogenicity of a HCV E2 DNA vaccine alone or with a protein vaccine boost in murine and porcine animal models. They found that this E2 DNA vaccine elicited E2-specific immune responses in mice and piglets and recombinant E2 protein vaccination boosting significantly increased the antibody response in piglets. The study is well designed and results are convincing. The presentation and readability of the manuscript is satisfactory.

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# Antimicrobial activity of *Sapindus mukorossi* and *Rheum emodi* extracts against *H pylori*: *In vitro* and *in vivo* studies

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## Abstract

**AIM:** To evaluate the antibacterial activity of *Sapindus mukorossi* (*S. mukorossi*) and *Rheum emodi* (*R. emodi*).

**METHODS:** Powders of *S. mukorossi* and *R. emodi* were extracted successively with petroleum ether, benzene, chloroform and ethanol and were concentrated in vacuum. The disk diffusion method was used for *in vitro* studies and *in vivo* studies were performed on male Wister rats. Thirty resistant clinical isolates of *H pylori*, as determined by their antibiotic sensitivity patterns by *E*-test, along with two Gram +ve (*S. aureus*, *B. subtilis*) and two Gram -ve (*E. coli*, *P. vugaris*) organisms were screened for their susceptibility patterns against these extracts.

**RESULTS:** In our screening, all 30 resistant isolates and the other four organisms (two Gram +ve *S. aureus*, *B. subtilis* and two Gram -ve, *E. coli*, *P. vugaris*) were sensitive to the test compounds. It was found that ethanol and chloroform extracts of *S. mukorossi* and ethanol and benzene extracts of *R. emodi* inhibited *H pylori* at very low concentrations. In the *in vitro* study, the isolates showed a considerable zone of inhibition at very low concentrations (10 μg/mL) for both the extracts. In the *in vivo* study, the *H pylori* infection was cleared with minimal doses of extracts of *S. mukorossi* (2.5 mg/mL) and *R. emodi* (3.0 mg/mL) given orally for seven days.

**CONCLUSION:** We can conclude from this study that the extracts of *S. mukorossi* and *R. emodi* inhibited the growth of *pylori in vitro* and, in *in vivo* studies, the *H pylori* infection cleared within seven days at very low concentrations. We also found that *H pylori* did not acquire resistance against these herbal extracts even after 10 consecutive passages.



## INTRODUCTION

*H pylori* has infected more than half the population of the world. Most people are unaware that they are infected because they remain asymptomatic throughout life and survive without any harmful infection-related clinical sequelae. However, some develop duodenal or gastric ulcers and a small proportion are diagnosed with MALT lymphoma or gastric malignancy[1]. Others suffer from non-specific dyspeptic symptoms with no obvious cause other than *H pylori* infection and its associated gastritis.

Global elimination of *H pylori* is a noble goal that would have a major impact on present and future world health. During the past decade effective treatment therapies have been developed to cure *H pylori* infection, following the work of Bazzoli *et al*[2] and Lamouliatte *et al*[3]. Current treatment is based on a combination of a proton pump inhibitor (PPI) with either amoxycillin or metronidazole. Large multicentric studies, especially MACH 1[4] and MACH 2[5], have confirmed the excellent results of pilot studies leading to eradication rates higher than 90%. Unfortunately, in subsequent years such good results have not been achieved, especially in South East Asia and Southern Europe, where resistance to antibiotics has become more prevalent. Complete eradication of *H pylori* is still in the initial stage. Resistance to amoxycillin, clarithromycin and metronidazole is widespread, and probably increasing as a result of the constant use of these drugs against *H pylori* infection. In our recent multicentric study, undertaken by the Indian *H pylori* study group, we assessed the Indian scenario of resistance[6]. The study showed higher resistance to antibiotics than in developed countries[7]. In this study, 100% resistance of *H pylori* towards metronidazole was reported in Hyderabad, which was much higher compared to other regions of India. If this trend continues the treatment with clarithromycin and metronidazole may become ineffective.

In view of the incomplete cure achieved with the

triple therapy described above and its possible side effects, alternative medicine cures are gaining much prominence and are found to be safe and effective eliminators of *H pylori* infection. Thus, in an endeavor to overcome increasing resistance, we continued to look for some selected herbal extracts that are capable of inhibiting the growth of *H pylori* with minimal or no side effects. In the present study, we selected two plants, namely *Sapindus mukorossi* and *Rheum emodi,* and investigated the inhibitory effect of these plant extracts against *H pylori in vitro* and *in vivo*.

*S. mukorossi* Gaerten (Sapindaceae), commonly known as Ritha or Aritha is found throughout India. The major constituents of its fruit are saponins (10%-11.5%), sugars (10%) and mucilage[8]. The fruit of the plant is reported to have expectorant, emetic, alexipharmic and abortificiant effects. It is also used for excessive salivation, epilepsy and chlorosis[9,10]. Saponins from this plant are known to be spermicidal *in vitro*[11]. This spermicidal property has been used in contraceptive cream[12]. The alcoholic extract of (*Sapindus trifoliatus Linn*) is reported to possess anti-implantation activity[11].

*R. emodi* (Polygonaceae), commonly known as Indian or Himalayan Rhubarb, is found in India. The major constituents of rhubarb rhizomes are anthraquinones. Rhubarb is used as a laxative and diuretic to treat kidney stones and it is used for gout and liver diseases characterized by jaundice. Externally, it is used to heal skin sores and scabs. Paradoxically, larger doses are used as a laxative, although small doses are used to treat dysenteric diarrhea[13]. The Chinese use rhubarb as an ulcer remedy, anti-helminthic and to treat cancer, upper intestinal bleeding (ulcers), fever, and headache. They consider it to be a bitter, cold dry herb used to "clear heat" from the liver, stomach and blood[14,15]. It is also used to treat toothache[16]. In Europe, rhubarb is a component of spring tonics or blood cleansing cures, including Swedish bitter[17]. Turkish or medicinal rhubarb is also one of the four major ingredients in the herbal cancer remedy.

We isolated extracts from both plants and carried out antimicrobial screening for various micro-organisms. Based on the above results, a study was designed using the obtained products of *S. mukorossi* and *R. emodi* to assess the antibacterial activity against *H pylori in vitro* and *in vivo*.

## MATERIALS AND METHODS

### Plant Materials

Authentic samples of *S. mukorossi* and *R. emodi* were obtained from an authorized supplier (M/s Munnalal Dawasas and Co. Hyderabad, Andhra Pradesh, India). The plants were identified and authenticated by experts in the Post Graduate and Research Department of Botany, Anwar-ul-loom College, Hyderabad, Andhra Pradesh, India.

### Bacterial strains

Thirty *H pylori* strains along with two gram +ve (*Staphylococcus aureus, Bacillus subtilis*) and two gram -ve (*Escerichia coli*, *Proteus vulgaris*) pathogenic bacteria were used in the study. The *H pylori* strains were isolated from gastric biopsy specimens [15 from duodenal ulcer (DU), 8 from gastric ulcer (GU), 4 from non-ulcer dyspepsia (NUD), 3 from gastric carcinoma (GC)], after informed consents were obtained from patients who underwent upper gastrointestinal endoscopy at Deccan College of Medical Sciences, Hyderabad, India. The other four pathogens were obtained in pure culture form from the Department of Microbiology, Deccan College of Medical Sciences, Hyderabad, A. P, India.

### E-test strips

E test strips were obtained from AB Biodisk, Solna, Sweden.

### Animals

Male Wister rats (175-200 g) were acclimated to the housing facilities for 5 d before initiation of the study. Free access to standard pellet chow was allowed throughout the experimental protocol, with the exception of overnight fasting before induction of the ulcer. All protocols were approved by the Animal Care and Use Committee of the Deccan College of Medical Sciences and Research Centre, Hyderabad, India where the study was conducted.

### Extraction, separation and purification of the compounds

For phytochemical analysis, approximately 100 g of fruit pericarp of *S. mukorossi* and rhizomes of *R. emodi* were collected and the materials were chopped, air dried at 35-40℃ and pulverized in an electric grinder. The powder obtained was successively extracted in petroleum ether (60-80℃), benzene, chloroform and ethanol.

The extracts were then powdered by using a rotary evaporator under reduced pressure. Fruit pericarp of *S. mukorossi* yielded 38g, 28g, 34g and 35g of powdered extracts with petroleum ether, benzene, chloroform and ethanol respectively. Rhizomes of *R. emodi* yielded 19 g, 17 g, 21 g and 22 g of powdered extracts. Extracts obtained by percolation using 70% of ethanol as a solvent at room temperature were processed according to process A of Farmacopeia dos Eastados Unidos do Brasil (1959) (AOAC 1990).

The extracts were evaporated at 40℃ under vacuum and the residue was freeze-dried. The dry extracts of the fruit pericarp of *S. mukorossi* and rhizomes of *R. emodi* were tested for the presence of saponins and anthraquinones. Each extract of the fruit pericarp of *S. mukorossi* (SM) and rhizomes of *R. emodi* (RE) were column chromatographed over a silica gel (200 mesh), eluted with $CHCl_3$-MeOH (70:30, 60:40, 50:50, 25:75) and compound fractions of (250 mL each) were collected and monitored by TLC. These column chromatographed compound fractions were further filtered to yield saponins and anthraquinones, which were separated by paper chromatography and preparative TLC to yield saponins [(SM-A (petroleum ether), SM-B (benzene), SM-C (chloroform) and SM-D (ethanol)] and anthraquinones [(RE-A (petroleum ether), RE-B (benzene), RE-C (chloroform) and RE-D (ethanol)] respectively.

All the filtrates obtained were dried by evaporation

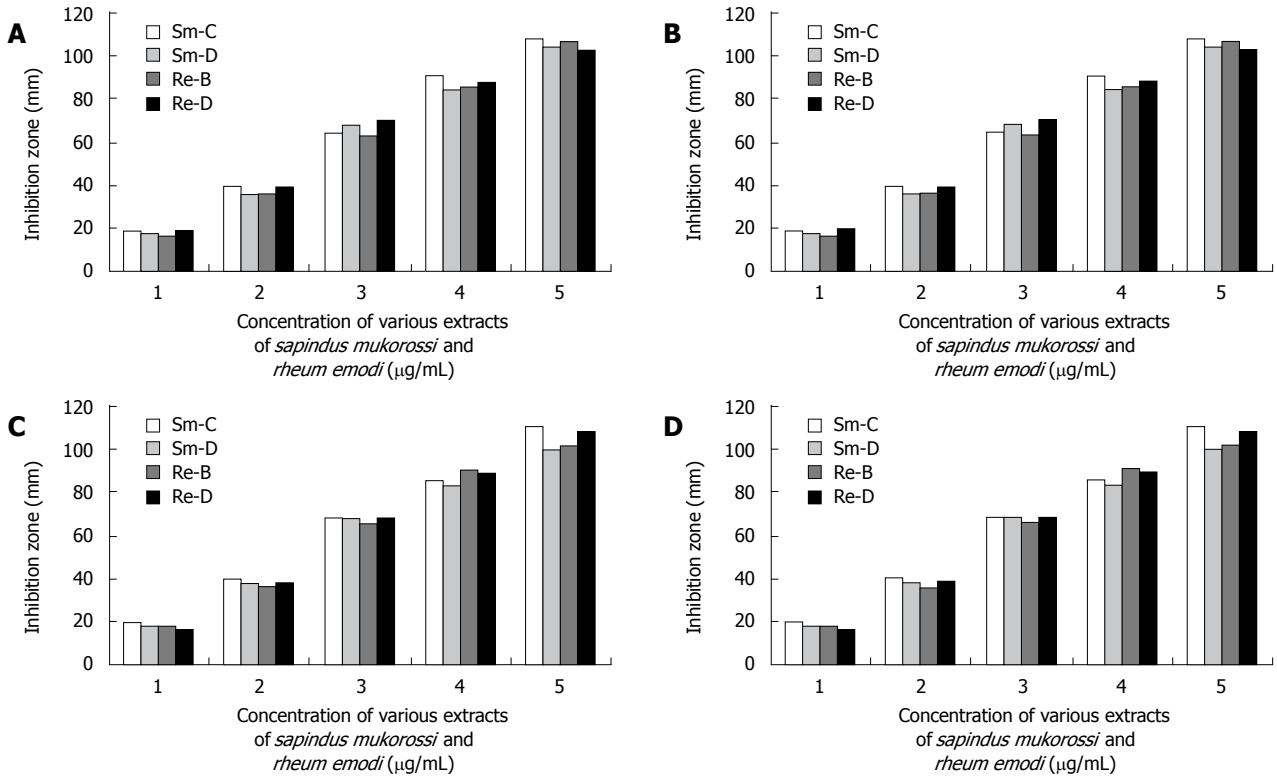


**Figure 1** Antibacterial activity of various extracts *Sapindus mukorossi* and *Rheum emodi* against *S. Aureus* (**A**), *B. Subtilis* (**B**),*E.Coli* (**C**), *P vulgaris* (**D**).

(Rotometer, 40℃) and the dried extracts were individually once again dissolved in 10 mL ethanol (95%). Then subjected to a complete drying process and weighed according to the AOAC (1990) method[18]. The products obtained were tested initially for antimicrobial activity against different gram +ve organisms (*S.aureus, B.subtilis*) and gram -ve organisms (*E.coli*, *P.vulgaris*). Effective antibacterial activity was noted (Figure 1A-1D).

### *Isolation of H pylori*

From every patient a total of three-biopsy specimens were collected, 1 in urea solution and two biopsies (1 from the antrum and the other from the corpus) were collected in Brucella broth supplemented with 2% fetal calf serum [FCS] (Gibco BRL, Germany) and 10% glycerol. The biopsy specimens were smeared directly onto Brucella agar (Difco Chemicals, Detroit, USA) supplemented with 7% (v/v) sheep blood, Vancomycin- 6 mg/mL, Amphoteracin B- 2mg/mL and Polymixin B- 2500 IU/mL (Sigma Chemicals Inc, Bangalore).

The agar plates were incubated at 37℃ for 3-5 d under microaerobic conditions (4% Oxygen, 5% Hydrogen, 5% Carbon-di-oxide, and 86% Nitrogen) in a candle jar dessicator. *H pylori* were identified based on the culture characteristics, such as small translucent colonies (2-4 mm) under a dissected microscope, gram -ve spiral organisms, positive for catalase, cytochrome oxidase and urease tests. Stocks of pure culture were preserved in Brucella broth (Difco Chemicals, Detroit, USA) supplemented with 10% FCS and 15% glycerol and stored in -80℃ and revived when required.

### *Antibiotic sensitivity tests*

The antibiotic sensitivity patterns of all the 30 *H pylori* strains towards commonly used antibiotics such as metronidazole, amoxycillin, tetracycline and clarithromycin were evaluated by the *E*-test method. All the strains were seeded on Brucella agar supplemented with 7% sheep blood and grown for 48 h under microaerobic conditions. Bacterial growth was scraped from the plates and resuspended in sterile saline. The inoculum was prepared to contain $10^8$ CFU/mL by adjusting the suspension to match the McFarland No-0.5 turbidity standard for MIC studies[19] and $2 \times 10^8$ CFU/mL of Mc Farland no- 1 standard for antibiotic sensitivity studies[20,21] (Table 1).

### *Determination of MIC of the herbal extracts on H pylori by disc diffusion method*

Growth inhibition was performed by the filter paper disc diffusion method[22] on brucella agar plates supplemented with 7% sheep blood under micro-aerobic conditions at 37℃. To check the antimicrobial activity of the extracts and to read the antibiotic sensitivity of the isolates, inoculum was prepared as described above and the culture was spread over the plates with the help of a sterile cotton swab.

Various concentration disks (10-200 μg/mL) of the plant extracts were prepared and the latter were placed on brucella agar plates with 7% sheep blood earlier inoculated with 50 μL bacterial suspension in brucella broth with 5% FCS ($10^8$-$10^9$ CFU/mL). This was done to evaluate the minimum concentration of the extracts at which *H pylori* growth was inhibited effectively. The MIC was determined

**Table 1 Antibiotic sensitivity patterns of *H pylori* strains as determined by *E*-test (*n* = 30)**

| Antibiotic | Sensitive *n* (%) | Resistance *n* (%) | Range (μg/mL) | MIC[1] (μg/mL) |
|---|---|---|---|---|
| Metronidazole | 7 (23.4) | 23 (76.6) | 0.0125 - > 256 | > 8 |
| Clarithromycin | 28 (93.3) | 2 (6.7) | < 0.016 - > 256 | > 2 |
| Amoxicillin | 28 (93.3) | 2 (6.7) | < 0.016 - > 256 | > 0.5 |
| Tetracycline | 28 (93.3) | 2 (6.7) | < 0.016 - 2 | > 4 |

[1]MIC- Minimum Inhibitory Concentration. (Values in parentheses are percentages).

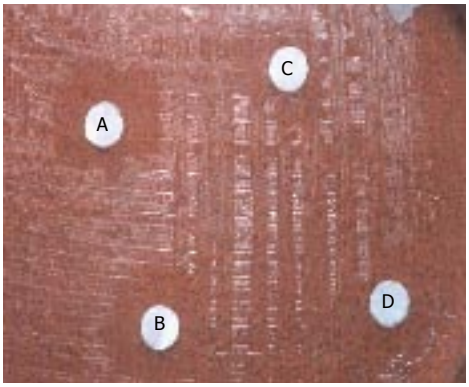


**Figure 2** Photograph showing inhibitory activity of *S. mukorossi* and *R. emodi* on *H pylori*. 'A' and 'C' represents ethanolic and chloroform extracts of *S. mukorossi*. 'B' and 'D' represents ethanolic and benzene extracts of *R. emodi*.

by measuring the zone of inhibition at each concentration after incubation at 37℃ for 3-5 d (Figure 2).

### *Determination of antimicrobial activity of the herbal extracts in an in vivo model*

**Induction of ulcers:** Ulcers were induced using a model modified from that described by Okabe and Pfeiffer[23]. Briefly, rats were given acetic acid (0.5 mL of 80% vol/vol) with a syringe instilled into the stomach and allowed to remain in contact with the stomach for 1 min, then it was aspirated and the area rinsed with sterile saline. The area exposed to acetic acid was 60 $mm^2$. The rats were killed by cervical dislocation and the stomach was removed and pinned out on a wax block. A paper grid with an area of 25 $mm^2$ was placed alongside the ulcer, which was then photographed (Figure 3). We then determined the ulcer area by planimetry, using × 5 enlargements of the photographs.

The area of ulceration in pixels was converted to square millimeters using the paper grid as a reference. Previous work using this ulcer model has revealed that ulcers induced by acetic acid are characterized by a thick layer of granulation tissue at the base and glandular disorganization at the ulcer margins. The ulcers involved the full thickness of the mucosa and penetrated into the muscularis mucosa. Perforation was not observed with this model. The rats were infected by giving the *H pylori* inoculum orally every alternate day for seven days.

**Bacterial content of ulcers:** To determine the bacterial content in gastric ulcers, animals were killed under aseptic

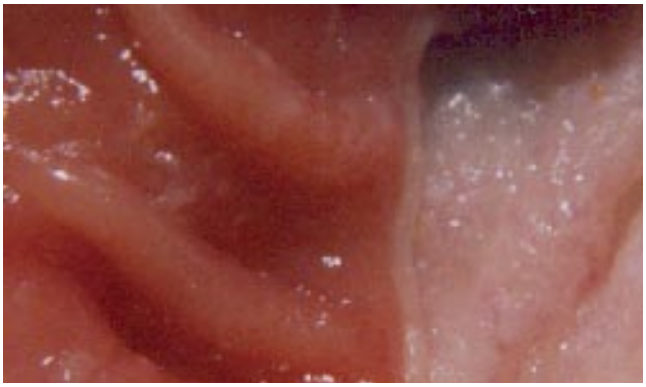

**Figure 3** Control Ulcer.

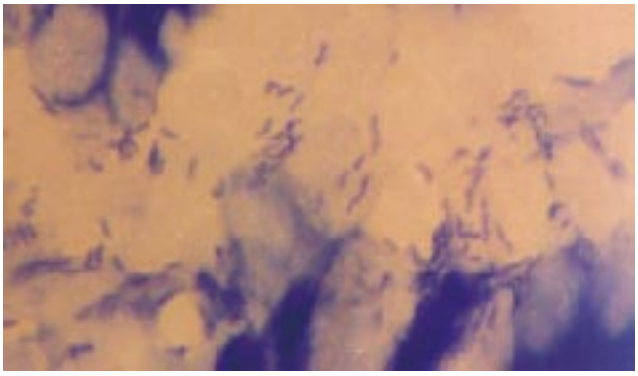

**Figure 4** Typical photograph showing gastric biopsy stained with Giemsa Stain showing tufts of spiral shaped *H pylori*.

conditions. Tissue (150 mg) was washed in sterile PBS and transferred to sterile, pre-weighed containers (to determine sample weight), sterile PBS was added, and then the sample was homogenized. Serial dilutions were then plated on Mc-Conkey agar and tryptic soy agar plates and incubated for 18-24 h under aerobic conditions. The colony-forming units (CFU) were determined using a Leica colony counter. Results are expressed as CFU per gram of tissue. Tissue samples of the ulcers and healthy tissues were embedded in plastic using a commercially available kit (JB-4 embedding kit; Polysciences, Warrington, PA). Thin sections (1-1.5 μm) were stained with methylene blue-fuchsin (basic) and examined under a light microscope (Figures 4 and 5).

Further, the stomach tissue was collected for determination of ulcer size, histopathology and molecular confirmation of *H pylori* using Polymerase Chain Reaction (PCR) (Figure 6).

The rats were then divided into two treatment groups, Group Ⅰ of 6 rats who orally received methanolic extract of *S. mukorossi* (Figure 7) and Group Ⅱ of 6 rats who orally received ethanolic extract of *R. emodi* (Figure 8). Group Ⅲ of 6 rats received saline or dextrose. The total daily intake of dried *Smukorossi* and *Remodi* was 2.5 mg/d and 3.0 mg/d respectively for seven days.

### *Effect of treatment*

The effect of treatment with the extracts of *S. mukorossi* and *R. emodi* was assessed. Ulcers were induced in the rats

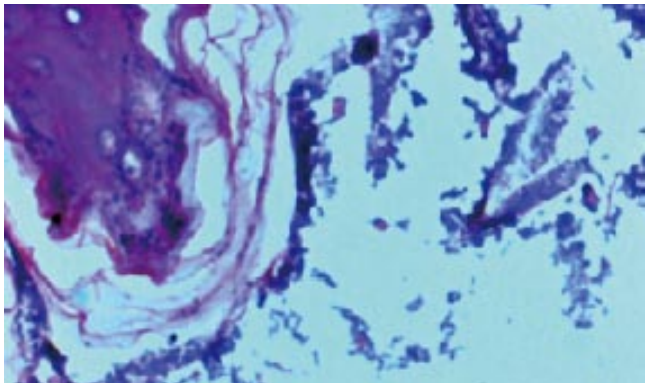

**Figure 5** Photograph showing colonization of *H pylori* in the upper gastric and esophageal region.

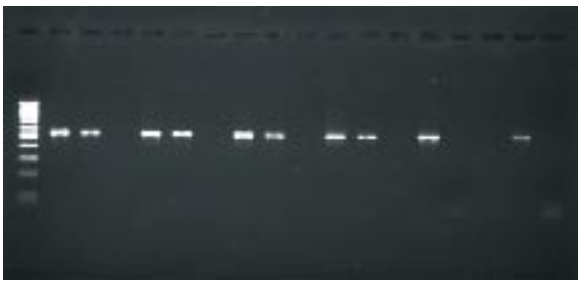

**Figure 6** Typical gel image showing 16S rRNA amplification of *H pylori*. Lane 1 represents 100bp Molecular weight marker, Lane 2 & 3, 5 & 6, 8 & 9, 11 & 12, 14 & 15 represents 16S rRNA amplification of *H pylori* DNA isolated from the culture. Lane 17 represents positive control of band at 534 bp. (ATCC26695), Lane 18 represents negative control.

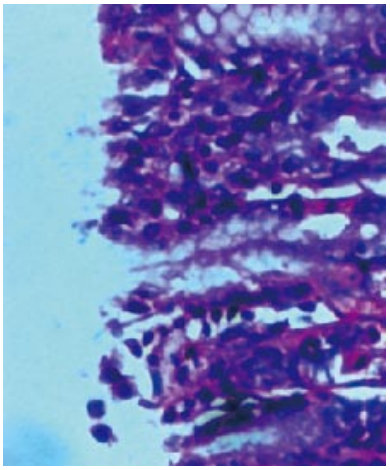

**Figure 7** Photograph of gastric mucosal section showing surface erosion and mucous depletion, *H pylori* can not be seen. Photograph of Post-infection treatment with the extract of *Sapindus mukorossi.*

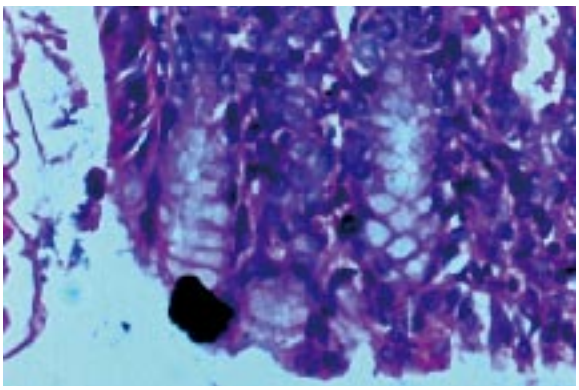

**Figure 8** Photograph of gastric mucosal section showing small foci surface erosion mucin secreting cells in pits are seen, No *H pylori* detected. Photograph of Post-infection treatment with the extract of *Rheum emodi.*

as described above. Seven days after ulcer induction and *H pylori* infection, both groups of rats were orally given the prescribed dose of both the extracts of 2.5 mg/mL and 3.0 mg/mL for seven days. The effect of treatment was checked by sacrificing the rats by cervical dislocation, removing the stomach and photographing it for ulcer determination, and taking tissue samples for bacterial culturing.

### *Statistical analysis*

The data were analyzed with a chi-square ($\chi^2$) test.

## RESULTS

In the *in vitro* study, we found that *S. mukorossi* and *R. emodi* extracts exert an inhibitory effect on *H pylori* (Figure 9). Ethanol and chloroform extracts of *S. mukorossi* and benzene and ethanol extracts of *R. emodi* with various concentrations ranging from 10-200 μg/mL were tested for their effect on *H pylori* growth by the disk diffusion method. In the *in vivo* study, we found that the extracts of *S. mukorossi and R. emodi* were able to clear *H pylori* infections in the rat models at concentrations of 2.5 mg/mL and 3.0 mg/mL post-infection (Figures 7 and 8).

### *MIC of the extracts as determined by Kirby Bauer's disc diffusion method*

It was found that, in the 30 isolates tested, the strains showed approximately 20 mm and 19 mm zones of inhibition with very low (10 μg/mL) concentrations of the ethanol extract of *S. mukorossi* and the benzene extract of *R. emodi*. The chloroform extract of *S. mukorossi* and the ethanol extract of *R. emodi* showed approximately 18 mm and 15 mm zones of inhibition at 10 μg/mL concentration. Maximum inhibition zones of approximately 98 mm and 94 mm were attained with the extracts of *S. mukorossi,* and maximum inhibitions zones of 92 mm and 90 mm were attained with the extracts of *R. emodi* at a concentration of 200 μg/mL. The details of the MIC of both the extracts against *H pylori* are listed in (Figure 9). *In vivo,* we found that the extracts of *S. mukorossi and R. emodi* were able to clear *H pylori* infections at the concentrations of 2.5 mg/mL and 3.0 mg/mL post-infection (Figures 7 and 8).

## DISCUSSION

Elimination of *H pylori* has been a major objective of treatment strategies worldwide. Unfortunately, none of them have been able to achieve 100% eradication rates[24]. The main reason underlying this failure could be the rapidly emerging resistance among many strains of *H pylori* towards various antibiotics. In addition to increased acquired resistance with the majority of

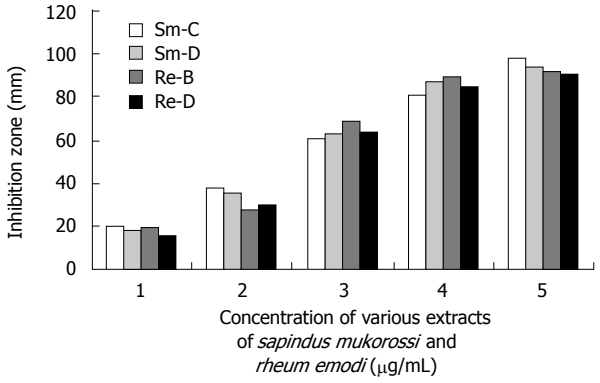


**Figure 9** Antibacterial activity of various extracts *Sapindus mukorossi* and *Rheum emodi* against *H pylori*.

antibiotic therapies, which are considered highly efficient for long-term treatment, studies have shown non-compliance among patients.

Apparently related to the incomplete cures achieved with the triple therapies and their possible side effects, herbal systems of medicine have become increasingly popular in recent years. According to one estimate, nearly 70% of synthetic drugs are derived from medicinal herbs and medicinal herbs have figured high in pharmaceutical industry research because of their high therapeutic activity. Herbal therapies have shown better efficiency and efficacy in inhibiting *H pylori* at both *in vitro* and *in vivo* levels[25,26]. Based on the above, we decided to look for plant extracts capable of inhibiting the growth of *H pylori*. The reason for selecting *S. mukorossi* and *R. emodi* to assess antibiotic activity against *H pylori* is their chemical nature. The chemistry of these herbs is very complex and involves large amounts of physiologically active compounds. Not all the constituents present in the plant have therapeutic activity. Physiological activity is largely dependent on the complete structure, including the glycosidic moiety[27]. This activity is enhanced several-fold due to the presence of sugars in these compounds[28]. *S. mukorossi* and *R. emodi* are saponins and anthraquinones in nature and it is because of this that these plants exhibit antibacterial activity.

Among various extracts of both these plants, we found chloroform and ethanolic extracts of *S. mukorossi* exerted strong antibacterial effects on all the gram +ve and gram -ve organisms, including *H pylori,* while the petroleum ether and benzene extracts did not show any effect. Similarly, benzene and ethanolic extracts of *R. emodi* were found to exhibit a strong inhibitory effect on *H pylori* and other organisms while the petroleum ether and chloroform extracts showed no potential affect either on *H pylori* or other gram +ve and -ve microbes. Our study is the first to report the antibacterial effect of these plant extracts on *H pylori*. In our screening, we found that all 30 *H pylori* isolates were sensitive to the test compounds. As evident from the results, the compounds tested were found to possess antibacterial activities on *H pylori* and were not affected by the resistance of the organism to antibiotics.

The sensitivity of *H pylori* to commonly used antibiotics by (*E*-test) was carried out to assess susceptibility patterns and the sensitivity of the compounds was studied to evaluate the anti-*H pylori* activity of our extracted compounds (10-200 μg/mL by disk diffusion method). We assessed the minimum inhibitory dose of the compounds at which the survival of *H pylori* was in jeopardy. As evident from Figure 9, the extracts of *S. mukorossi* and *R. emodi* are highly effective even at a very minimal dose of 10 μg/disk.

Further, we found that none of the isolates acquired resistance even after 10 passages at sub-inhibitory concentrations. We found that *S. mukorossi* extracts and *R. emodi* extracts of 10 μg/mL concentration proved to be the MIC for almost all the isolates (except MS-24 and MS-28, which were found to be completely inhibited at 8 μg/mL). The reason behind this could be strain variation (data not shown).

*S mukorossi and R. emodi* extracts were found to inhibit all strains of *H pylori* (both sensitive and resistant) at a concentration of 10 μg/mL. This inhibitory concentration is comparable with the previously reported allicins 6-12 μg/mL, garlic oil 8-32 μg/mL[29] and ajoenes 10-25 μg/mL of oil macerated garlic extract. Our compounds proved to be more potent than thiosulfinate 40 μg/mL, vinyldithiins < 100 μg/mL, epigallocatechin gallate in Chinese lung chen tea 50-100 μg/mL and garlic powder 250 μg/mL[29]. Further more, the organism did not develop any resistance to the test compound even after 10 subsequent passages, grown at sub-inhibitory concentrations, whereas *H pylori* strains acquired resistance to amoxycillin and clarithromycin after 10 sequential passages[30,31]. In addition, we found that our extract had almost equivalent bacterial activities against both antibiotic susceptible and resistant *H pylori* strains. Animal studies suggest that an extract of *S. mukorossi and R. emodi* taken orally may help prevent stomach ulcers and it is of interest to note that *S. mukorossi and R. emodi* appear to be both anti-inflammatory and anti-ulcerogenic. However, future studies concerning these properties must be carried out to justify their anti-ulcerogenic properties.

In conclusion, the results presented here indicate that the extracts of *S. mukorossi and R. emodi*, which were screened for their anti-bacterial activity against *H pylori,* are active both *in vitro* and *in vivo.* In addition, the *in vivo* studies also proved to be highly efficient in terms of dosage, tolerability and curing active *H pylori* infection. Future studies will assess the mechanism by which these extracts effect the survival of *H pylori.*

## ACKNOWLEDGMENTS

We are very grateful to Dr. CM Habibullah for his patronage, encouragement, guidance and financial support during the course of the study.

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*BASIC RESEARCH*

# Elenoside increases intestinal motility

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## Abstract

**AIM:** To study the effects of elenoside, an arylnaphthalene lignan from *Justicia hyssopifolia*, on gastrointestinal motility *in vivo* and *in vitro* in rats.

**METHODS:** Routine *in vivo* experimental assessments were catharsis index, water percentage of boluses, intestinal transit, and codeine antagonism. The groups included were vehicle control (propylene glycol-ethanol-plant oil-tween 80), elenoside (i.p. 25 and 50 mg/kg), cisapride (i.p. 10 mg/kg), and codeine phosphate (intragastric route, 50 mg/kg). *In vitro* approaches used isolated rat intestinal tissues (duodenum, jejunum, and ileum). The effects of elenoside at concentrations of 3.2 x $10^{-4}$, 6.4 x $10^{-4}$ and 1.2 x $10^{-3}$ mol/L, and cisapride at $10^{-6}$ mol/L were investigated.

**RESULTS:** Elenoside *in vivo* produced an increase in the catharsis index and water percentage of boluses and in the percentage of distance traveled by a suspension of activated charcoal. Codeine phosphate antagonized the effect of 25 mg/kg of elenoside. *In vitro*, elenoside in duodenum, jejunum and ileum produced an initial decrease in the contraction force followed by an increase. Elenoside resulted in decreased intestinal frequency in duodenum, jejunum, and ileum. The *in vitro* and *in vivo* effects of elenoside were similar to those produced by cisapride.

**CONCLUSION:** Elenoside is a lignan with an action similar to that of purgative and prokinetics drugs. Elenoside, could be an alternative to cisapride in treatment of gastrointestinal diseases as well as a preventive therapy for the undesirable gastrointestinal effects produced by opioids used for mild to moderate pain.



**Key words:** Elenoside; Gastrointestinal motility; Small intestine preparation

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## INTRODUCTION

A great number of arylnaphthalene lignans have been isolated from different species of *Justicia*, many of them exhibiting diverse biological activities including antitumoral[1-4], antiviral[5-7], insecticidal[8], cardiotonic[9,10], antiulcerogenic[11] and anti-inflammatory properties[12,13], and an ability to inhibit lipid peroxidation[14]. Interesting activities also include platelet activating factor antagonism and central nervous system action[15]. Recently it has been observed that magnolol and honokinol, two neolignans, obtained from *Magnolia officinalis* Rehd. et Wils, inhibited the contractility of isolated gastric fundus strips from rats treated with Ach or 5-HT and of isolated ileum from guinea pigs treated with Ach or $CaCl_2$; in each case, the neolignans behaved as non-competitive muscarinic antagonists[16]. However, clinical or experimental studies on the effects of the gastrointestinal activity of lignans obtained from *Justicia* species have not, to our knowledge, been performed.

In a previous paper[17] we reported the isolation from *Justicia hyssopifolia L.* of an arylnaphthalene lignan and its aglycone, now called elenoside and elenin respectively, the former being a β-D-glucoside. Elenoside is a cytotoxic arylnapthalene lignan (NSC 644013-W/1) that displayed cytotoxic activity in the human tumor cell line panel of the US National Cancer Institute[18]. Preliminary screening results suggest that elenoside exerts a sedative, hypnotic barbiturate-type effect[18,19]. In addition, we have found that elenoside has digitalis-like activity similar to that of mammalian lignans[20]. Recently we have also observed that elenoside has central sedative effects and a possible application in anxiety conditions[21]. The current study was designed to investigate the effects of elenoside on gastrointestinal motility.

## MATERIALS AND METHODS

### Plant material and chemicals

*Justicia hyssopifolia L.* belongs to the family *Acanthaceae* and is an endemic species in the Canary Islands. Leaves of *J. hyssopifolia* were collected in April 2002 at Punta Cangrejo,

Adeje, Tenerife. A voucher specimen was deposited at the Herbarium of the Department of Botany, Faculty of Biology, University of La Laguna (TFC-28938). The elenoside was extracted and identified in *Centro de Productos Naturales y Agrobiologia, CSIC,* of Tenerife. It was suspended in a mixture of propylene glycol-ethanol-plant oil-Tween80 (40:10:50:2) and administered intraperitoneally in a total volume of 0.5 mL. The control group received vehicle only. An activated charcoal suspension indicator was prepared with 10 g activated charcoal (Panreac, Barcelona, Spain) and 2.5 g Arabic gum dissolved in 100 mL of distilled water. Cisapride was also used (Janssen-Cilag, S.A. Madrid, Spain).

### Animals and treatment

Male Sprague Dawley rats weighing 200-250 g were used. The rats were housed under normal laboratory conditions at 22℃ on a standard light-dark schedule (12:12; lighted from 8 am-8 pm) and had free access to standard laboratory chow and water. Animal care complied with the Guide for the Care and Use of Laboratory Animals in accordance with the Guiding Principles in the Use of Animals in Pharmacology. The study protocol was approved by the Local Ethical Committee for animal experimentation at the University of La Laguna.

The standard dosage treatment protocol for each set of experiments described is as follows: rats assigned to randomized groups of 10 each receiving intraperitoneally (i.p.) a solution of propylene glycol-ethanol-plant oil-Tween 80 (40:10:50:2) (vehicle control group), 25 or 50 mg/kg of elenoside in vehicle, or 10 mg/kg cisapride.

### Catharsis index

The established index of catharsis is the number of humid boluses produced in 24 h. Masri *et al* found that the majority of humid boluses are produced in the first hours following administration of the substance[22]. Thus, we used their method to study the effect of elenoside. Rats were fasted for 24 h prior to the experiment. Following this period, the rats received the standard dosage treatment protocol ($n$ = 10 per dosage group). Rats were then individually placed in metabolism cages, and the boluses were collected for a 6 h period above blotting paper to facilitate counting.

### Water percentage of boluses

Using the standard dosage protocol described above, we tested the water percentage of the boluses. Rats ($n$ = 10 per dosage group) were individually placed in metabolism cages, and the boluses were collected in a methacrylate container for a period of 6 h and deposited in a watch glass. Afterward, the samples were weighed and placed in a heater at 100℃ for 3 h, followed by another weighing to establish the percentage of water in each.

### Intestinal transit

This experimental method allows the evaluation of drug action on intestinal transit speed, by measuring the distance travelled by a suspension of activated charcoal when it has been administered intragastrically (po). Rats ($n$ = 10 per dosage group) were fasted for 24 h prior to the experiment. At 15 min following treatment with the standard dosage protocol, the rats received 2 mL of a suspension of activated charcoal po. After 20 min, the rats were anesthetized and sacrificed; their intestines were removed from the pylorus through the ileocecal junction. The migration of activated charcoal from the pylorus to the most distal point of migration was expressed as distance (cm) migration using the stain. The percentage of distance travelled by the activated charcoal suspension established the intestinal transit. This percentage was expressed as % = 100 × l/L, in which *"l"* is the migration distance of the activated charcoal and *"L"* is the distance between the pylorus and the ileocecal junction.

### Intestinal transit and codeine antagonism

Using the standard treatment protocol, we studied the effect of elenoside in the presence of codeine phosphate, an opioid with inhibitory activity on intestinal motility. Following administration of the standard dosage treatment, the rats immediately received 50 mg/kg of codeine phosphate po. The method used for the measurement of intestinal transit was the same as that described in the preceding section.

### Isolated rat intestine

Rats were anesthetized and sacrificed. The abdomen was opened and a length of duodenum, jejunum and ileum was removed and placed in Tyrode´s solution. Pieces of smooth muscle (1-2 cm) were dissected free from surrounding tissues and mounted in an organ bath with Tyrode´s solution at 37℃, pH 7.4, through which a mixture of 50 mL/L $CO_2$ and 950 mL/L $O_2$ bubbled continuously[23]. The isotonic contractions of the preparation were recorded on a Grass Model 7D Polygraph through a Statham force displacement transducer. The load applied to the lever was 1 g. After stabilization, the effect of elenoside was studied for 5 min at the following concentrations: $3.2 \times 10^{-4}$, $6.4 \times 10^{-4}$ and $1.2 \times 10^{-3}$ mol/L, and cisapride at $10^{-6}$ mol/L. Ten samples each of duodenum, jejunum, and ileum were used for each concentration.

### Statistical analysis

Statistical analysis was performed using the Prism program with two-way analysis of variance (ANOVA) (between group-factor: dose repeated measures factor: time), followed by Tukey´s Multiple Comparison *post-hoc* test. For nonparametric analysis, the Kruskal-Wallis test, was followed by the Mann-Whitney test for catharsis index, water of boluses, intestinal transit, and intestinal transit and codeine antagonism. A probability of 5% or less was considered to indicate a significant difference.

## RESULTS

### Catharsis index

Table 1 shows the results of cathartic activity for the vehicle control group and groups receiving elenoside at doses of 25 or 50 mg/kg or cisapride at dose of 10 mg/kg. Elenoside produced an increase in the catharsis index compared to the control group. The results of 50 mg/kg group and controls ($P < 0.01$) and of the 50 mg/kg and 25

**Table 1 Effect of vehicle control, elenoside (E) and cisapride (C) on catharsis index in rats ($n$ = 10) per treatment group**

| | Rat weight | Catharsis index |
|---|---|---|
| Control | 239 ± 4 | 0.1 |
| E-25 mg/kg | 240 ± 3 | 1.5 |
| E-50 mg/kg | 238 ± 3 | 2.16[a] |
| C-10 mg/kg | 236 ± 2 | 2.04[a] |

Values are mean ± SE for 10 rats. [a]$P < 0.05$ *vs* control.

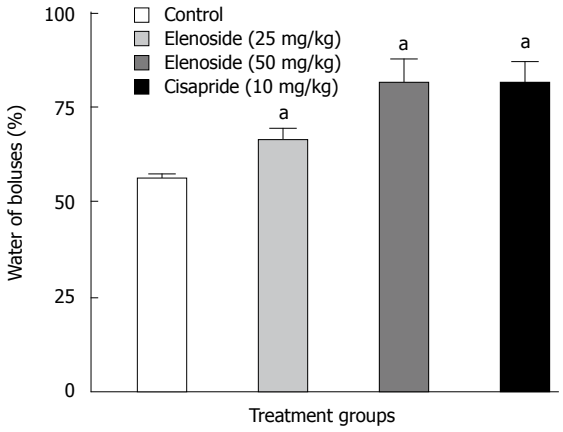


**Figure 1** Percentage increase in water of boluses in vehicle control, elenoside, and cisapride. Values are mean ± SE for 10 rats/group. [a]$P < 0.05$ *vs* control.

mg/kg elenoside treatments, respectively, were significantly different from one another. Cisapride produced an increase in the catharsis index compared to the control group ($P < 0.05$), but it did not differ significantly from 50 mg/kg of elenoside in its effects.

### Water percentage of boluses

Figure 1 shows the results of the water percentage of boluses analysis for control group, elenoside at doses of 25 and 50 mg/kg, and cisapride at 10 mg/kg. Both concentrations of elenoside produced an increase in water percentage *vs* vehicle control (*vs* 25 mg/kg elenoside, $P < 0.01$; *vs* 50 mg/kg elenoside, $P < 0.01$). Cisapride caused a significant increase in the water percentage versus the control group ($P < 0.01$), an effect similar to that produced by 50 mg/kg elenoside.

### Intestinal transit

Figure 2 shows the results of the analysis of the effects of elenoside and cisapride on small intestinal transit (SIT) speed. Both concentrations of elenoside produced an increase in the percentage of distance the suspension of activated charcoal travelled *vs* the vehicle control (*vs* 25 mg/kg elenoside, $P < 0.01$; *vs* 50 mg/kg elenoside, $P < 0.01$). Cisapride produced a significant increase in SIT (control *vs* cisapride 10 mg/kg, $P < 0.05$). There was no difference between cisapride and the 50 mg/kg of elenoside.

### Intestinal transit and codeine antagonism

Figure 3 shows the results of SIT analysis for elenoside with codeine phosphate. Codeine phosphate antagonized

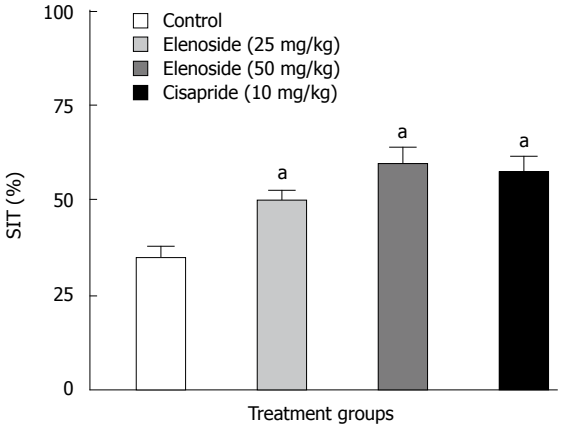


**Figure 2** Small intestinal transit (SIT) in vehicle control and elenoside-treated and cisapride-treated animals. Values are mean ± SE for 10 rats/group. [a]$P < 0.05$ *vs* control.

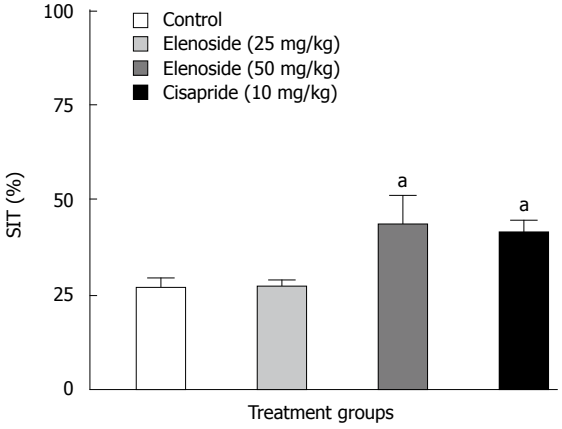


**Figure 3** Codeine phosphate induced delay in small intestinal transit (SIT) in vehicle control, elenoside, and cisapride groups. Values are mean ± SE for 10 rats/group. [a]$P < 0.05$ *vs* control.

the effect of 25 mg/kg of elenoside. Moreover, codeine phosphate at 50 mg/kg produced an antagonistic effect on 50 mg/kg of elenoside and 10 mg/kg of cisapride, although this effect was not complete (control *vs* 50 mg/kg elenoside, $P < 0.05$; control *vs* 10 mg/kg cisapride, $P < 0.05$)

### Isolated rat intestine

Figure 4 A-C show the results of the concentration force analysis; effects of elenoside and cisapride on duodenum, jejunum and ileum are depicted in Figures 4A, 4B and 4C, respectively. There was a clear dose-effect relationship over the three concentrations of elenoside.

At one minute, elenoside produced a decrease in the contraction force, which continued with increasing concentration from $3.2 \times 10^{-4}$ mol/L ($P < 0.01$) to $6.4 \times 10^{-4}$ mol/L ($P < 0.01$) to $1.2 \times 10^{-3}$ mol/L ($P < 0.01$) during a five-minute assessment. Cisapride produced an effect similar to that of the $6.4 \times 10^{-4}$ mol/L of elenoside. Elenoside at a concentration of $1.2 \times 10^{-3}$ mol/L produced a significant increase in contraction force *vs* $3.2 \times 10^{-4}$ mol/L at 2 ($P < 0.01$), 4 ($P < 0.01$) and 5 ($P < 0.01$) min respectively (Figure 4A).

Elenoside applied to jejunum produced a decrease in the contraction force at one minute, followed by an

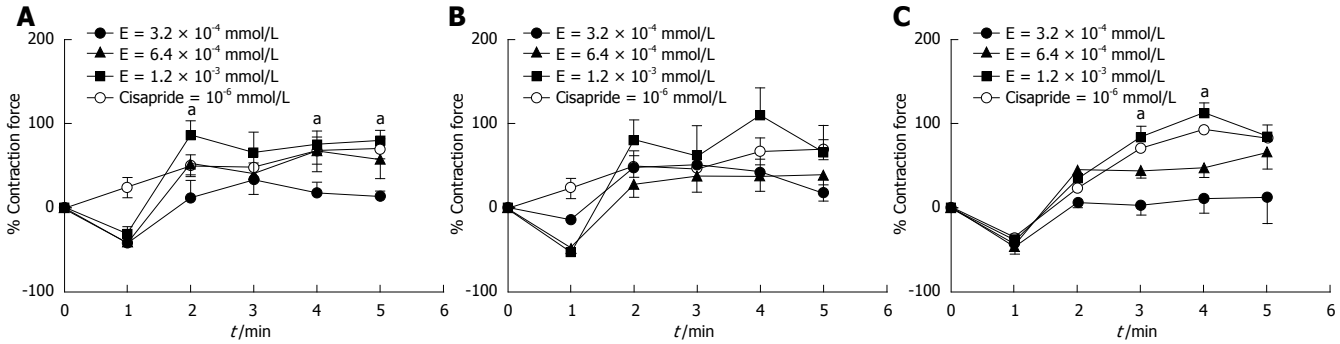


**Figure 4** **A**: Time-course of the effects of elenoside and cisapride on contraction force in rat duodenum vs baseline. Values are mean ± SE for 10 duodenum samples during 6 min; $^aP < 0.05$. **B**: Time-course of the effects of elenoside and cisapride on contraction force in rat jejunum vs baseline. Values are mean ± SE for 10 jejunum samples during 6 min; $^aP < 0.05$. **C**: Time-course of the effects of elenoside and cisapride on contraction force in rat ileum vs baseline. Values are mean ± SE for 10 ileum samples during 6 min; $^aP < 0.05$.

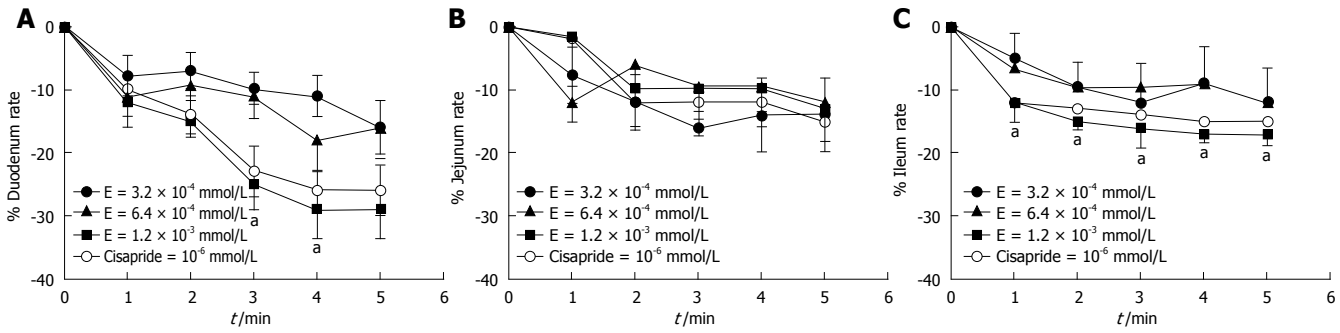


**Figure 5** **A**: Time-course of the effects of elenoside and cisapride on intestinal frequency in the rat duodenum vs baseline. Values are mean ± SE for 10 duodenum samples during 6 min; $^aP < 0.05$. **B**: Time-course of the effects of elenoside and cisapride on intestinal frequency in the rat jejunum vs baseline. Values are mean ± SE for 10 jejunum samples during 6 min; $^aP < 0.05$. **C**: Time-course of the effects of elenoside and cisapride on intestinal frequency in the rat ileum vs baseline. Values are mean ± SE for 10 ileum samples during 6 min; $^aP < 0.05$.

increase at concentrations of $3.2 \times 10^{-4}$ mol/L ($P < 0.01$), $6.4 \times 10^{-4}$ mol/L ($P < 0.05$), and $1.2 \times 10^{-3}$ mol/L ($P < 0.01$) at 5 min. Cisapride produced an effect similar to that obtained with elenoside at a concentration of $3.2 \times 10^{-4}$ mmol/L (Figure 4B).

When elenoside was applied to ileum, a decrease in the contraction force was produced a minute later. In the next minute, the contraction force increased and continued to do so up to five minutes with elenoside concentrations of $6.4 \times 10^{-4}$ mol/L ($P < 0.01$) and $1.2 \times 10^{-3}$ mol/L ($P < 0.01$). There was no effect on contraction force with the mid-range concentration of elenoside. Cisapride induced an effect similar to that of elenoside at a concentration of $1.2 \times 10^{-3}$ mol/L. Elenoside at a concentration of $3.2 \times 10^{-4}$ mol/L produced a significant increase in the contraction force *vs* $1.2 \times 10^{-3}$ mol/L elenoside at 3 ($P < 0.05$) and 4 ($P < 0.05$) min, respectively (Figure 4C).

Figure 5A-5C depicts results from the time-course analysis of the effects of elenoside on intestinal frequency of duodenum, jejunum, and ileum respectively. Elenoside produced a decrease in the intestinal frequency of duodenum at concentrations of $6.4 \times 10^{-4}$ mo/L ($P < 0.05$) and $1.2 \times 10^{-3}$ mo/L ($P < 0.01$) during the time-course. Cisapride produced an effect similar to that of elenoside at a concentration of $1.2 \times 10^{-3}$ mo/L. Elenoside at a concentration of $1.2 \times 10^{-3}$ mo/L produced a significant decrease in the intestinal frequency *vs* $3.2 \times 10^{-4}$ mo/L at 3 ($P < 0.01$) and 4 ($P < 0.01$) min, respectively (Figure 5A).

Elenoside decreased intestinal frequency in jejunum at all concentrations used during the time-course. No statistical differences among the concentrations of elenoside were observed (Figure 5B).

Elenoside produced a decrease in intestinal frequency in ileum at all concentrations, but only the concentration of $1.2 \times 10^{-3}$ mo/L ($P < 0.01$) produced a statistically significant decrease (Figure 5C).

## DISCUSSION

In this study, elenoside, has been shown to act as a stimulant of gastrointestinal motility. It produced an increase in the catharsis index and in the number of humid boluses at a dose of 50 mg/kg. The water volume of the boluses was also enhanced after treatment with elenoside at the two doses used. These effects of elenoside on the gastrointestinal tract suggest its actions similar to that of purgative drugs.

Another effect of elenoside was the increase in the distance traveled by the charcoal suspension at doses of 25 and 50 mg/kg. The distance traveled by the charcoal suspension has been used in the evaluation of drugs to determine their effects on gastrointestinal motility[24-26]. Elenoside elicited gastrointestinal activity similar to that effected by cisapride at a dose of 10 mg/kg. This effect exerted by elenoside suggested an action similar to that of cisapride, a commonly used prokinetic drug

that acts *via* a mechanism that facilitates cholinergic neurotransmission[27], and of other prokinetic drugs, such as metoclopramide, domperidone, erythromycin, and mosapride[28,29], ghrelin and GHRP-6[30], betanecol[31], and magnolol and honokiol[16]. Other lignans can exert similar effects: Podophyllotoxin and picropodophyllotoxin, lignans isolated from *Podophyllum* species, and produce diarrhea when administered to animals[32]. Herbal remedies with prokinetic activity similar to elenoside are peppermint oil[33], *Cocculus hirsutus*[34], *Zingiber officinale*[35], *Indigofera dendroides*[25], Shundao granules[36], and Banxia-houpo-tang HKT[37].

Codeine phosphate totally antagonized this prokinetic action at the lowest dose of elenoside and partially antagonized it at the higher elenoside dose. In addition, it antagonized the prokinetic action of cisapride. Codeine phosphate is an opium alkaloid with an activity similar to but weaker than that of morphine. It is given mainly orally in the treatment of mild to moderate pain[38]. However, although opioids are given to relieve pain, they exert undesirable gastrointestinal side effects such as nausea, vomiting, and a decreased gastrointestinal transit[39].

Elenoside could be an alternative together with cisapride in the treatment of the undesirable gastrointestinal effects produced by opioid drugs. Increased peristaltic and cathartic activity are parameters of irritant cathartics, according to Fling classification[40].

In isolated rat duodenum, jejunum, and ileum, elenoside produced a decrease in the contraction force followed by an increase. Elenoside applied to duodenum and ileum decreased intestinal frequency, but no significant changes in jejunum were found. Other lignans have been shown to affect contraction force and intestinal frequency[41-43]. Lignans obtained from *Podophylum* species caused a decrease in the initial rate and amplitude of contractions of isolated intestinal preparations followed by an increase in the force of contraction[32]. 2, 3-dibenzil-butirolactone, a lignan obtained from *Carthamus tinctorus* L. (Compositae), was responsible for the cathartic activity of this plant[44]. *Curcuma longa* relaxed spontaneous contractions in isolated rabbit jejunum[45]. On the other hand, some lignans used in the treatment of diarrhea, produced an inhibition in the normal intestine propellant movement[46]. Magnolol and honokiol obtained from *M. officinalis* induced a decrease in contractility of guinea pig ileum[16].

Traditional herbal remedies are used because they can improve the symptoms of gastrointestinal diseases, such as dyspepsia, nausea, vomiting, and abdominal distension. Elenoside obtained from *J. hyssopifolia* could be used to treat these symptoms of gastrointestinal diseases. It produced an increase in the catharsis index or number of humid boluses; an enhancement of the water volume of boluses; and an increase in the distance traveled by the charcoal suspension. In addition, codeine phosphate totally antagonized its prokinetic action; in isolated rat duodenum, jejunum, and ileum, elenoside elicited a decrease in contraction force followed by an increase; and elenoside caused a decrease in intestinal frequency in duodenum and ileum, but not in jejunum.

In conclusion, elenoside in the gastrointestinal tract exhibits activities similar to that of purgative and prokinetics drugs. Elenoside could be an alternative to cisapride in the treatment of gastrointestinal diseases as well as a preventive of the undesirable gastrointestinal effects produced by opioids used for mild to moderate pain.

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*BASIC RESEARCH*

# Effect of Kaiyu Qingwei Jianji on the morphometry and residual strain distribution of small intestine in experimental diabetic rats

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## Abstract

**AIM:** To investigate the effect of a Chinese medicine, Kaiyu Qingwei Jianji (KYQWJJ) used for diabetic treatment, on the morphometry and residual strain distribution of the small intestine in streptozotocin (STZ) -induced diabetic rats. Correlation analysis was also performed between the opening angle and residual strain with the blood glucose level.

**METHODS:** Forty-two male Wistar rats weighing 220-240 g were included in this study. Thirty-two STZ-induced diabetic rats were subdivided into four groups ($n$ = 8 in each group), i.e. diabetic control group (DM); high dose of KYQWJJ (T1, 36g/kg per day); low dose of KYQWJJ (T2, 17 g/kg per day) and Gliclazide (T3, 50 mg/kg per day). Another ten rats were used as non-diabetic control (CON). The medicines were poured directly into stomach lumen by gastric lavage twice daily. The rats of CON and DM groups were only poured the physiological saline. Blood glucose and plasma insulin levels were measured. Experimental period was 35 d. At the end of experiment, three 5-cm long segments were harvested from the duodenum, jejunum and ileum. Three rings of 1-2 mm in length for no-load and zero-stress state tests were cut from the middle of different segments. The morphometric data, such as the circumferential length, the wall thickness and the opening angle were measured from the digitized images of intestinal segments in the no-load state and zero-stress state. The residual strain was computed from the morphometry data. Furthermore, the linear regression analysis was performed between blood glucose level with morphometric and biomechanical data in the different intestinal segments.

**RESULTS:** The blood glucose level of DM group was consistent 4-fold to 5-fold higher than those in CON group during the experiment (16.89 ± 1.11 *vs* 3.44 ± 0.15 mmol/L, $P < 0.001$). The blood glucose level in the T1 (16.89 ± 1.11 *vs* 11.08 ± 2.67 mmol/L, $P < 0.01$) and T3 groups (16.89 ± 1.11 *vs* 13.54 ± 1.73 mmol/L, $P < 0.05$), but not in T2 group ($P > 0.05$) was significantly lower than those in DM group. The plasma insulin levels of DM, T1, T2 and T3 groups were significantly lower than those in CON group (10.98 ± 1.02, 12.52 ± 1.42,13.54 ± 1.56,10.96 ± 0.96 vs 17.84 ± 2.34 pmol/L respectively, $P < 0.05$), but no significantly difference among the groups with exception of CON group. The wet weight/cm and total wall thickness of duodenum, jejunum and ileum in DM group were significantly higher than those in CON group (wet weight (g/cm): duodenum 0.209 ± 0.012 *vs* 0.166 ± 0.010, jejunum 0.149 ± 0.008 *vs* 0.121 ± 0.004, ileum 0.134 ± 0.013 *vs* 0.112 ± 0.007; Wall thickness (mm): duodenum 0.849 ± 0.027 *vs* 0.710 ± 0.026, jejunum 0.7259 ± 0.034 *vs* 0.627 ± 0.025, ileum 0.532 ± 0.023 *vs* 0.470 ± 0.010, all $P < 0.05$), T1 and T3 treatment could partly restore change of wall thickness, but T2 could not. The opening angle and absolute value of inner and outer residual stain were significantly smaller in duodenal segment (188 ± 11 degrees, -0.31 ± 0.02 and 0.35 ± 0.03 *vs* 259 ± 15 degrees, -0.40 ± 0.02 and 0.43 ± 0.05) and larger in jejunal (215 ± 20 degrees, -0.30 ± 0.03 and 0.36 ± 0.06 *vs* 172 ± 19 degrees, -0.25 ± 0.02 and 0.27 ± 0.02) and ileal segments (183 ± 20 degrees, -0.28 ± 0.01 and 0.34 ± 0.05 *vs* 153 ± 14 degrees, -0.23 ± 0.03 and 0.29 ± 0.04) in DM group than in CON group ($P < 0.01$). T1 and T3 treatment could partly restore this biomechanical alteration, but strong effect was found in T1 treatment (duodenum 243 ± 14 degrees, -0.36 ± 0.02 and 0.42 ± 0.06, jejunum 180 ± 15 degrees, -0.26 ± 0.03 and 0.30 ± 0.06 and ileum 163 ± 17 degrees, -0.23 ± 0.03 and 0.30 ± 0.05, compared with DM, $P < 0.05$). The linear association was found between the glucose level with most morphometric and biomechanical data.

**CONCLUSION:** KYQWJJ (high dose) treatment could partly restore the changes of blood glucose level and the remodeling of morphometry and residual strain of small intestine in diabetic rats. The linear regression analysis

demonstrated that the effect of KYQWJJ on intestinal opening angle and residual strain is partially through its effect on the blood glucose level.



**Key words:** Diabetes; Intestine; Kaiyu Qingwei Jianji; Residual strain; Rat

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## INTRODUCTION

Dysfunction of upper gastrointestinal tract (GI) is common among diabetic patients[1]. As many as 75% of patients visiting diabetes clinics report significant GI Symptoms[2,3]. Common complaints include dysphagia, early satiety, reflux, abdominal pain, nausea, vomiting, and diarrhea[2,3]. As with other complications of diabetes, the duration of the disorder and poor glycemic control seem to be associated with more severe GI problems[2,3]. Histologically, many experiments have demonstrated prominent proliferation of GI wall layers, especially mucosa in the small intestine and esophagus of diabetes[4-9]. Many studies have shown that DM causes morphological changes and biomechanical remodeling in the esophagus[10,11], stomach[12] and small intestine[13,14]. Recently, Frøkjær et al in a human study found an increase in esophageal wall thickness and altered deformation to the distension with reduced longitudinal shortening and the radial stretch in insulin dependent diabetes mellitus (IDDM) patients (unpublished data). The biomechanical remodeling of the GI tract likely plays an important role in the GI disorder of diabetic patients[1]. Therefore, it is important to study the biomechanical properties of the small intestine in diabetic animals. Remodeling of the structural and biomechanical properties can be measured as changes in the residual strain and stress-strain distributions. Residual strain is defined as the strain in the no-load state (where external forces are zero) in reference to the zero-stress state.

To the best of our knowledge, only few studies related to the residual strain in diabetic small intestine have been studied[13,15]. Previously we have demonstrated that gliclazide treatment could partially restore the changes of biomechanical parameters of small intestine in the diabetic rats[15]. Regarding the Kaiyu Qingwei Jianji (KYQWJJ), several previous studies have demonstrated that it could decrease blood glucose level, decrease blood lipid level, increase sensitivity of insulin and decrease resistance of insulin[16-18]. However, no study has been performed about the effect of KYQWJJ on the morphometric and biomechanical properties of small intestine in diabetes. Therefore, the goal of the present study was to investigate the effect of KYQWJJ on the remodeling of the zero-stress state of the small intestine. The gliclazide is serving as positive control in the present study.

## MATERIALS AND METHODS

### Animal model and groups

Fifty male Wistar rats weighing 220-240 g were included in this study. Forty rats were made diabetic by a single intraperitoneal injection of 60 mg/kg streptozotocin (STZ, Sigma Company). This dose resulted in a fasting serum glucose level greater than 11.1 mmoL/L in 80% of rats after 4 d of injection; the remaining 20% were finally not used in this study. Another 10 rats of similar age and body weight from the same vendor were used as non-diabetic control (CON). Thirty-two diabetic rats were subdivided into four groups ($n$ = 8 in each group), i.e. diabetes mellitus control group (DM); high dose of KYQWJJ (T1); low dose of KYQWJJ (T2) and Gliclazide (T3).

### Drugs and administration methods

KYQWJJ was composed of Radix Bupleuri, Radix et Rhizoma Rhei, Rhizoma Pinelliae and Rhizoma Coptidis, and provided by China-Japan Friendship Hospital. Gliclazide was purchased from Hua Ju pharmaceutical factory (Tanjun, China). The medicines were injected directly into stomach lumen by gastric lavage twice daily and the dosage was 36 g/kg for T1, 17 g/kg for T2 and 50 mg/kg for T3 respectively. The rats of CON and DM groups were only poured the physiological saline.

### Experimental procedures

Weight and blood glucose levels were measured 5 d, three weeks and five weeks after initiating the experiment. The plasma insulin level was measured when the experiment finished. Experimental period was 35 d.

After 35 d, the animals were anesthetized with sodium pentobarbital (50 mg/kg ip). Following laparatomy, the calcium antagonist, papaverine (60 mg/kg) was injected into the lower thoracic aorta through a cannula (22 G/25 mm) in order to abolish contractile activity in the GI tract. After obtaining smooth muscle relaxation, the whole small intestine was harvested. The most proximal segment was close to the pylorus whereas the most distal segment was close to the ileo-cacal valve. The length of duodenum and whole jejunum and ileum in vitro was measured. Within a short time, the residual contents in the lumen were gently cleared using saline and the wet weight of duodenum, jejunum and ileum was measured. Three 6 cm-long segments from duodenum, jejunum and ileum were harvested. The duodenum was taken from the descending part, 1 cm down the pylorus; the jejunum from 5 cm distal to the ligament of Treitz, and the ileum from 5 cm proximal to the ileo-cecal valve. Then the segments were placed immediately into cold aerated Krebs solution containing 6% dextran. Three rings, 1-2 mm in length, were cut from the middle part of each segment for no-load and zero-stress state tests. The rings were photographed in the no-load state using a video camera (SONY CCD Camera, Japan) and then cut radially on the anti-mesenteric side to obtain their zero-stress state. A 60-min-period

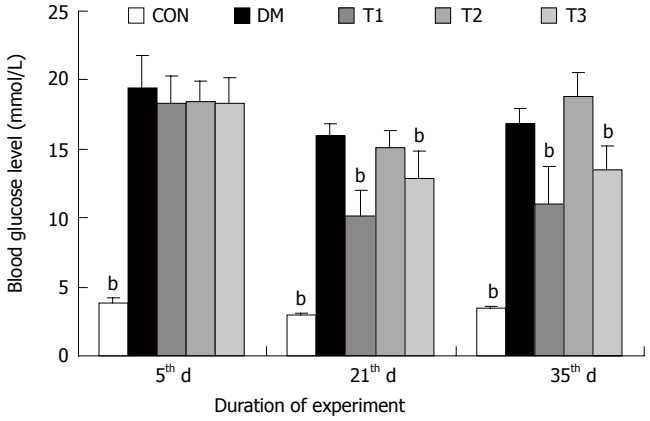


**Figure 1** The blood glucose levels in different groups, compared with DM group ($^bP < 0.01$).

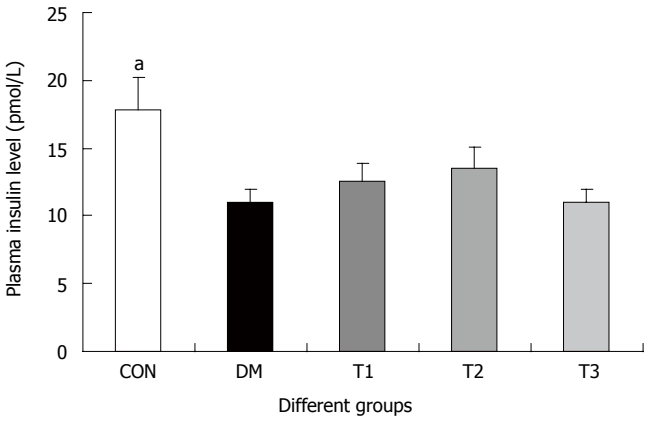


**Figure 2** The serum insulin levels in different groups, compared with DM group ($^aP < 0.05$).

was allowed for equilibration and the specimens were photographed again. The selection of this time period was based on pilot experiments.

### Data analysis

The morphometric data were obtained from the digitalized images of the photographs of the segments in the zero-stress and no-load states. Measurements were done using SigmaScan software (Jandel Scientific, Germany). The following data were measured from each specimen: the circumferential length (C), the wall thickness (h), and the opening angle at the zero-stress state. The subscripts i, o, n, and z refer to the inner (mucosal) surface, outer (serosal) surface, no-load state and zero-stress state condition. The data from triplet of rings were averaged before further analysis.

The measured data was used for computation of residual strains defined as:

Residual Green's strain at the mucosal surface:

$$e_i = \frac{(\frac{C_{i-n}}{C_{i-z}})^2 - 1}{2} \quad (1)$$

Residual Green's strain at the serosal surface:

$$e_o = \frac{(\frac{C_{o-n}}{C_{o-z}})^2 - 1}{2} \quad (2)$$

Thus, from the circumferential lengths at the no-load and zero-stress state, we can compute the circumferential residual strain, at the mucosal and serosal surfaces in the sense of Green strain. Green strain is used when large deformations are encountered as in this study. Negative strain implies that the tissue is in compression whereas positive strain implies extension.

The association between the glucose level with opening angle and residual strain in different groups with referenced to the DM group were performed using the linear regression analysis.

### Statistical analysis

The data were representative of a normal distribution and accordingly the results were expressed as mean ± SD unless otherwise stated. Analysis of variance was used to detect difference among the different groups (Sigmastat 2.0TM). Linear regression analysis was used to demonstrate possible association between the blood glucose levels and opening angle and residual strain (Sigmastat 2.0TM). The results were regarded as significant when $P < 0.05$.

## RESULTS

### The blood glucose and plasma insulin level

The changes of the blood glucose level during the development of diabetes are shown in Figure 1. The blood glucose level of DM group was consistent 4-fold to 5-fold higher than those in CON group during the experiment ($F = 19.4$, $P < 0.001$). Compared with DM group, significant lower of blood glucose level was found in the T1 and T3 groups ($F = 16.1$, $P < 0.01$ and $F = 5.8$, $P < 0.05$), but not in T2 group ($F = 0.8$, $P > 0.05$). The plasma insulin level was shown in Figure 2. The plasma insulin levels of DM, T1, T2 and T3 groups were significantly lower than those in CON group ($P < 0.05$), but no significantly difference was found among the groups with exception of CON group ($P > 0.05$).

### The intestinal weight and wall thickness (Figures 3 and 4)

The wet weight per centimeter and the wall thickness were increased during diabetes in the duodenum, jejunum and ileum ($P < 0.01$). After treatment with T1, T2 and T3, the wet weight per centimeter of different segments did not change significantly. However, T1 and T3 partly inhibited the wall thickness increase, in which the effect of T1 was stronger ($P < 0.05$).

### Biomechanical data

The opening angles and residual strains of the different intestinal segments were shown in Figures 5 and 6. In brief, the opening angles and absolute value of residual strain were smaller in duodenum and larger in jejunum and ileum in the DM group when compared to those in CON group ($P < 0.01$). T1 and T3 treatment could partly restore this biomechanical alteration, in which the effect of T1 was stronger ($P < 0.05$). No significant effect in T2 treatment was found.

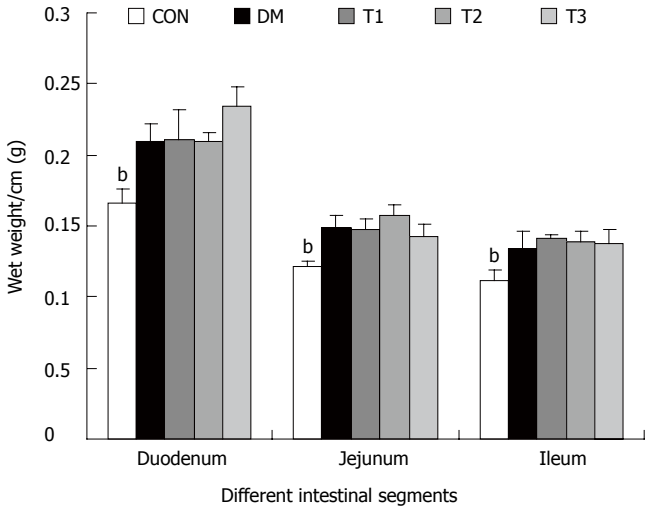


**Figure 3** The intestinal wet weight of different segments per centimeter long in different groups, compared with DM group ($^bP < 0.01$).

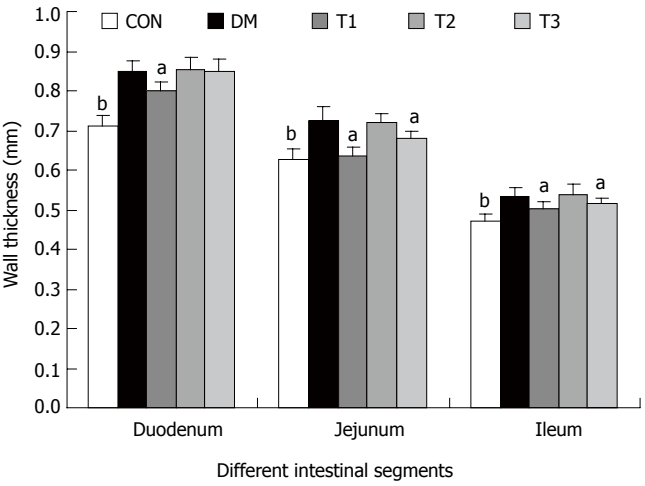


**Figure 4** The intestinal wall thickness of different segments in different groups, compared with DM group ($^aP < 0.05$; $^bP < 0.01$).

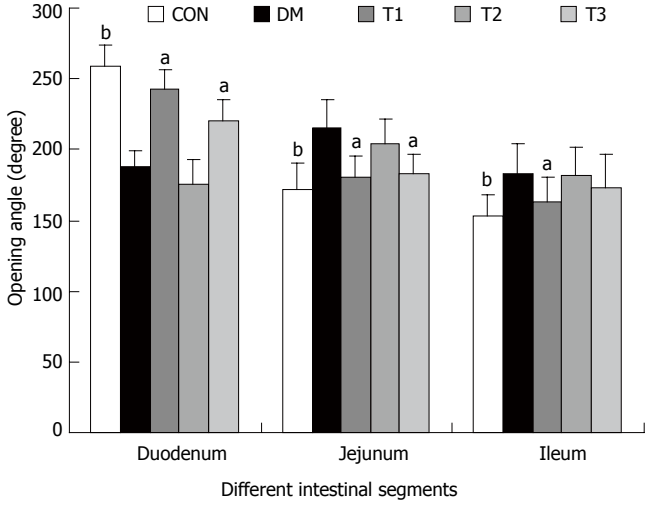


**Figure 5** The opening angles of different intestinal segments in different groups, compared with DM group ($^aP < 0.05$; $^bP < 0.01$).

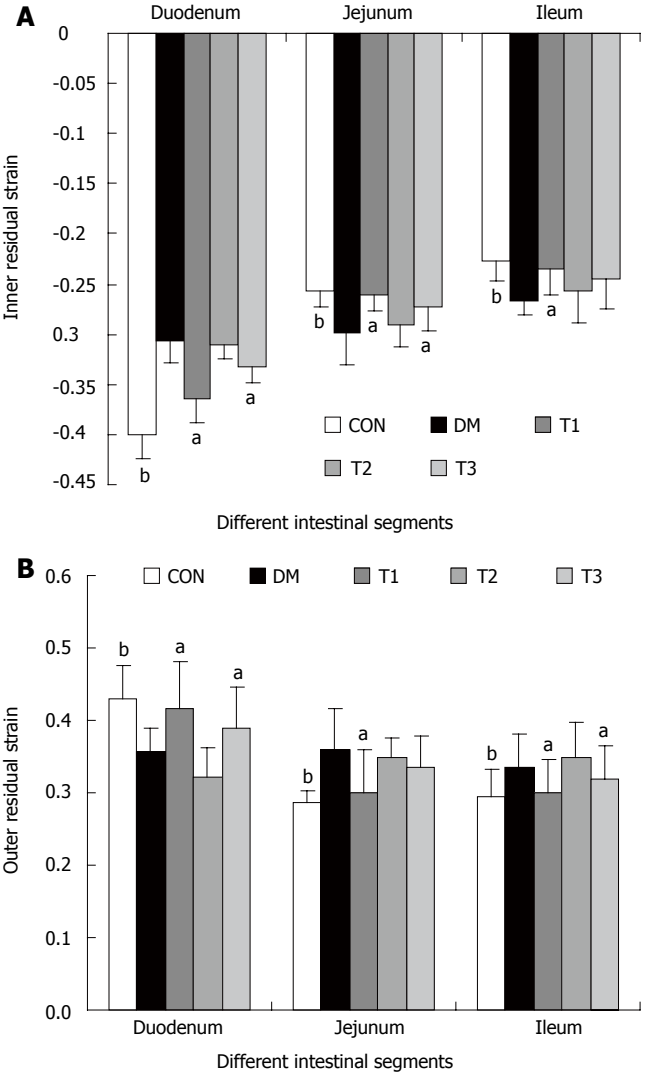


**Figure 6** The inner (**A**) and outer (**B**) residual strain distribution of different intestinal segments in different groups, compared with DM group ($^aP < 0.05$; $^bP < 0.01$).

### *Association between blood glucose level with morphometric and biomechanical data*

Linear regression analysis indicated that the linear association existed between blood glucose level with most morphometric and biomechanical data (Table 1). The examples of association between the glucose with opening angle and inner residual strain in duodenal segment were shown in the Figure 7. The high linear association existed between blood glucose level with opening angle ($r = 0.657$, $P < 0.001$, Figure 7A) and inner residual strain ($r = 0.653$, $P < 0.001$, Figure 7B).

## DISCUSSION

Studies of the gut in the streptozotocin model of diabetes have almost entirely involved use of the rat. The form of diabetes mellitus induced by parenteral streptozotocin administration in rats is insulin-dependent (type I). Without treatment with insulin, STZ-induced diabetic rats become hyperglycaemic, polyphagic, polidipsic, poliuric and undernourished. In our studies, the blood glucose level was 4-5 times higher in diabetic rats than in non-

**Table 1 Association between blood glucose level with morphometric and biomechanical data**

| | Duodenum | | | Jejunum | | | Ileum | | |
|---|---|---|---|---|---|---|---|---|---|
| | | *F* | *P* | | *F* | *P* | | *F* | *P* |
| Opening angle | y = -4.937x + 281.6<br>R = 0.656 | 28.7 | < 0.001 | y = 4.253x + 131.7<br>R = 0.591 | 20.4 | < 0.001 | y = 2.244x + 142.6<br>R = 0.33 | 4.7 | 0.038 |
| Inner residual strain | y = 0.007x - 0.422<br>R = 0.653 | 28.2 | < 0.001 | y = -0.003x - 0.236<br>R = 0.447 | 9.5 | 0.004 | y = 0.001x - 0.286<br>R = 0.13 | 0.7 | 0.418 |
| No-load wall thickness | y = 0.007x + 0.707<br>R = 0.575 | 18.3 | < 0.001 | y = 0.004x + 0.617<br>R = 0.37 | 5.9 | 0.02 | y = 0.003x + 0.485<br>R = 0.32 | 4.4 | 0.042 |

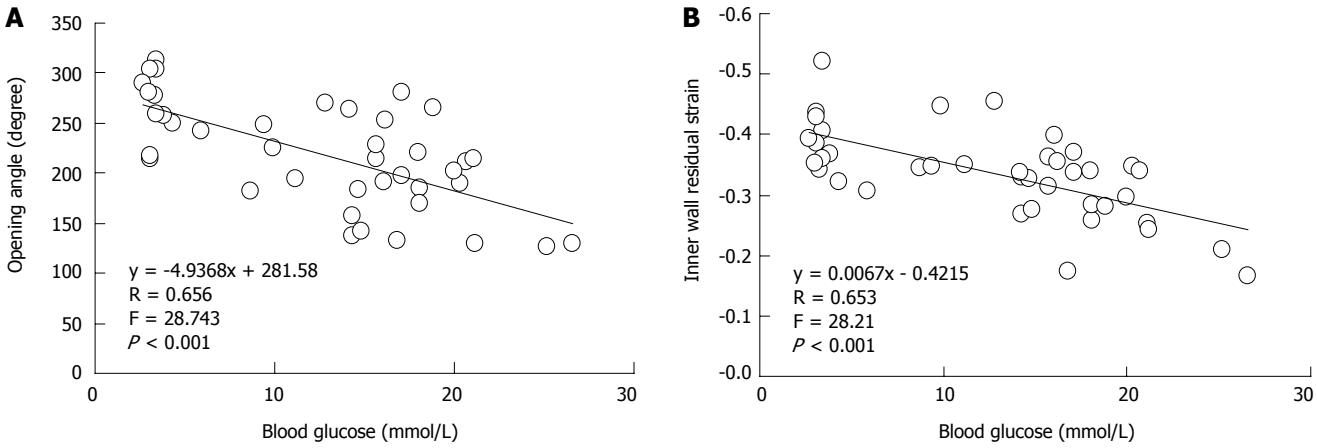


**Figure 7** The examples of association between the glucose with opening angle (**A**) and inner residual strain (**B**) in duodenal segment.

diabetic rats. The major findings in our studies were that the opening angle and residual strain were smaller in the duodenum and larger in the jejunum and ileum in diabetic rats compared to normal rats. After gliclazide and high dose KYQWJJ treatment, these biomechanical parameters trend to back to normal state. Furthermore, we confirmed previous data that the intestinal weight per-unit length prominently increased in the diabetic rats[9,12-14], gliclazide and high dose KYQWJJ treatment can partly reverse the remodeling of intestine. High dose KYQWJJ seems better than gliclazide to reverse the morphometric and biomechanical remodeling of intestine in STZ -induced diabetic rats.

The small intestine is the longest section of the digestive tract and consists of three segments, namely the duodenum, jejunum and ileum, forming a passage from the pylorus to the large intestine. The main functions of the small intestine can be roughly divided into transport, absorption and secretion. The movement of luminal contents is facilitated by changes in the geometry of the wall and lumen due to the motility. It is well known that a bolus propelled in front of a peristaltic contraction distends the gut wall[19]. Therefore, it is important to know the passive mechanical wall properties devoid of any neurohumoral regulatory mechanisms. The biomechanical properties of the small intestine depend on its structure. Because significant structural remodeling of the intestine is occurred in the diabetes[3-9,12], therefore, the biomechanical properties of the small intestine may be also changed. To our understanding of knowledge, we consider the zero-stress state and residual strain as the most relevant quantitative aspect of remodeling because it is a measure of the non-uniformity of growth or resorption in different parts of the intestinal wall. Furthermore, the structural components of the intestinal wall must be measured at the zero-stress state because in this state the morphology and sizes of the cells and extracellular matrix are not distorted by stress and strain. In our present experiment, during the development of diabetes, the opening angles and absolute value of residual strains were decreased in the duodenum and increased in the jejunum and ileum when compared to those in the controls. High dose KYQWJJ and Gliclazide treatment partly restored this biomechanical alteration, the effect of the former is stronger. In the previous study we have discussed the possible mechanism of effect of Gliclazide on the diabetic intestinal remodeling[15]. Regarding the KYQWJJ, several previous studies have demonstrated that it could decrease blood glucose level, decrease blood lipid level, increase sensitivity of insulin and decrease resistance of insulin[16-18]. We confirmed in the present study that KYQWJJ could decrease blood glucose level in the diabetic rats. The linear regression analysis demonstrated that the linear association existed between blood glucose level with most morphometric and biomechanical data. Therefore, the effect of KYQWJJ on intestinal opening angle and residual strain is partially through its effect on the blood glucose level. These seem to be the part mechanism for the effect of KYQWJJ on the diabetic intestinal remodeling. However, regarding more detail mechanisms of effect of KYQWJJ on the diabetic intestinal remodeling, further studies should be done.

The changes in the opening angle of intestine during the development of diabetes shown in this study suggest that the morphological and biomechanical remodeling of different layers are an important determinant of growth and remodeling of the zero-stress state. Fung's hypothesis of non-uniform remodeling suggested that if the inner wall grows more than the outer wall, the opening angle will increase; whereas if the outer wall grows more than the inner wall, the opening angle will decrease[20]. In this experiment, since the changes in jejunum and ileum primarily are in the mucosa layers, the inner wall grows more than the outer wall and hence the opening angle increased. Correspondingly, the outer residual strain became more tensile whereas the inner residual strain became more compressive in these two segments. But in the duodenal segment, the opening angle and absolute value of residual strain were decreased during the diabetes. We have demonstrated that the all layers of duodenum increased after experimental diabetes and the wall became stiffer[13], at this condition the biomechanical properties of layers may determine the remodeling of the zero-stress state. Because the wall becomes stiffer, the opening angle may become smaller.

The GI wall structure or deformation changes in the diabetes may alter the relative positions of the mechanosensitive afferents (zero setting of the mechanosensitive afferents)[21]. The biomechanical remo-deling in the diabetes such as alterations of residual strain and stress distribution[15] and increase the wall stiffness[13,14] will alter the tension and stress distribution of the mechanosensitive afferents[22,23]. As results, the perception and motility of the GI tract will change as well. Hence, the morphological changes and biomechanical remodeling of GI tract in the diabetes is likely to affect the function of mechanosensitive afferents in the GI wall and further affect the motor and sensory function. After KYQWJJ treatment, the structural remodeling of different segments was partly improved, accordingly the biomechanical remodeling of these segments was partly reversed. Therefore, the KYQWJJ should have the effect to improve motor and sensory dysfunction of diabetic small intestine.

In conclusion, Chinese medicine of KYQWJJ treatment could partly restore the changes of blood glucose and insulin levels and remodeling of morphometry and residual strain of small intestine in STZ-induced diabetic rats. The linear regression analysis demonstrated that the effect of KYQWJJ on intestinal opening angle and residual strain is partially through its effect on the blood glucose level. Therefore, it is possible to develop some Chinese herbs, such as KYQWJJ, to improve the intestinal dysfunction and further may be applicable in the clinics.

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*CLINICAL RESEARCH*

# Noninvasive evaluation of hepatic fibrosis in children with infant hepatitis syndrome

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## Abstract

**AIM:** To elucidate the impact of hemodynamic parameters on ultrasonography and serum fibrosis markers for the assessment of liver fibrosis in the children with infant hepatitis syndrome (IHS).

**METHODS:** Forty-one children with IHS and 46 healthy infants were examined by ultrasonography, and several hemodynamic indices such as peak systolic velocity (PSV) and resistant index (RI) of proper hepatic artery (PHA) were measured. Serum fibrosis markers including hyaluronic acid (HA), pre-collagen type-Ⅲ (PC-Ⅲ), collagen type Ⅳ (C-Ⅳ), and laminin (LN) were assayed by radioimmunoassays. In children with IHS, liver tissues were obtained either by ultrasound-guided liver biopsy ($n$ = 35) or in the course of operation ($n$ = 6). The stages of hepatic fibrosis were scored as mild ($S_1$ and $S_2$), moderate ($S_3$), or severe ($S_4$) according to liver histological diagnosis. Multiple groups comparative and Spearman correlative analyses were carried out.

**RESULTS:** Histopathologically, 39 children (95.1%) were found to have hepatic fibrosis, 12 of them stage S1 or S2, 12 stage S3, and 15 stage S4. PSV, RI of the PHA, and serum HA showed a consecutive increase from mild to severe hepatic fibrosis and a close positive correlation with hepatic fibrosis in IHS group ($r$ = 0.717, 0.745 and 0.712, respectively, $P$ = 0.001). The Doppler waveform of HV was also positively correlated with the degree of hepatic fibrosis in IHS group ($r$ = 0.783, $P$ < 0.001).

**CONCLUSION:** Combination of ultrasonic studies on the hepatic hemodynamics with the evaluation of serum HA may provide an indicator for hepatic fibrosis in patients with IHS. This may be a useful noninvasive method for the diagnosis and evaluation of the prognosis of IHS.



**Key words:** Infant hepatitis syndrome; Hepatic fibrosis; Doppler ultrasonography; Hemodynamics; Hyaluronic acid

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## INTRODUCTION

Infant hepatitis syndrome (IHS)[1] is defined as elevation of serum levels of conjugated bilirubin in the first 12 mo of life, may be due to infectious, genetic, metabolic, or undefined abnormalities, giving rise either to mechanical obstruction of bile flow or to functional impairment of hepatic excretory function and bile secretion. Main symptoms are prolonged jaundice, hepatomegaly or hepatosplenomegaly, and impairment of the liver focused. Although IHS has multiple etiologies, hepatic fibrosis is one of the main pathologic features. Histology is the gold standard for assessing the degree of hepatic fibrosis and for estimating prognosis[2-6]; however, there are several limitations in the usage of liver biopsy in general clinical practice due to the invasive nature of the examination, especially in infant age group. So there is a need for a non-invasive examination, which is clinically useful and accurately reproducible.

In adults, two-dimensional ultrasonography (2DUS)[2-5,7,8], Doppler blood stream[7-10,11-14,26], and determination of serum fibrosis markers[15-19,21-23,25,26] have been performed widely for the noninvasive diagnosis of hepatic fibrosis and therapeutic surveillance[20,24], and they were also useful in the assessment of cirrhosis in the pediatric age group[27-30]. But until recently, only a few studies[31,32] focussed on infants, especially on infants with IHS.

This prospective study investigated the association

between the hepatic fibrosis in infants with IHS and hepatic hemodynamic parameters and serum fibrosis markers, correlated the results with the histological findings, and evaluated the clinical relevance of the noninvasive methods.

## MATERIALS AND METHODS

### Patients

Forty-one infants (27 boys and 14 girls; mean age 5.2 ± 3.6 mo) who were admitted in the Department of pediatrics in our hospital from January 2000 to December 2003 were included. The patients were diagnosed as infant hepatitis syndrome (IHS) according to the diagnosis criteria of the national infant virus hepatitis prevention and cure symposium[1]. Infants presented with jaundice, hepatomegaly or hepatosplenomegaly, and impairment of the liver function. They didn't have any contraindication for liver biopsy. Those with clinically overt heart failure were excluded. Etiologies included viral infection, metabolic disorders, familial cholestasis, or idiopathic neonatal hepatitis. Etiologies were categorized on the basis of their clinic and pathologic diagnosis (Table 1). Forty-six infants (34 boys and 12 girls; mean age 4.6 ± 3.6 mo) who presented with normal liver size and function, negative HBV status, and without any history of clinical signs of liver disease served as healthy controls.

### Ultrasonography

Ultrasonography was performed on all children (healthy and those with IHS) by two physicians, who were unaware of the results of the liver biopsy and any laboratory finding at the time of the examinations. All children were held in the arms of their mothers and studied after a fasting period of 2-4 h. Some of them were examined during normal sleep, some were helped by use of a sugar nipple, if necessary, and some were given a sedative drug before the examination. GE-LOGIQ9 ultrasound scanner (GE, Milwaukee, and American) equipped with an 8-MHz electric curved scanning transducer or HP 8500 ultrasound scanner (HP, Andover, American) with a 7.5-MHz electric curved scanning transducer were used for the examination. Evaluation of liver parenchyma includes the assessment of the size, configuration, homogeneity, contour, and the vascular structure. Spleen and visibility of the gallbladder were also evaluated and compared with normal data. Color Flow Mapping(CFM) was used to determine the direction of flow of the hepatic blood vessels, and Pulse Wave Doppler (PW) was used to measure the Doppler waveforms of the PHA, portal vein (PV), and hepatic vein (HV). During the PW examination, the angle between the Doppler beam and longitudinal axis of the blood vessels was kept at 30°, and Doppler sample volume was smaller than the inner diameter of vessels. In each individual, the Doppler waveforms of the PHA, PV, and HV were measured. Doppler waveform analysis was calculated from 3-5 consecutive waveforms on the Doppler tracing in each subject. Flow in the middle hepatic vein (MHV) was evaluated at subcostal or intercostals, a distance of at least 2 cm from the inferior vena cava (IVC). This distance was important to rule out the possibility that the MHV flow pattern was influenced more by the flow pattern of the IVC than by the surrounding liver parenchyma.The waveforms of the MHV were classified into 3 types[11,14]: Type 1 (HV0), regular triphasic waveforms (with a short phase of reversed flow) which were considered to be normal flow, whereas type 2 (HV1), biphasic waveforms (without reversed flow) and type 3 (HV2), flat waveforms (with continuous blood flow) were considered to be abnormal.

**Table 1 Etiology of IHS**

| Etiology | *n* | % |
|---|---|---|
| Cytomegalovirus hepatitis (CMVH) | 18 | 43.90 |
| CMVH + Glucose-6-phosphate dehydrogenase | 3 | 7.32 |
| CMVH + congenital choledochocele | 2 | 4.88 |
| CMVH + intrahepatic biliary atresia | 1 | 2.44 |
| CMVH + hemangioma | 1 | 2.44 |
| Glycogen storage disease | 5 | 12.20 |
| Infant Hepatitis Syndrome (undefined) | 8 | 19.51 |
| Congenital deformity of biliary tract (Extrahepatic biliary atresia) | 1 | 2.44 |
| Congenital choledochocele | 1 | 2.44 |
| Drug-induced hepatitis | 1 | 2.44 |
| Total | 41 | 100.00 |

### Laboratory tests

Serum total bilirubin (T-Bil), indirect bilirubin (I-Bil), direct bilirubin (D-Bil), aspartate aminotransferase (AST), alanine aminotransferase (ALT), γ-glutamyl transpeptidase (γ-GTP), alkaline phosphatase (AKP), total protein, albumin, globulin, the ratio of albumin to globulin (A/G), and prothrombin time were measured by routine methods. The etiologic differential diagnosis was made according to the presence of serological markers for viral hepatitis type B, toxoplasma, rubella, cytomegalovirus, herpes simplex, and syphilis (TORCHS) (positive IgM or more than a fourfold rise in IgG titer), and urine cytomegalovirus (CMV) culture. The serum specimens for fibrosis indices, including HA, PC-Ⅲ, C-Ⅳ and LN, were determined by commercially available radioimmunoassays (RIA kits; Shanghai Navy Medical Institution, Shanghai, China). Samples of peripheral venous blood of the subjects were collected, immediately separated, and preserved at -40℃ until they could be assayed.

### Liver biopsy and histological analysis

Liver biopsy was performed in 41 children with IHS, 35 cases underwent US-guidance liver biopsies and 6 cases underwent operation biopsies. Intercostals or subcostal ultrasound-guided liver biopsies were performed during the scanning with an 18G biopsy-cut (CR Bard, Inc., Covington, GA, USA) needle driven by the spring-loaded automatic biopsy gun (CR Bard, Inc., MAGNER, USA). The Bard biopsy gun consists of a hand-held device that triggers rapid firing of an 18G cutting needle. When the gun is fired, an inner trocar with its 1.9 cm sample notch thrusts forward, followed by its outer cannula which shears a core of tissue with minimum crushing of the specimen. For a satisfactory sampling, an average of two specimens

was obtained in each ultrasound-guided core-needle biopsy case. All biopsy specimens were 0.8-1.9 cm in length and were fixed in 10% buffered formalin. Sections were stained with hematoxylin-eosin; Masson's trichrome stain was used specially for staining of fibrous tissue components.

The stage of hepatic fibrosis was scored according to the diagnostic criteria of viral hepatitis in Xian, China, 2000[33]. S0 corresponds to the absence of fibrosis, S1 to fibrosis within portal tract areas, S2 to fibrosis around the portal tract areas with formation of fibrosis segregation while maintain lobular structure, S3 to the formation of fibrosis segregation and disorder of lobular structure without hepatic cirrhosis, and S4 to early or late stage cirrhosis. Then these patients were divided into three groups: mild (S1-S2 grade), moderate (S3 grade) and severe (S4 grade).

### Statistical analysis

The one-way ANOVA was used to compare numerical variables between multiple groups. The chi-square test and Fisher exact test were used to compare categorical variables. Association between the ranked values was evaluated using Spearman rank correlation. $P < 0.05$ was considered statistically significant. All statistical analyses were done with SPSS for windows, version 10.0 (SPSS Inc. American).

## RESULTS

In the IHS group, there was an increase in T-Bil which reached up to 810 μmol/L. D-Bil increased in almost all except for 5 children (I-Bil was higher than D-Bil). There was an increase in serum ALT activity in all cases, the highest value was 598 U/L, mean 126.9 U/L. Serum AST activity increased in all except for 3 cases, and the highest value was 2816 U/L, mean 282.4 U/L. The AST/ALT ratio was 3.5 (normal 1.15), the ratio of albumin to globulin (A/G) decreased in 29 cases, and inversed in 5 cases. Pathology showed liver fibrosis in 39 cases (95.1%), 12 cases with S1 or S2, 12 cases with S3, and 15 cases with S4. Further findings were ballooning change ($n$ = 35, 85.4%), hepatocyte necrosis ($n$ = 30, 73.2%), cholestasis in liver cells ($n$ = 26, 63.4%), cholestasis in the hepatic lobules ($n$ = 21, 51.2%), cholestasis in portal area ($n$ = 7, 17.1%), and cytomorphosis of giant corpuscle ($n$ = 20, 48.8%).

### 2DUS findings

In the 39 cases of liver fibrosis, 15 cases with S4 stage liver fibrosis (confirmed by histological diagnosis) showed that the liver parenchyma was more echogenic and coarse than normal, among which 6 severe cases with obvious echogenic changes showed nodularity of the liver edge and ascites by two-dimensional ultrasonography (2DUS). The other 24 cases with S1-S3 stage liver fibrosis (confirmed by histological diagnosis) were all hepatomegaly but the liver parenchyma was normal, except for 5 cases of Glycogen Storage Disease (1 case in S0 stage, 2 in S2 stage and 2 in S3 stage) presented with significant hepatomegaly, increased echogenicity, and slightly increased attenuation. Additionally, we found that proper hepatic arteries (PHA) in the IHS group were enlarged and even were easy to display on the 2DUS.

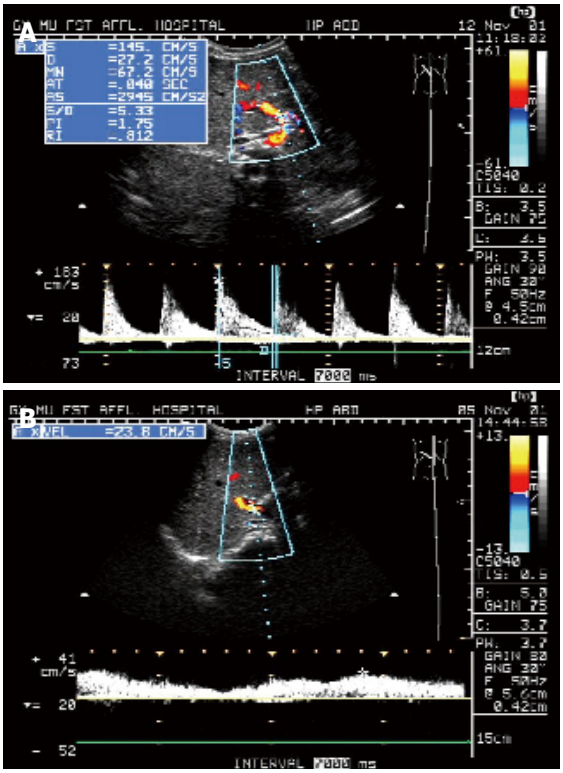


**Figure 1** **A**: Duplex Doppler Sonogram of case with IHS showed the PHA was easy to see as well as PSV and RI of the PHA increased significantly; **B**: Duplex Doppler Sonogram of the normal subject showed normal waveform of PV, and PHA cannot display clearly.

### CDUS findings

We found that PHA in the IHS group can be displayed easily on the B-Mode, as well as on the Color Flow Mappings and even better (Figure 1A). PHA can be seen in 100% of patients in IHS group but only in 58.7% of the control subjects, there was significant difference between IHS group and control group ($P < 0.001$). And then, PW parameters showed an increase in PSV, EDV, and RI (Figure 1A). PSV and RI in IHS group were significantly higher than in the control group ($P < 0.05$), as well as, with development of hepatic fibrosis from mild to severe, PSV and RI of the PHA increase gradually (Figure 2A and 2C), and EDV of the PHA decreased gradually (Figure 2B), there was a significant difference between groups ($P < 0.05$). Furthermore, PW waveform of the MHV developed from HV1 (damped biphasic waveform without reversed flow) to HV2 (flat waveform) with the severity of the hepatic fibrosis (Table 2).

### Serum fibrosis index

Serum concentration of fibrosis markers was higher in the IHS than in the control group ($P < 0.05$). Especially serum HA increased gradually from the mild to the severe group along with the severity of the hepatic fibrosis (Figure 2D), and it was significantly higher in IHS group than in the control group ($P < 0.05$). For PC-Ⅲ, C-Ⅳ and LN, there were also significant differences between infants with severe hepatic fibrosis and the control group ($P < 0.05$). When infants of the IHS group with mild or moderate

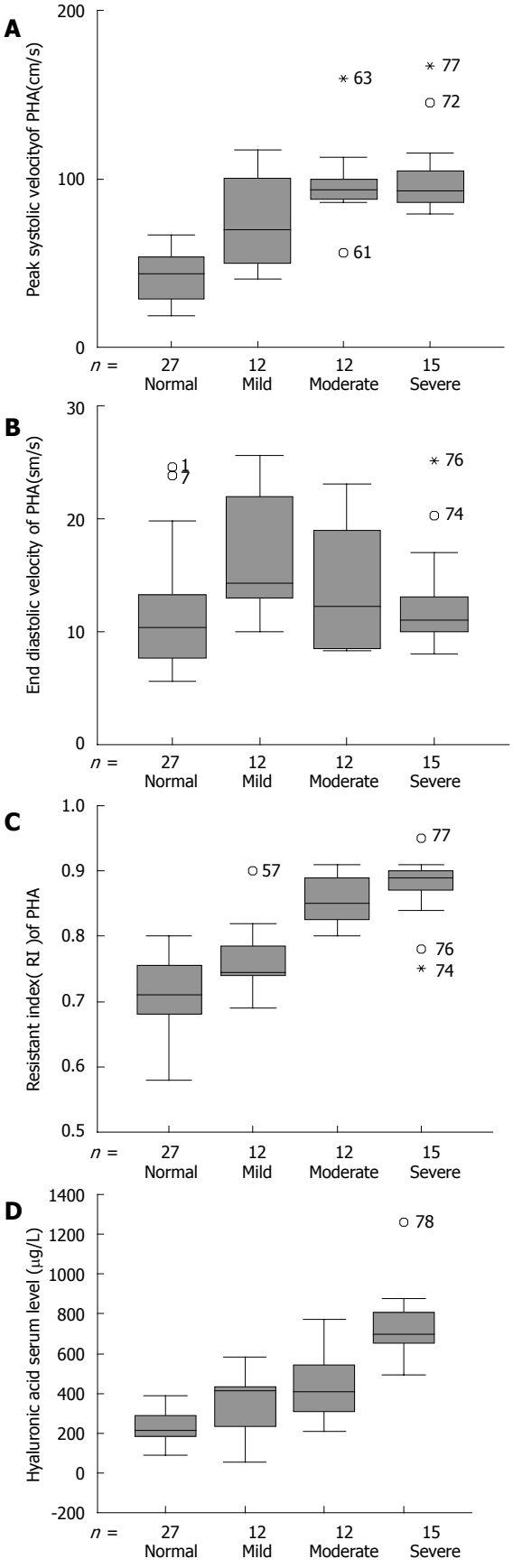


**Figure 2** Boxplots showing **A**: Significantly higher PSV of PHA in patients with advanced liver fibrosis; **B**: Decreased gradually EDV of PHA in patients with advanced liver fibrosis; **C**: Significantly higher RI of PHA in patients with advanced liver fibrosis; **D**: Significantly higher serum HA level in patients with advanced liver fibrosis.

**Table 2 Doppler sonograms in hepatic veins in patients with different fibrosis stage of IHS *n* (%)**

| Group | Patients (*n*) | HV0 | HV1 | HV2 | Total |
|---|---|---|---|---|---|
| Control group | 46 | 43 (93.50) | 3 (6.50) | 0 (0.00) | 46 (100) |
| Mild group | 12 | 2 (16.67) | 10 (83.33) | 0 (0.00) | 12 (100) |
| Moderate group | 12 | 1 (8.33) | 10 (83.4) | 1 (8.33) | 12 (100) |
| Severe group | 15 | 1 (6.67) | 10 (66.67) | 4 (26.67) | 15 (100) |

Chi-square test, $\chi^2 = 37.67$, $P < 0.001$.

fibrosis were compared to the healthy group, differences did not reach statistical significance (Table 3).

### *Correlative analyses*

We found close positive correlations between the serum HA and hepatic fibrosis ($r = 0.712$, $P < 0.001$), between PSV of PHA and hepatic fibrosis ($r = 0.717$, $P = 0.001$), and between RI of PHA and hepatic fibrosis ($r = 0.745$, $P = 0.001$). The changes of hepatic vein waveforms were also positively related to liver fibrosis ($r = 0.783$, $P < 0.001$). In contrast, liver size, the size of the spleen and hepatic capsule, the echogenicity of the liver parenchyma, the inner diameter of the PV, nor the velocity of the PV were found to be useful for the diagnosis of hepatic fibrosis in IHS patients.

## DISCUSSION

The basic pathological changes of chronic liver disease in the adults have been recognized more accurately and profoundly[33-36]. According to a new pathological classification method which was carried out in the adults in our country[33], staging of hepatic fibrosis can help us to recognize the development of the hepatic fibrosis in infants with IHS in our study. In the IHS group, 39 cases (95.1%) showed to have hepatic fibrosis by pathology. Further findings were ballooning change ($n = 35$, 85.4%), hepatocyte necrosis ($n = 30$, 73.2%), cholestasis in liver cells ($n = 26$, 63.4%), cholestasis in the hepatic lobules ($n = 21$, 51.2%), cholestasis in portal area ($n = 7$, 17.1%), and cytomorphosis of giant corpuscle ($n = 20$, 48.8%) of. Our findings showed that hepatic fibrosis was also one of the main pathologic features in the patients of this age group. We found no significant changes between the mild and the moderate hepatic fibrosis in 2DUS examination, it is compatible with other reports in the literature about adults[2,3,5-8].

PSV and RI of the PHA were significantly higher in infants with IHS than in the control group ($P < 0.05$). This phenomenon might be due to shrunken intrahepatic blood vessel space and the correspondingly increased peripheral resistance of the artery microvasscular bed, which increased the PSV and RI. This might also be the result of the severity of diffuse liver parenchyma impairment, hepatocellular degeneration and necrosis, inflammatory cell infiltration and fibrous connective tissue development[9,10]. Our findings also showed that PSV, RI of

**Table 3 Serum fibrosis indexes in patients with different fibrosis stage of IHS (mean ± SD, μg/L)**

| Group | Patients (*n*) | HA | PC-Ⅲ | C Ⅳ | LN |
|---|---|---|---|---|---|
| Control group | 46 | 225.18 ± 80.58 | 432.32 ± 166.68 | 129.69 ± 33.08 | 141.63 ± 34.63 |
| Mild group | 12 | 352.11 ± 157.33[a] | 445.73 ± 191.11 | 149.85 ± 49.27 | 151.22 ± 36.57 |
| Moderate group | 12 | 447.48 ± 175.57[b] | 480.77 ± 217.15 | 141.91 ± 47.51 | 132.56 ± 41.29 |
| Severe group | 15 | 745.32 ± 231.12[b,d] | 580.36 ± 147.79[a] | 185.00 ± 45.82[b] | 167.90 ± 29.41[a] |

HA, Serum hyaluronic acid; PC-Ⅲ: Pre-Collagen type Ⅲ; C-Ⅳ: Collagen type Ⅳ; LN: Laminin. [a]$P < 0.05$, and [b]$P < 0.01$ *vs* control group; [d]$P < 0.01$, *vs* mild and moderate group.

the PHA increased gradually and EDV decreased gradually with advanced liver fibrosis, and there were significant differences between their groups ($P < 0.05$). PSV and RI of the PHA were positively correlated with the severity of liver fibrosis ($r = 0.717$ and 0.745, respectively $P = 0.001$). Hemodynamic changes of chronic liver disease in adults[7, 9,10] and in the pediatric age group[29] have been described by other researchers. Some results of our study were inconsistent with the findings of Xue *et al*[10] whose work was focused on chronic liver disease and confirmed cirrhosis in adults. One point was EDV of PHA elevating in IHS group instead of declining, another was velocity of PHA increasing without deficiency of PV. These may be concerned with a relatively short course of disease, abundant hepatic blood flow, active liver fibrosis and inflammation, or vasodilatation induced by inflammatory substances in patients with IHS.

In our findings, the changes in the waveforms of the hepatic vein were positively correlated with the severity of liver fibrosis ($r = 0.783$, $P < 0.001$). Other groups described a damped waveform in the hepatic veins of patients with liver cirrhosis, and our results were consistent with the studies of Bolondi *et al*[14]. This may be due to increased parenchyma stiffness, which impaired the compliance of the hepatic veins and caused the waveforms to change from HV0 to HV1. With the development of the hepatic fibrosis, diffuse fibrosis can cause disorganization of lobular architecture. Therefore, the hepatic veins were stenosis by pressure of the fibrosis tissues, pseudo lobule and the hepatic capsule. Furthermore, a damped waveform (HV2) in the hepatic veins was associated with focal hepatic vein stenosis[11-14].

Many groups addressed serum indexes such as HA, PC-Ⅲ, LN and C-Ⅳ, which reflected the stage of hepatic fibrosis. There is agreement that the four serum fibrosis indexes are relevant for the serodiagnosis of hepatic fibrosis, either alone[2,5,12,15,17-19,21,24,31,32] or in combination[4,16, 20,22,23,25-28]. In our study, although we found some cases were inconsistent with pathological diagnosis, even with a high stage of hepatic fibrosis, the four serum fibrosis indexes were still around normal range, but statistical results indicated that the serum fibrosis index HA increased gradually from the mild to severe group as severity of liver fibrosis increased, and was significantly higher in each of fibrosis groups than in the control group. And then, the serum fibrosis index HA has a high positive correlation with the severity of hepatic fibrosis in IHS group. For the other serum fibrosis indices, PC-Ⅲ, C-Ⅳ and LN, there was only a significant difference between the severe hepatic fibrosis group and control group. And PC-Ⅲ, C-Ⅳ had a low positive correlation with the severity of hepatic fibrosis in IHS group. There was no correlation between LN and the severity of hepatic fibrosis in IHS group. One researcher's result showed the rate of inconsistency between the four serum fibrosis indexes and stage of hepatic fibrosis: HA was 29.2%, C-Ⅳ 31.9%, PC-Ⅲ 34.5% and LN 38.1%, the rate of consistency between serum HA and stage of hepatic fibrosis was the highest one, which was followed by C-Ⅳ. This suggested that among the four serum fibrosis indexes, HA was the most ideal index for the diagnosis of hepatic fibrosis[15]. Our results were also consistent with other reports in the literature[5,15-17,22]. Furthermore, infants less than one year old belong to a special group in the mostly active developing phase, the concentration of serum fibrosis indices was affected by many factors. In particular, serum PC-Ⅲ is very high in neonates, but quickly decreases by one year of age showing a marked negative correlation with age[30].

Liver biopsy guided by ultrasound in infants in 35 cases were performed during the scanning with an 18 G biopsy-cut needle driven by spring-loaded automatic biopsy gun, satisfactory sampling were get in all cases without any complication. A level of serum total bilirubin ⩽ 200 μmol/L( former 136 μmol/ L) was indicated for liver biopsy in IHS group. According to our results, we consider that in clinical application, ultrasound-guided liver biopsy is also a valuable technology, secure, rapid, with high achievement ratio and no complication in the infant age group.

Our findings showed that young age, short course of disease, incompatibility with age and degree of liver fibrosis were the features of IHS group, parameters such as serum HA, PSV, and RI of PHA as well as the changes in the waveforms of the hepatic veins were more sensitive and reliable indicators in evaluating hepatic fibrosis in patients with IHS. Combination of ultrasonic studies on the hepatic hemodynamics with the evaluation of serum HA may provide an indicator for revealing hepatic fibrosis in children with IHS. This may be a useful noninvasive method for the diagnosis and estimation of the prognosis of IHS.

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*RAPID COMMUNICATION*

# Relationship between Crohn's disease, infection with *Mycobacterium avium* subspecies *paratuberculosis* and *SLC11A1* gene polymorphisms in Sardinian patients

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## Abstract

**AIM:** To study the association between Crohn's disease (CD), *Mycobacterium avium* subspecies *paratuberculosis* (MAP), and genetic factors by examining the role of natural resistance-associated macrophage protein 1 (*NRAMP1*) gene polymorphisms (now *SLC11A1*) in Sardinian patients with CD and controls.

**METHODS:** Thirty-seven CD patients and 34 controls with no inflammatory bowel disease (IBD) were recruited at the University of Sassari after giving written consent. Six *SCL11A1* polymorphisms previously reported to be the most significantly associated with IBD were searched. *M. paratuberculosis* was identified by IS900 PCR and sequencing. Logistic regression was used to calculate odds ratios (OR) for the associations among CD, presence of MAP, and 6 loci described above.

**RESULTS:** For the first time, a strong association was observed between polymorphisms at *NRAMP1* locus 823C/T and CD. While CD was strongly associated with both *NRAMP1* and MAP, *NRAMP1* polymorphisms and MAP themselves were not correlated.

**CONCLUSION:** Combined with previous work on the *NOD2/CARD15* gene, it is clear that the interplay of genetic, infectious, and immunologic factors in the etiology of CD is complex.



**Key words:** *Mycobacterium avium* subspecies *paratuberculosis*; Crohn's disease; *SCL11A1* polymorphisms

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## INTRODUCTION

Natural resistance-associated macrophage protein 1 (NRAMP1), now strictly referred to as SLC11A1 (Solute carrier 11a1) and the gene which encodes for it is recognized as having a role in the susceptibility of men and animals to a number of mycobacterial infections. In human beings, the *NRAMP1* gene is located on human chromosome region 2q35. It is composed of 15 exons and covers at least 16 kb of DNA. It encodes an integral membrane protein of 550 amino acids that is expressed exclusively in the lysosomal compartment of monocytes and macrophages[1]. The promoter region of the *NRAMP1* gene possesses a polymorphism within a possible enhancer element containing a Z-DNA-forming dinucleotide repeat[2,3].

It has been proposed that *NRAMP1* polymorphisms play a role in susceptibility to mycobacterial infections[4,5]. To date, studies have yielded contradictory results[2]. In a West African population, NRAMP1 variants have been associated with susceptibility to *Mycobacterium tuberculosis*[6]. Malik *et al*[7] reported that variants of NRAMP1 are associated with tuberculosis (TB) in children. *NRAMP1*

gene polymorphisms have been linked with genetic susceptibility to infection with *M. tuberculosis* and progression of TB into severe clinical forms in eastern China[8]. Abe *et al*[9] reported that genetic variation in the *NRAMP1* gene is associated with tuberculous cavitation of the lungs in Japanese patients. Kim *et al*[10] found that *NRAMP1* polymorphisms are associated with tuberculous pleurisy.

Various studies have been carried out to establish a connection between *NRAMP1* variants and diseases caused by mycobacteria other than the TB bacilli, such as *Mycobacterium ulcerans* causing Buruli ulcers[11] and *Mycobacterium leprae*[12]. A recent study[5] showed that the *NRAMP1* promoter region polymorphism are positively associated with leprosy but not with the Mitsuda reaction (intradermal injection of lepromin), and that variants of the *NRAMP1* gene favour microbial survival inside the macrophages by blocking the efficient transport of iron. It was reported that the *NRAMP1* gene may have a role in some autoimmune diseases, including rheumatoid arthritis (RA)[13]. It was also reported that *NRAMP1* 823 C/C prevents the development of rheumatoid nodules in RA patients[14], type 1 diabetes[15] and multiple sclerosis[16]. Variants of the *NRAMP1* gene have been associated with improved response to Bacillus Calmette-Guerin immunotherapy for superficial bladder cancer[17]. Hofmeister *et al* showed that *NRAMP1* variants are specifically associated with Crohn's disease (CD)[18].

In a previous study, we found that CD is associated with polymorphisms of the *NOD2* gene and the pathogen MAP in Sardinian patients[19]. NOD2 protein is an intracellular protein that activates NFkB upon binding to microbial peptidoglycan. MAP is the etiological agent of Johne's disease, a granulomatous enteritis of ruminants and other monogastric animals[20]. Although the association between MAP and CD (a human equivalent of paratuberculosis) has been postulated for a long time, only recent studies have made improvements in isolation and genomic techniques have allowed this link to be firmly established in a number of different populations[21-24].

As a part of our ongoing studies with *NOD2/CARD15*, we also looked at the role of other genes, especially *NRAMP1*, which may be associated with the survival of intracellular pathogens such as MAP. The role of NRAMP1 in regulating microbial survival inside phagosomes is related to iron transport, although the mechanism has not yet been completely elucidated[5,25].

## MATERIALS AND METHODS

Patients were recruited at the University of Sassari after giving written consent. Using a case-control design, we analyzed 37 CD patients and 34 controls with no inflammatory bowel disease (IBD).

We searched for polymorphisms in 6 loci previously reported to be the most significantly associated with IBD[13]. The *SCL11A1* polymorphisms that we analyzed included a (GT)n microsatellite in the promoter region, (-)237 C/T; 469 + 14G/C, INT4; a non-conservative base substitution at codon 543 (D543N); 823 C/T; and a 4-bp TGTG deletion locating 55 nucleotides downstream of the last codon in exon 15 (1729 + del55del4).

Amplification was performed as previously reported[13] using 100 ng of template genomic DNA previously extracted from intestinal tissues. The primer sequences that we used were previously reported[13]. Detection of MAP also was performed as previously reported[24].

Logistic regression was used to calculate odds ratios (OR) for the associations among CD, presence of MAP, and the 6 loci described above. Saturated and reduced models were computed and their comparability was assessed using the likelihood ratio test. The reduced model was assessed for goodness of fit using the covariate patterns as groups. In addition, the actual and model-predicted probabilities of CD according to levels of the independent variables in the final model were calculated.

**Table 1 Full and reduced logistic regression main effects models for the association between Crohn's disease, MAP infection, and the presence of a number of genetic polymorphisms of the NRAMP1 gene**

| | | Full model | | Reduced model[2] | |
|---|---|---|---|---|---|
| | | Odds Ratio | *P* | Odds Ratio | *P* |
| MAP | Negative | 1.0[1] | | 1.0[1] | |
| | Positive | 45.5 | < 0.001 | 42.4 | < 0.001 |
| 1729 + 55del4 | +/+ | 1.0[1] | | 1.0[1] | |
| | +/DEL, DEL/DEL | 5.6 | 0.014 | 5.6 | 0.011 |
| 823 C/T | CC | 1.0[1] | | 1.0[1] | |
| | CT | 75.6 | 0.001 | 50.8 | 0.001 |
| D534R | GG | 1.0[1] | | | |
| | GC | 1.1 | 0.913 | | |
| (-)237C/T | CC | 1.0[1] | | | |
| | CT, TT | 0.5 | 0.350 | | |
| INT4 | GG | 1.0[1] | | | |
| | GC, CC | 2.5 | 0.224 | | |
| GT (*n*) | Allele1 | 1.0[1] | | | |
| | Allele2 | 1.2 | 0.807 | | |
| | Allele3 | 0.6 | 0.673 | | |

[1]Reference category; [2]Model statistics: Likelihood ratio test for equivalence with full model: $P = 0.436$; Pearson goodness-of-fit test: $P = 0.407$; Area under Receiver-Operating Characteristic curve: 0.886.

## RESULTS

Table 1 shows the full and reduced logistic regression models computed for this study. The full model showed significant associations between CD and the presence of MAP, the 1729 + 55del4 deletion polymorphism and the 823 C/T CT polymorphism. No significant associations were found between CD and the INT4, D534R, GT (n) and (-) 237 C/T loci. These non-significant loci were dropped in the reduced model and likelihood ratio testing showed no difference between the full and reduced models (likelihood ratio test: $P = 0.436$). Furthermore, the reduced model showed good fit (Pearson goodness-of-fit test: $P = 0.407$) and predictive power. We tested for significant interactions between MAP infection and each of the genetic loci, none of which was statistically significant. We therefore selected the reduced main effect model as our final model. No association was found between MAP and the 823 C/T or 1729 + 55del4 polymorphisms.

Figure 1 shows the actual and final model-predicted probabilities of CD by MAP infection and the two statistically significant loci, 823 C/T and 1729 + 55del4. The close similarity between the actual and predicted probabilities reflected the good fit of our final model to the data. Strong independent effects of MAP infection and each of the loci on the probability of CD were observed (Figure 1). Even with no mutant polymorphism, MAP infection was highly predictive of CD. Having the CT polymorphism at the 823 locus, the probability of CD was greatly increased both among patients infected with MAP and among patients not infected with MAP. The effect of deletions at the 1729 locus was more moderate. It should be noted that these probabilities were sensitive to the ratio of cases to controls in the study, which were shown here to illustrate the particularly strong association among CD, MAP infection and the 823 C/T locus as well as the close fit between the experimental data and our chosen statistical model.

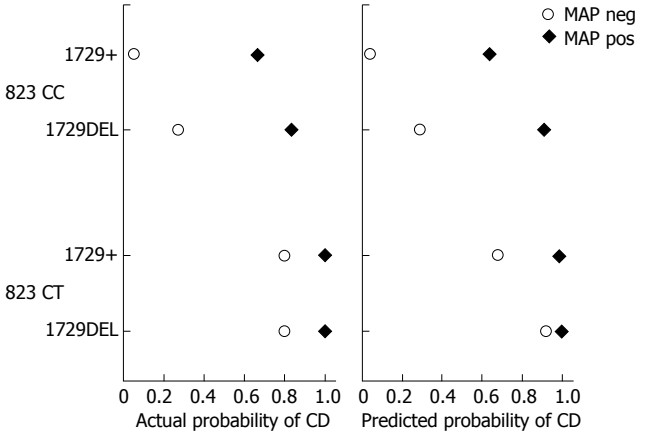


**Figure 1** Actual probability of CD and predicted probability of CD showing strong independent effects of MAP infection and each of the loci on the probability of CD.

## DISCUSSION

For the first time, a strong association has been observed between CD and polymorphisms at the 823C/T and 1729 + 55del4 loci in the *NRAMP1* gene in Sardinians.

Although the combination of MAP infection and 823 CT mutation could perfectly predict CD, generalizations were limited by the small sample size in our study. Only 8 patients in this study simultaneously had MAP infection, 823 CT mutation, and CD. It would be worthwhile to follow up this finding in a larger study to determine if these two factors are a useful prognostic index for the development of CD.

It was reported that polymorphisms in the *NOD2* gene and the presence of MAP are strongly associated with CD[19]. Both *NOD2* polymorphisms and MAP infection had a strong independent association with CD, and are also associated with one another, suggesting that susceptibility to MAP infection may be influenced by *NOD2* polymorphisms[26]. NOD2 protein is involved in the activation of NFkB factor and failure of its priming signal causes failure of pathogen clearance, possibly explaining the abnormal adaptive immune response to pathogens[27].

Our findings agree and disagree with some previous genetic studies of various *NRAMP1* loci and CD. Our finding which is lacking of association between GT (*n*) alleles and CD is consistent with the failure to find an association between any of these alleles and IBD in Americans[28]. Stokkers *et al*[29] found that mutations at the 823C/T or (-) 274C/T loci mutations are not associated with CD, which is in agreement with our findings at (-) 274C/T locus in this study. However, we have found a very strong association between CD and the 823CT polymorphism.

Although previous studies have suggested that NRAMP1 mutations may favour microbial survival, the fact that this study failed to find any association between *NRAMP1* polymorphisms and MAP infection does not support that viewpoint. Interestingly, although a previous study[6] showed allele 2 of this polymorphism is associated with mycobacterial infections, we did not find such an association between this allele and MAP infection.

The lack of association between *NRAMP1* polymorphisms and MAP infection is particularly curious because NRAMP1 is thought to directly impact microbial survival inside of macrophages. NRAMP1 is thought to either deny needed iron in intraphagosomal microbes, or to increase transphagosomal iron needed to create bactericidal hydroxyl radicals via the Haber-Weiss/Fenton reaction[25].

The 823CT and 1729 + 55del4 polymorphisms have been associated with autoimmune disease[14], but not with another mycobacterial disease, tuberculosis[30], which probably does not have an autoimmune etiology. In the present study, these polymorphisms were found to be associated with CD which probably does have an autoimmune component, but not associated with MAP infection. These findings, combined with the strong association between MAP infection and CD, suggest that *NRAMP1* polymorphisms may not cause CD by affecting the survival of MAP in intestinal tissue, but rather work together with MAP infection to cause CD *via* an autoimmune mechanism. In other words, one possible interpretation may be that these data support the aetiology of CD with both infectious and autoimmune components. At least, one study has elucidated a mechanism by which NRAMP1 affects the survival of intracellular pathogens and is simultaneously involved in autoimmune responses[25].

A further step of the study is to test the derived model on a large cohort of patients and controls.

In conclusion, CD may be strongly influenced by both NRAMP1 and MAP, although *NRAMP1* polymorphisms and MAP infection are not themselves correlated. Combined with previous work on the *NOD2/CARD15* gene, a complex interplay of genetic, immunologic and infectious factors may play a role in the aetiology of CD.

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*RAPID COMMUNICATION*

# Trocar-related abdominal wall bleeding in 200 patients after laparoscopic cholecistectomy: Personal experience

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## Abstract

**AIM:** To determine the complications and incidence of the first and second access-related vascular injuries induced by videolaparoscopic cholecistectomy.

**METHODS:** We retrospectively reviewed vascular injuries in 200 consecutive patients who underwent videolaparoscopic cholecistectomy from 2003 to 2005. One hundred and one patients with placement of radial expanding trocars were assigned into group A and 99 patients with placement of pyramidal tipped trocars into group B. All the patients were submitted to open access according to Hasson for the first trocar.

**RESULTS:** Bleeding did not occur at the intraoperative cannula-site in group A. However, it occurred at the intraoperative cannula-site of 7 patients (7.1%) in group B, with a statistically significant difference ($P < 0.01$). No mortality was registered. More vascular lesions were found in group B.

**CONCLUSION:** The advantage of Hasson technique is that peritoneal cavity access is gained under direct vision, preventing most severe injuries. The open technique with radial expanding trocars is recommended for secure access to the abdominal cavity in videolaparoscopy. Great care should be taken to avoid major complications and understanding the abdominal wall anatomy is important for reducing bleeding during or after s placement of trocars.



**Key words:** Videolaparoscopy; Minor vascular complications; Trocars; Prevention

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## INTRODUCTION

As minimally invasive techniques become more popular in modern surgery, laparoscope is now an instrument used by almost all surgical disciplines[1].

Primary and secondary accesses to the peritoneal cavity are the most crucial phases of laparoscopy. In fact vascular and visceral injuries (0.003%-6%) have been reported secondary to incisions made for positioning the laparoscopic cannulae or ports[1-3].

These injuries usually occur during the initial phase of operation or during placement of secondary cannulae, which should be placed under direct vision and with prior transillumination of the abdominal wall[4]. The technique of open laparoscopy developed by Harrith M. Hasson in 1970[4] combines safety of minilaparotomy with versatility of laparoscopy. This open access technique has also been introduced with the aim of reducing such injuries[3].

The aim of this study was to evaluate our laparoscopic experience with a focus on access-related vascular injuries and compare the effectiveness of two different types of trocar.

## MATERIALS AND METHODS

We retrospectively reviewed the vascular injuries in 200 consecutive patients who underwent videolaparoscopic cholecystectomy in our university hospital from 2003 to 2005, with the approval of the ethics committee of our hospital.

One hundred and one patients with placement of radial expanding trocars (Ethicon Endo-Path Excel Endo-Surgery©) were assigned into group A and 99 patients with placement of pyramidal tipped trocars (Ethicon Endo-Path Tristar Endo-Surgery©) into group B. All the patients were submitted to open access according to Hasson for the first trocar, and then the following trocars were introduced under direct vision, after abdominal wall puncture and transillumination. We even used "open access" as previously described[4].

Carbon dioxide was insufflated into the intra-abdominal cavity with a pressure of 10-12 mmHg. We did not formally evaluate the time needed to achieve

abdominal entry and distension during open laparoscopy, but we noted that it generally varied from 3 to 10 min. Time variations depended on patient weight, abdominal wall strength, a well-developed Richet's fascia and post-operative fibrosis.

The diameter of Hasson, subxyphoid, and hypocondriac trocars was 10, 5, and 10 mm, respectively. The "primary access-related complications" were defined as those caused by the introduction of the first trocar and the "secondary access-related complications" as those caused by the introduction of the second or following trocars in the open access using Hasson's technique.

## RESULTS

The age, gender, clinical history, comorbidity and American Society of Anesthesiology (ASA) classification of the patients were similar in both groups (Table 1).

Primary access-related complications occurred in 7 cases (3.5%), all in group B. No first-trocar lesions (Hasson-related) were registered in the two groups. Secondary access-related complications occurred in 6 cases of epigastric vessels injuries, all of which were treated with direct suture by the end of general anaesthesia (in all cases the bleeding started at the disinflation of pneumoperitoneum). One case (0.5%) of large abdominal wall haematomas was managed conservatively. No bleeding occurred at the intra-operative cannula-site in group A. However, bleeding occurred in 7 patients (7.1%) of group B, with a statistically significant difference ($P < 0.01$). Statistical analysis was performed by $\chi^2$ test and $P < 0.01$ was considered statistically significant. No intraoperative morbidity or mortality was registered in our study.

**Table 1 Personal experience**

| | Group A | Group B | *P* |
|---|---|---|---|
| Equipe position | French | French | - |
| ASA class Ⅰ / Ⅱ / Ⅲ | 10/64/27 | 8/72/19 | NS |
| Bleeding comorbidities | 4% | 1% | NS |
| Type of trocar | Conical | Pyramidal | - |
| Patients | 101 | 99 | NS |
| M:F ratio | 0.6:1 | 0.6:1 | NS |
| Age (mean) | 62 | 64 | NS |
| Body weight (mean, Kg) | 72.7 | 70.8 | NS |
| Bleeding (7 cases = 3.5%) | 0 | 7 (7.1%) | < 0.01 |
| Relaparotomy | 0 | 0 | 0 |
| Mortality | 0 | 0 | - |

## DISCUSSION

Between 1997 and mid-2002, FDA received more than 1300 trocar-associated injury reports, including 30 deaths. Hemorrhage due to vessel injury and infection secondary to bowel injury, especially when diagnosis is delayed, are the most serious complications which most likely result in death. Most data suggest that the rate of trocar-related complications is about 0.003%-6% and the average incidence of trocar-related vascular injuries is approximately 0.1%[5,6].

Minor vascular injuries are referred to the injuries to vessels of lesser importance than the aorta, inferior vena cava and iliac vessels. By far, the most common minor vascular injury of the inferior epigastric vessels occurs in up to 2.5% of laparoscopic cholecystectomies[3,7].

The major advantage of the open access technique using a step-by-step entrance to all layers of the abdominal wall is that peritoneal cavity access is gained under direct vision, preventing severe injuries[5].

The "11 secured steps" for the safe insertion of trocars using conical disposable trocars are recommended[8]. A randomized study comparing the conventional cutting with radial expanding trocars at all port sites reported that there is a significant difference in cannula-site bleeding and post-operative wound complications, which is in favour of using radial expanding trocars[9].

In a rabbit model, the relative risk of vessel injury induced by pyramidal tipped trocars is significantly increased compared with that induced by conical tipped trocars, especially if larger diameter trocars are used. The smooth contour of the conical tip stretches and separates the tissue layers with little damage, whereas the sharp-edged pyramidal tip lacerates any tissue it encounters, including vessels, even if the trocar is inserted directly over (in 88% of cases, partial interruption of vessel) or at 1-2 mm from the vessel (no injuries)[10,11].

In 1999, Balzer KM *et al*[11] dissected the abdominal walls of 21 cadavers transversally and measured the morphometric distances as well as determined the distances of the epigastric arteries and the ascending branch of the deep circumflex iliac artery from the midline and lateral edge of rectus muscle. The results showed that In order to minimize the danger of lesions of abdominal vessels, trocars should be placed in the ventral midline or in a 5 cm wide zone to the lateral border of the rectus sheath.

If prevention fails, it is possible to try to tamponade by applying pressure to the abdominal wall with a balloon trocar or a Foley catheter pulled against the abdominal wall and attached to a clamp (the temporary pressure usually stops bleeding from small vessels or veins), which should not be used to stop the bleeding at the trocar site.

If significant bleeding occurs, ligation is needed using Keith or Reverdin needle and large suture that can be removed in the recovery room when the patient awakens. Occasionally, in catastrophic bleeding from the abdominal wall, an incision should be made to dissect down the bleeding vessels and control the bleeding by ligation[12].

In conclusion, the risk of vessel injury varies markedly with the different trocars used. The open technique with radial expanding trocars is recommended for access to the abdominal cavity during videolaparoscopy. However, open laparoscopy cannot prevent small bowel injury or post-operative herniation, but can eliminate failed laparoscopy attempts, inappropriate insufflation, gas embolism, peritoneal, stomach, colon and vessel injury.

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*RAPID COMMUNICATION*

# Protective effects of amphetamine on gastric ulcerations induced by indomethacin in rats

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## Abstract

**AIM:** To study the effects of amphetamine, an indirect-acting adrenomimetic compound on the indomethacin-induced gastric ulcerations in rats.

**METHODS:** Male Wistar-Bratislava rats were randomly divided into four groups: Group 1 (control), received an ulcerogenic dose of indomethacin (50 μmol/kg) and Groups 2, 3 and 4, treated with amphetamine (10, 25 and 50 μmol/kg). The drug was administered simultaneously with indomethacin and once again 4 h later. The animals were sacrificed 8 h after indomethacin treatment. The stomachs were opened and the incidence, the number of lesions and their severity were evaluated. The results were expressed as percentage and as mean ± standard error (mean ± SE).

**RESULTS:** The incidence of ulceration in the control group was 100%. Amphetamine, at doses of 10, 25 and 50 μmol/kg, lowered the incidence to 88.89%, 77.78% and 37.5% respectively. The protection ratio was positive: 24.14%, 55.17% and 80.6% respectively. The total number of ulcerations/rat was 12.44 ± 3.69 in the control group. It decreased to 7.33 ± 1.89, 5.33 ± 2.38 and 2.25 ± 1.97 under the effects of the above-mentioned doses of amphetamine.

**CONCLUSION:** Amphetamine affords a significant dose-dependent protection against the indomethacin-induced gastric ulcerations in rats. It is suggested that the adrenergic system is involved in the gastric mucosa protection.



**Key words:** Amphetamine; Gastric ulceration; Indomethacin; Rats

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## INTRODUCTION

The involvement of the adrenergic system in some models of gastric ulcerations is well documented[1-4]. Our previous investigations have shown that amphetamine, an indirectly acting sympathomimetic amine has a protective activity on gastric ulcerations induced by reserpine and immobilization in rats[5]. These findings were interpreted as an evidence of a protective activity of the adrenergic mechanisms on the integrity of the gastric mucosa[3,4,6].

Other models of gastric ulcers imply the administration of some nonsteroidal anti-inflammatory agents such as acetylsalicylic acid[7], phenylbutazone[8] or indomethacin[9]. The genesis of these ulcerations arises from both the local depression of prostanoid biosynthesis[10-12] and a local injury[13]. However, the participation of the adrenergic system in this experimental setting is not excluded[14,15].

In this paper, we studied the effect of amphetamine on the indomethacin-induced gastric ulcerations in albino rats.

## MATERIALS AND METHODS

### *Animals and ulcer model*

The experiments were carried out on male Wistar albino rats, weighing 144-255 g (from the Animal Center of University of Medicine and Pharmacy Iuliu Haţieganu, Cluj-Napoca). The animals were kept in a room with a constant temperature (20-22℃) and humidity, under a natural light-dark regime. They were fed common rat chow and received water ad libitum. The food was withdrawn 12 h prior to administration of the ulcerogenic agent. The rats were randomly divided in four groups.

Control group (Group Ⅰ) was administered intraperitoneally (ip) with indomethacin 50 μmol/kg. Groups Ⅱ, Ⅲ and Ⅳ were simultaneously ip injected with

**Table 1 Effect of amphetamine on incidence and severity of indomethacin-induced gastric ulcerations (%)**

| Groups | | TU | LU | PR |
|---|---|---|---|---|
| I | $I_{50}$ | 100 | 100 | |
| II | $I_{50} + A_{10}$ | 88.89 | 77.778 | (+) 24.138 |
| III | $I_{50} + A_{25}$ | 77.78 | 66.667 | (+) 55.172[1] |
| V | $I_{50} + A_{50}$ | 37.5[a] | 12.5[a] | (+) 80.603[1] |

The subscript indices represent the doses expressed as μmol/kg. I : Indomethacin; A: Amphetamine; TU: Total number of ulcerations; LU: Large ulcerations (> 1 mm); PR: Protection ratio. [a]$P < 0.05$ *vs* group I (Fischer's exact probability and median's test); [1]Significant at PR > (+) 33.33%.

**Table 2 Effect of amphetamine on number and severity of indomethacin-induced gastric ulcerations (mean ± SE)**

| Groups | | Number of ulcerations/stomach | | Severity |
|---|---|---|---|---|
| | | TU | LU | UI |
| I (9) | $I_{50}$ | 12.44 ± 3.69 | 10.11 ± 3.74 | 1.61 ± 0.31 |
| II (9) | $I_{50} + A_{10}$ | 7.33 ± 1.89 | 5.44 ± 1.52 | 1.22 ± 0.25 |
| III (9) | $I_{50} + A_{25}$ | 5.33 ± 2.38 | 3.11 ± 1.9 | 0.72 ± 0.21[a] |
| IV (8) | $I_{50} + A_{50}$ | 2.25 ± 1.91[c] | 1.0 ± 1.0[c] | 0.31 ± 0.19[b] |

The subscript indices represent the doses expressed as μmol/kg. I: Indomethacin; A: Amphetamine. TU: Total number of ulcerations; LU: Large ulcerations (> 1 mm); UI: Ulcer index. [a]$P < 0.05$, [b]$P < 0.01$, [c]$P < 0.05$ *vs* group I .

indomethacin and amphetamine sulphate, and 4 h later, only with amphetamine. The doses of amphetamine were 10, 25 and 50 μmol/kg , respectively.

During the ulcerogenesis period, the rats were fasted, but water was allowed ad libitum. Eight hours after indomethacin administration, the animals were sacrificed; the stomachs were removed, opened along the curvatura maior and rinsed with saline. They were examined immediately, with a magnifying glass. The following parameters were recorded: the body weight (g), the incidence of ulcerations/group, the total number of ulcerations (TU), the number of large ulcerations (LU) i.e. ulcerations larger than 1 mm and the severity of ulcerations estimated according to a scale between 0 and 4 (ulcer index-UI)[3].

### Drugs

**Indomethacin:** Powder supplied by Terapia SA, Cluj-Napoca, Romania, was suspended in a mixture of 2% methylcellulose and 1% glycerin, ten times diluted with saline. The concentration was 0.025 mol/L (8.945 g/L) and the dose, 50 μmol (17.89 mg)/kg.

**Amphetamine sulphate (racemate):** It was prepared as an aqueous solution 0.025 mol/L (9.21 g/L) for the dose of 50 μmol (18.425 mg)/kg. Doses of 10 μmol (3.685 mg)/kg and 25 μmol (9.2105 mg)/kg were adapted after dilution. All drugs were ip administered in a volume of 2 mL/kg.

### Ethical issues

The study was conducted in accordance with the Helsinki's Declaration on Animal's Studies and approved by the local ethics committee.

### Statistical analysis

All values are expressed as means and standard errors (mean ± SE). The indicators of ulcerogenesis were analyzed initially by a one-way ANOVA test. The significance of differences between groups was observed by the bilateral Student's *t* test and by multiple comparison tests of Newman-Keuls and Scheffé. This last test allows comparisons between blocks of groups. These procedures were followed by non-parametric Kruskal-Wallis, Mann-Whitney, median's and Fischer's exact probability tests[16,17]. The statistical significance of the differences was admitted if $P < 0.05$.

The protection ratio (PR) was calculated according to the formula:

$$PR\% = (UI_{control} - UI_{treated}) / UI_{control} \times 100$$

Statistical significance for PR was considered for values outside (-) 33% → (+) 33% range[3].

## RESULTS

The body weight of the rats was a homogenous parameter, i.e. there were no significant differences between groups (data not included in Tables). The doses of 25 and 50 μmol/kg of amphetamine induced a stereotyped behavior. It was not affected by indomethacin.

Indomethacin induced ulcerations in 100% of the rats. The total number of ulcerations was 12.4 ± 3.69, most of them (10.1 ± 3.74) were larger than 1 mm. The UI was 1.61. When amphetamine was administered, all the parameters diminished in a dose-dependent manner (Tables 1 and 2). In addition, a dose-dependent increase of the PR was found (Table 1).

Significant differences between the control group and the groups treated with 25.0 μmol/kg and 50.0 μmol/kg of amphetamine were found in most tests. Some tests showed significant differences between the 10.0 μmol/kg amphetamine administered group and the group that received 50.0 μmol/kg (Tables 1 and 2). For the parameters of ulcerogenesis (TU, LU, and UI), an increase of significance was found in the order: total ulcerations (TU) < ulcerations larger than 1 mm (LU) < ulcer index (UI).

## DISCUSSION

These results seem at first glance rather surprising, since, according to most authors, the ulcerations produced by nonsteroidal anti-inflammatory agents are due to an impaired prostanoids biosynthesis in the gastric mucosa[10,18,19], and to a local injury as well[13].

It is possible that other mechanisms are involved in the ulcerogenesis caused by indomethacin: the direct cytotoxic effect[20], the promotion of apoptosis of gastric mucosal cells[21-23] as well as the induction of interactions between polymorphonuclear leukocytes and endothelium of gastric mucosa vessels[24-28]. The effects of amphetamine may be related to any of these mechanisms. However, only the links between prostaglandins and adrenergic system are firmly established[29]. Prostaglandins, particularly those

from E series inhibit catecholamines release[30,31]. On the other hand, adrenergic stimulation evokes an increase of prostaglandin release[32,33].

It is well known that amphetamine acts mainly via the release of dopamine and noradrenaline from both central and peripheral sites[34,35]. The question arises whether dopamine or noradrenaline or both are involved in the protection of gastric ulcerations afforded by amphetamine. Our previous results have shown that dopaminergic mechanisms were not involved in the protective activity of amphetamine and catecholamines in restraint and reserpine-induced gastric ulcerations[3,5,7]. We did not investigate the involvement of dopamine receptors in indomethacin-ulcerations.

On the other hand, there are data in the literature indicating that the dopaminergic system may be also involved in the genesis of the ulcerations induced by nonsteroidal anti-inflammatory agents[36,37]. So, dopaminergic agonists, such as apomorphine and bromocriptine, diminished significantly the incidence and the severity of the ulcerations evoked by indomethacin in albino rats. The protective activity of apomorphine was antagonized by haloperidol[36]. This agent and other dopaminergic antagonists aggravated gastric ulcerations induced by indomethacin[36], reserpine[37] and stress[38,39]. These findings suggest that dopamine would have a protective activity in various models of experimental ulcers[37,40-42]. It was also shown that gastric pentadecapeptide BPC 157 markedly affects the central and peripheral dopaminergic system. It blocks the acute stereotype??? elicited by amphetamine[43]. At the same time, it completely prevents the gastric ulcerations induced by haloperidol[37,44].

More recently, a cocaine- and amphetamine-regulated transcript (CART) messenger RNA was isolated from the brain[45,46], peripheral nerves[47] and tissues[48,49]. Its corresponding polypeptide inhibits after central injection, food intake, the gastric emptying and gastric acid secretion in 24 h fasted rats. However, CART polypeptide, ip injection does not have the same effect. It was also shown that the intracisternal injection of CART greatly reduces the gastric acid output elevated by subcutaneous indomethacin administration[50]. These data are consonant with the view that the adrenergic system has a protective action on gastric mucosa.

In conclusion, amphetamine protects gastric mucosa against the damaging effect of indomethacin. The mechanism of action is still obscure, it might involve both the central and peripheral segments of the adrenergic system.

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*RAPID COMMUNICATION*

# Non-invasive diagnosis of gastric mucosal atrophy in an asymptomatic population with high prevalence of gastric cancer

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## Abstract

**AIM:** To validate a non-invasive method to detect gastric mucosal atrophy in a Chilean population with high prevalence of gastric cancer and a poor survival rate.

**METHODS:** We first determined the optimal cut-off level of serum pepsinogen (PG)-1, PG-1/PG-2 ratio and 17-gastrin in 31 voluntary symptomatic patients (mean age: 66.1 years), of them 61% had histologically confirmed gastric atrophy. Then, in a population-based sample of 536 healthy individuals (209 residents in counties with higher relative risk and 327 residents in counties with lower relative risk for gastric cancer), we measured serum anti-*H pylori* antibodies, PG and 17-gastrin and estimated their risk of gastric cancer.

**RESULTS:** We found that serum PG-1 < 61.5 μg/L, PG-1/PG-2 ratio < 2.2 and 17-gastrin > 13.3 pmol/L had a high specificity (91%-100%) and a fair sensitivity (56%-78%) to detect corpus-predominant atrophy. Based on low serum PG-1 and PG-1/PG-2 ratio together as diagnostic criteria, 12.5% of the asymptomatic subjects had corpus-predominant atrophy (0% of those under 25 years and 20.2% over 65 years old). The frequency of gastric atrophy was similar (12% *vs* 13%) but *H pylori* infection rate was slightly higher (77% *vs* 71%) in the high-risk compared to the low-risk counties. Based on their estimated gastric cancer risk, individuals were classified as: low-risk group (no *H pylori* infection and no atrophy; *n* = 115; 21.4%); moderate-risk group (*H pylori* infection but no atrophy; *n* = 354, 66.0%); and high-risk group (gastric atrophy, with or without *H pylori* infection; *n* = 67, 12.5%). The high-risk group was significantly older (mean age: 61.9 ± 13.3 years), more frequently men and less educated as compared with the low-risk group.

**CONCLUSION:** We propose to concentrate on an upper gastrointestinal endoscopy for detection of early gastric cancer in the high-risk group. This intervention model could improve the poor prognosis of gastric cancer in Chile.



**Key words:** Gastric cancer; *H pylori*; Gastric atrophy; Non-invasive diagnosis; Pepsinogen; Gastrin

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## INTRODUCTION

Gastric cancer (GC) is still highly prevalent in Chile and the world[1]. With the exception of Japan, which has implemented early detection strategies through the population screening, the diagnosis of GC is usually late and its prognosis is very poor. In Chile, a study showed that a global survival rate at 5 years after diagnosis was less than 15%, although it surpassed 90% when early stage tumors were treated with curative resection[2].

In spite of the identification of *H pylori* infection as a relevant etiological factor, this has not yet led to significant changes in the diagnostic or therapeutic strategy. The initial pathogenic role of *H pylori* infection is currently undisputed, explaining between 70% and 90% of GC risk in a given population[3,4]. As first postulated by Correa[5], *H pylori* produces an inflammation of the gastric mucosa (gastritis) that, evolving asymptomatically through many years, progresses in a decreasing proportion to gastric atrophy (GA), intestinal metaplasia, dysplasia, and finally to well differentiated adenocarcinoma. Other diverse environmental and genetic factors modulate this chain of

events. Atrophy and intestinal metaplasia of the gastric mucosa are doubtfully reversible lesions that determine a significant increase in the risk of developing GC[5,6].

The "gold standard" for the diagnosis of gastric atrophy is the histological study of biopsies obtained during an upper gastrointestinal (GI) endoscopy, an invasive method hardly suitable for population screening. Non-invasive strategies, applicable to an asymptomatic population, would allow having better knowledge of the frequency and natural history of gastric mucosal atrophy, testing prevention strategies and diagnosing more effectively the progression to (early) gastric cancer.

The possibility to detect gastric atrophy by means of some serum markers, such as pepsinogen (PG)-1, PG-1/PG-2 ratio and 17-gastrin, has recently been described[7-9]. However, the apparent geographic or even racial variability in the diagnostic performance of these tests precludes its simple extrapolation to our milieu.

The general objective of this study was to validate a non-invasive diagnostic tool for gastric mucosal atrophy and then to apply it to an asymptomatic population in order to identify the population groups at greater risk for developing GC. The specific objectives were: (1) to determine the diagnostic performance of PG-1, PG-1/PG-2 and 17-gastrin in serum for the diagnosis of gastric atrophy in a sample of symptomatic patients, compared to the histology as a gold standard; (2) to determine serologically the frequency of gastric atrophy in a sample of asymptomatic subjects residing in counties with different risks of developing GC; and (3) to non-invasively categorize the estimated risk of developing GC in the same sample.

## MATERIALS AND METHODS

### Subjects

For the diagnostic validation study, we included voluntary patients older than 40 years old, with clinical indications for upper GI endoscopy. Exclusion criteria were: use of proton pump inhibitors within the last month, gastric cancer, active peptic ulcer or previous gastric resection. The Ethics Committee of our institution approved the study.

For the population study, we used the sera of 536 subjects selected from the 3600 subjects that constituted a random Chilean population sample in which a National Health Survey was performed[10]. This sub-sample was obtained by ordering the subjects from the original sample according to mortality risk for GC in their county of residence (described below) and then selecting subjects from the extreme counties on the list (those with greater and lower relative risk of GC, respectively) until reaching the number of available determinations.

### Diagnostic validation study

After obtaining informed consent from the patients, an upper GI endoscopy was performed. Two sets of biopsies from the gastric corpus and the antrum (both the gastric wall and greater curve) were sent separately for histological analysis. An additional antral biopsy was destined to a rapid urease test (ProntoDry®, Medical Instrument Corp, Brignais, France). Serum from a 10-mL blood sample was separated and stored at -20℃ until processing.

### Population study

Blood samples were obtained from all over the country during the 2003 National Health Survey, sent to regional hospitals and then to our center, where the sera were stored at -80℃ until processing. The categorization of relative risk (RR) of GC for each subject was calculated according to the mortality rates for GC registered in his/her county of residence during 1985-2002, corrected for age, sex and population size, and adjusted through a hierarchical Poisson regression model, considering the extra-Poisson structural variability, estimated by Bayesian analysis[11,12]. The counties with a RR of mortality for GC > 1 were considered high-risk counties.

### Analytical determinations

For anti-*H pylori* serology, the commercial bioelisa assay HELICOBACTER IgG (BioKit®, Barcelona, Spain) was used. In previous studies, we determined that the most appropriate cut-off level for the diagnosis of *H pylori* infection in the Chilean adult population was 72.8 arbitrary units (AU)/mL.

Determination of serum levels of PG-1, PG-2 and 17-gastrin was performed using a commercial ELISA assay (Gastropanel®; (BioKit®, Helsinki, Finland), following the manufacturer's instructions.

### Histological study

The formalin-fixed and paraffin-embedded samples were cut into thin sections and stained with hematoxylin-eosin (H&E). The modified Sydney classification[13] was used to classify the histological findings in the gastric corpus and the antrum separately. We used previously described criteria for histological categorization[14]. Non-atrophic chronic gastritis was defined as the presence of chronic inflammation (score $\geqslant$ 1), with or without acute inflammation, with no atrophy (score = 0) in the corpus or the antrum. Chronic atrophic gastritis was diagnosed in the presence of atrophy (Sydney score $\geqslant$ 1), with or without intestinal metaplasia, associated with chronic inflammation (score $\geqslant$ 1). The patients with atrophic gastritis were classified as antrum predominant when the atrophy score was greater in the antrum than in the corpus, corpus predominant when the score was greater in the corpus and multifocal when the score was the same in the both sites.

### Statistical analysis

The Student's *t* test, ANOVA or Kruskal-Wallis test were used for univariate analysis of the discrete variables (age and serum levels of PG-1, PG-1/PG-2, 17-gastrin) and the chi-square test or the Fisher exact test for categorical variables (sex, endoscopy variables and levels of PG-1, PG-1/PG-2 and 17-gastrin above or below the respective cut-off levels) associated with the presence of histological atrophy. Through ROC (receiving operator characteristic) curves, the best cut-off levels were determined for the serum levels of PG-1, PG-1/PG-2 and 17-gastrin for the detection of the antrum and corpus atrophy (Table 1). To evaluate the diagnostic performance of the serologic

**Table 1 Correlation between the serum levels of pepsinogen and gastrin with the type and topography of histological gastritis (median)**

| | Chronic non-atrophic gastritis ($n$ = 10) | Antrum-predominant atrophic gastritis ($n$ = 10) | Corpus-predominant atrophic gastritis[1] ($n$ = 9) |
|---|---|---|---|
| PG-1 (μg/L)[a] | 115.6 | 103.3 | 40.9 |
| PG-1/PG-2[a] | 4.39 | 5.62 | 2.22 |
| 17-gastrin[b] (pmol/L) | 6.2 | 3.7 | 36.8 |

[1]Including 1 patient with multifocal atrophic gastritis; [a]$P < 0.05$ or [b]$P < 0.01$ between the three groups (Kruskal-Wallis Test).

**Table 2 Diagnostic performance of serology (pepsinogen and 17-gastrin) to diagnose gastric atrophy**

| | Cut-off level | Sensitivity % (95% CI) | Specificity % (95% CI) | LR[5]+ | LR- | Youden's J index |
|---|---|---|---|---|---|---|
| PG-1 (μg/L)[3] | < 25[1] | 44 (12-77) | 95 (87-100) | 8.8 | 0.59 | 0.40 ± 0.17 |
| | ⩽ 61.5[2] | 78 (40-97) | 91 (71-99) | 8.6 | 0.24 | 0.69 ± 0.15 |
| PG-1/PG-2[3] | < 2.5[1] ⩽ 2.2[2] | 56 (21-86) | 100 (84-100) | ∞ | 0.44 | 0.56 ± 0.17 |
| 17-gastrin (pmol/L) | < 2[1,4] | 30 (16-58) | 86 (70-100) | 2.1 | 0.81 | 0.16 ± 0.16 |
| | ⩽ 7.5[2,4] | 90 (56-98) | 52 (30-74) | 1.89 | 0.19 | 0.42 ± 0.14 |
| | > 13.3[2,3] | 67 (30-92) | 96 (77-99) | 14.7 | 0.35 | 0.62 ± 0.16 |

[1]Suggested by the manufacturer; [2]Determined by ROC curves; [3]To detect corpus-predominant atrophic gastritis; [4]To detect antrum-predominant atrophic gastritis; [5]LR = likelihood ratio.

determinations, we used the likelihood ratio (LR) and Youden's J index[15] (Table 2). Nominal logistic regression was used to perform a multivariate analysis of variables associated to the groups with different estimated risks of GC (Table 3). A $P$ value less than 0.05 was considered statistically significant. The statistical analyses were performed using Epi Info version 3.2 (Epidemiology Program Office, CDC, Atlanta, GA, USA) and SPSS version 14 (SPSS Inc, Chicago, Illinois, USA) computer programs.

## RESULTS

### Diagnostic validation of the serologic methods

Thirty-one patients (21 women, 68%) with an average age of 66.1 (range: 42-90) years were included. Endoscopy was normal in 12 (39%) patients, compatible with gastric atrophy in 8 (26%) patients, showed erosive esophagitis in 2 (6.45%) patients and erosive antropathy in 1 (3.22%) patient. Using histology, serology and rapid urease test, *H pylori* infection was found in 13/31 (42%) patients. The serum level of PG-1 was significantly greater in the *H pylori*-infected patients (136.7 ± 60.8 μg/L) as compared with the non-infected patients (78 ± 68.2 μg/L) ($P < 0.05$). The serum levels of PG-2 and 17-gastrin and the PG-1/PG-2 ratio were similar in the both groups.

### Histological features of the gastric specimens

According to the Sydney classification[13], chronic gastritis was found in 29 (94%) patients, 19 (61%) of them were atrophic. The atrophy was antrum-predominant in 10 (53%) patients, corpus-predominant in 8 (42%) and multifocal in 1 (5%) patient. Glandular atrophy was scored as moderate or severe (score ⩾ 2) in 47% of the cases. The frequency of *H pylori* infection was higher in the patients with chronic non-atrophic or antrum-predominant atrophic gastritis compared to the patients with corpus-predominant atrophic gastritis (60% and 11%, respectively; $P = 0.054$).

### Correlation between histology and serum levels of pepsinogen and gastrin

The results are summarized in Table 1. The serum level of PG-1 was significantly lower in the group with the corpus atrophy, and the PG-1/PG-2 ratio was also lower in this group, although without attaining statistical significance. In comparison with non-atrophic gastritis, the average serum level of 17-gastrin was significantly lower in the patients with the antrum atrophy and significantly higher in those with the corpus atrophy. Table 2 shows the diagnostic performance of the different determinations for the detection of the corpus or antrum gastric atrophy, using the cut-off levels recommended by the manufacturer and those determined through ROC curves. The latter slightly improves sensitivity for the diagnosis of the corpus atrophy, conserving high levels of specificity, which determines LR+ in ranges close to those considered clinically useful (> 10)[16] and a slight improvement in the Youden's J index. The diagnosis of the antrum atrophy through the 17-gastrin level was not reliable. In contrast, the 17-gastrin level over 13.3 pmol/L had a diagnostic performance comparable to PG-1 and the PG-1/PG-2 ratio to diagnose the corpus atrophy.

### Population study

Of the 536 subjects, 209 resided in counties at high risk for GC (mean RR = 1.25) and 327 in counties at low risk for GC (mean RR = 0.8). The frequency of *H pylori* infection was 72.9% (95% CI: 70%-76%). The frequency

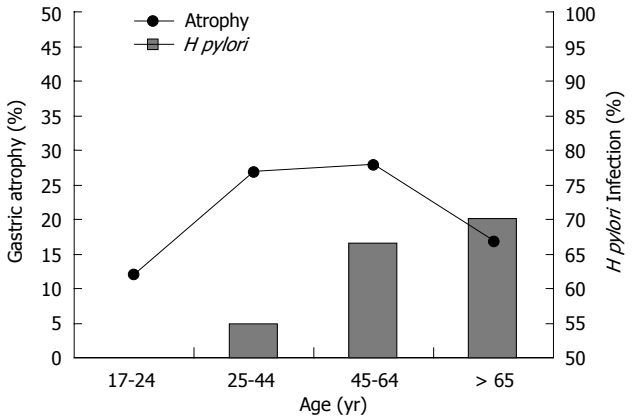


**Figure 1** Frequency of gastric atrophy and *H pylori* infection in 536 asymptomatic subjects by age.

of the corpus atrophy was 26.5% when using serum level of PG-1 ⩽ 61.5 μg/L as a diagnostic criterion, and 12.5% when using the more restrictive combination of PG-1 ⩽ 61.5 μg/L and PG-1/PG-2 ⩽ 2.2[17], which was used for the following analyses.

The frequency of *H pylori* infection was higher in the counties with high RR for GC (76.6%) compared to those with low RR (70.6%), although did not reach a statistical significance. The frequency of gastric atrophy was similar between the both groups (11.5% and 13.1%, respectively). The gastric atrophy was significantly correlated with age (Figure 1). No atrophy was detected in the subjects younger than 25 years, while 20.2% of those older than 65 years had PG-1 level and PG-1/PG-2 ratio compatible with the corpus atrophy, which probably explained the reduction in the serological evidence of *H pylori* infection observed in this group.

Using serological data, we divided the samples into three groups: Group A = no *H pylori* infection and no gastric atrophy (low GC risk); Group B = *H pylori* infection but no gastric atrophy (moderate GC risk); and Group C = serological evidence of gastric atrophy, with or without *H pylori* infection (high GC risk). The demographic characteristics and relevant comparisons among the three groups are shown in Table 3. There were significant differences in the distribution of some variables classically related with GC risk, such as age and educational level. Similar to the samples of the symptomatic patients, *H pylori* infection was associated with a significant elevation of PG-1 (87.4 ± 53.2 in non-infected group *vs* 116.6 ± 51.2 μg/L infected group; $P < 0.05$). Similarly, the corpus atrophy was associated with a significant elevation in the serum level of 17-gastrin (Table 3). The proportion of the subjects residing in counties with a high RR of GC was not significantly different among the three groups, although due to the relatively low frequency of gastric atrophy, dispersion of this parameter was wide (95% CI: 25.3%-43.3% for Group A; 36.1%-46.6% for Group B; and 24.5%-48.5% for Group C). In a multivariate analysis, using Group A as a reference, the only variable associated with *H pylori* infection (Group B) was 17-gastrin (OR: 1.05 (95% CI: 1.005-1.10), $P < 0.05$). For the gastric atrophy, the most significant variable was 17-gastrin (OR: 1.13 (95% CI: 1.07-1.18), $P < 0.001$), and also male (OR: 2.08 (95% CI: 1.07-4.04), $P < 0.05$) and older age [OR: 1.02 (95% CI: 1.004-1.05), $P < 0.05$] were significant risk factors, while educational level was a protective factor [OR: 0.9 (95% CI: 0.84-0.99), $P < 0.05$].

**Table 3 Characteristics of the asymptomatic population samples according to the estimated gastric cancer risk**

| Characteristics | Estimated gastric cancer risk | | | Total ($n$ = 536) |
|---|---|---|---|---|
| | Low (A) ($n$ = 115) | Medium (B) ($n$ = 354) | High (C) ($n$ = 67) | |
| Corpus atrophy[1] | Absent | Absent | Present | |
| *H pylori* infection[2] | Absent | Present | Present or absent | |
| Men, *n* (%) | 46 (40.0) | 168 (47.5) | 35 (52.2) | 249 (46.5) |
| Age (yr)[a], (mean ± SD) | 49.6 ± 22.2 | 48.6 ± 18.0 | 61.9 ± 13.3 | 50.5 ± 19.0 |
| Educational level (yr)[a], (mean ± SD) | 8.8 ± 5.2 | 8.2 ± 4.3 | 5.8 ± 4.2 | 8.0 ± 4.6 |
| Rurality, *n* (%) | 27 (23.5) | 99 (28.0) | 22 (32.8) | 148 (27.6) |
| Living in high-risk counties, *n* (%) | 39 (33.9) | 146 (41.2) | 24 (35.8) | 209 (39.0) |
| 17-Gastrin[a] (pmol/L), (mean ± SD) | 4.1 ± 6.8 | 5.6 ± 7.1 | 14 ± 14.3 | 6.3 ± 8.8 |

[1]As determined by serum level of PG-1 ⩽ 61.5 μg/L and PG-1/PG-2 ratio ⩽ 2.2. [2]Serology (+) for *H pylori* (ELISA). [a]Significant variables in the multivariate analysis ($P < 0.05$): B *vs* A: 17-gastrin (OR 1.05); C *vs* A: sex (OR 2.08); age (OR 1.02); education years (OR 0.9); 17-gastrin (OR 1.13).

## DISCUSSION

In spite of the advances in the knowledge of gastric carcinogenesis, including the role of *H pylori* infection, early diagnosis and opportune time for surgical intervention will continue to be the basis of effective treatment. Digestive symptoms are late and non-specific events[18] such that in order to diagnose early stage tumors, it is necessary to identify high-risk asymptomatic subjects. This work intends to validate a non-invasive method to advance in this direction.

Even though the detection of *H pylori* infection identifies a group that concentrates virtually all the subjects at risk to develop GC in a population[4], the low incidence of GC in the infected and the generally high frequency of infection in countries with high GC frequency, such as Chile and other Andean countries of South America, determine a limited localizing effect. Additionally, the loss in serological evidence of infection detected in the old-aged group in this population, probably as a consequence of gastric atrophy, would imply missing the group with the highest GC risk. Moreover, the eradication of *H pylori* infection does not seem to prevent the development of GC in subjects that have already developed gastric atrophy or intestinal metaplasia[19].

Gastric atrophy is generally accepted the major risk factor for GC development. A prospective study by Uemura *et al*[4] showed that the patients with severe atrophy had 5 times greater RR than those without atrophy. Other Japanese studies have shown that in *H pylori*- infected subjects, gastric atrophy is associated with up to 90 times greater risk for GC[6,20,21].

The serological diagnosis of gastric atrophy by means of serum PG is based on the fact that a small proportion of the diverse isoforms of this digestive enzyme, produced in the mucosa of the upper digestive tract, enters the blood. The isoforms corresponding to PG-1 are synthesized only in the gastric corpus, while PG-2 isoforms are synthesized in the whole gastric mucosa and even in the proximal duodenum. In the presence of corpus atrophy, the serum level of PG-1 diminishes, while PG-2 is stable or diminishes very slightly. However, published studies are relatively heterogeneous. While most studies seem to validate the diagnostic performance of the method[22-27], other studies show a much less reliable performance[28,29] and even suggest the influence of racial factors[30]. A recent meta-analysis suggested that additional studies are required in diverse populations to determine its real value[17].

Our results showed that in this population, low level of serum PG-1 or low PG-1/PG-2 ratio were able to detect the presence of the corpus atrophy with moderate sensitivity (56%-78%) and high specificity (91%-100%). The latter minimizes the risk of false positives and makes the method especially appropriate to be used in a low prevalence setting, such as expected in an asymptomatic population. The diagnostic performance of low levels of 17-gastrin to detect the antrum atrophy was clearly unsatisfactory in these samples, probably due to the antagonistic effect of concurrent corpus atrophy, which determines a significant elevation of the gastrin level. In contrast, an elevated level of 17-gastrin was highly reliable for the diagnosis of corpus atrophy in the group with confirmed histological diagnosis (Table 1) and also showed a significant correlation with the gastric atrophy in the group with serological diagnosis (Table 3), which supports the validity of this finding. A recent multi-ethnic non-European study, including subjects from Japan, China, Tanzania and the Dominican Republic, and using diagnostic criteria very similar to ours, showed wide variation in serum 17-gastrin levels, related to sex, age and the country of origin, and also demonstrated a significant positive correlation between the level of 17-gastrin and the presence of gastric atrophy[31]. If these results are confirmed through correlation with histology, 17-gastrin may be as useful as PG-1 level to diagnose corpus-predominant gastric atrophy in some populations.

The serum level of PG-1 is also affected by antagonistic influences. Our study and other previous studies[32] confirm that *H pylori* infection, and also the use of proton pump inhibitors[33], raise the level of serum PG-1, which could mask the diminishment determined by gastric atrophy and thereby explain the limited sensitivity of the method.

It is pertinent to ask whether the diagnostic performance demonstrated in the initial sample, constituted of selected, symptomatic, old-aged patients with a high frequency of gastric atrophy (60%) and therefore a relatively low frequency of *H pylori* infection, can be extrapolated to an asymptomatic population sample. To dispel this doubt, it would be necessary to certify histologically the frequency of gastric atrophy in a representative sub-sample of the latter group, which we expect to do soon. In the meantime, we restricted the diagnosis of gastric atrophy only to those subjects who simultaneously had PG-1 levels and PG-1/PG-2 ratios below the previously determined cut-off levels, a more demanding and restrictive diagnostic criterion that has been suggested to minimize the risk of false positive[17]. There is no reliable data with respect to the frequency of gastric atrophy in the Chilean population. A recent study from Japan, a country with which we share an elevated risk for GC, using the same methodology, demonstrated the frequencies of *H pylori* infection and gastric atrophy for the age groups very similar to ours[34].

The demographic analysis of the three proposed risk groups, that were determined serologically, showed that they differed significantly in some variables recognized as associated with GC risk, such as age[35], gender and educational level[36], which indirectly supports the validity of this categorization. Our suggestion is that the group with evidence of gastric atrophy should be followed up and studied preferentially through upper GI endoscopy and biopsies. This non-invasive assessment of GC risk has been evaluated in a recent Japanese study[37], showing that the subjects with serologically detected gastric atrophy, either with or without *H pylori* infection, had annual incidence of gastric cancer significantly higher than those subjects without atrophy. As has been recommended[38], focusing invasive and more expensive diagnostic methods on high-risk groups significantly increases the chance of detecting early GC, that in our country comprised less than 10% of the diagnosed cases[2,39] and merely increased to 15% in the only population-based study performed by Llorens[40] between 1978 and 1986 in non-selected volunteers. As long as the precocity of the diagnosis is not modified, it will not be possible to change the disappointing prognosis of GC in Chile, with a 5-year survival rate after diagnosis, ranging between 3% and 12%[2,39]. In contrast, a recent Japanese population screening study, using serum PG and radiology as diagnostic the methods, detected 88% of early GC with a much lower cost than the conventional screening and concluded that this type of program would be very beneficial for high-risk populations[41].

It is known that Chile has one of the highest mortality rates for GC in the world[42]. Nevertheless, the RR is markedly heterogeneous at the regional and county level, ranging between 0.26 and 2.25. Apparently, the greater part of this difference is explained by the higher frequency of *H pylori* infection, mainly in young people, in high-risk counties (Ferreccio *et al* submitted). The eradication of *H pylori* in this stage, before the development of gastric atrophy, would probably reduce the incidence of GC, as has been demonstrated in a Chinese population[19], although it is a strategy of doubtful practical feasibility given the Chilean population's high frequency of infection (73%). The frequency of gastric atrophy, a late consequence of infection, was expected to be greater in the high-risk counties which could not be confirmed in this study, probably due to an insufficient sample size.

In summary, our results confirmed the diagnostic usefulness of the serum levels of pepsinogen and gastrin for the non-invasive detection of corpus-predominant gastric atrophy in the symptomatic patients, showed its

feasibility in an asymptomatic population and suggested that the method could be useful to identify the groups with higher risks of developing GC, in which preventive and control measures can be focused. It is probable that successive PG and/or 17-gastrin determinations (annually or biannually), confirming low levels or showing a decreasing tendency, would lead to even better diagnostic performance. The incorporation of this method to preventive health examination in a selected population (i.e., men, older than 50, residents in high-risk counties) could probably increase the proportion of GC diagnosed in the early or incipient phase, the only method to improve the prognosis of this serious and frequent disease.

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*RAPID COMMUNICATION*

# Ultrasound guided percutaneous cholecystostomy in high-risk patients for surgical intervention

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## Abstract

**AIM:** To assess the efficacy and safety of ultrasound guided percutaneous cholecystostomy (PC) in the treatment of acute cholecystitis in a well-defined high risk patients under general anesthesia.

**METHODS:** The data of 27 consecutive patients who underwent percutaneous transhepatic cholecystostomy for the management of acute cholecystitis from January 1999 to June 2003 was retrospectively evaluated. All of the patients had both clinical and sonographic signs of acute cholecystitis and had comorbid diseases.

**RESULTS:** Ultrasound revealed gallbladder stones in 25 patients and acalculous cholecystitis in two patients. Cholecystostomy catheters were removed 14-32 d (mean 23 d) after the procedure in cases where complete regression of all symptoms was achieved. There were statistically significant reductions in leukocytosis, ($13.7 \times 10^3 \pm 1.3 \times 10^3$ μg/L *vs* $13 \times 10^3 \pm 1 \times 10^3$ μg/L, $P < 0.05$ for 24 h after PC; $13.7 \times 10^3 \pm 1.3 \times 10^3$ μg/L *vs* $8.3 \times 10^3 \pm 1.2 \times 10^3$ μg/L, $P < 0.0001$ for 72 h after PC), C -reactive protein (51.2 ± 18.5 mg/L *vs* 27.3 ± 10.4 mg/L, $P < 0.05$ for 24 h after PC; 51.2 ± 18.5 mg/L *vs* 5.4 ± 1.5 mg/L, $P < 0.0001$ for 72 h after PC), and fever (38 ± 0.35℃ *vs* 37.3 ± 0.32℃, P < 0.05 for 24 h after PC; 38 ± 0.35℃ vs 36.9 ± 0.15℃, $P < 0.0001$ for 72 h after PC). Sphincterotomy and stone extraction was performed successfully with endoscopic retrograde cholangio-pancreatography (ERCP) in three patients. After cholecystostomy, 5 (18%) patients underwent delayed cholecystectomy without any complications. Three out of 22 patients were admitted with recurrent acute cholecystitis during the follow-up and recovered with medical treatment. Catheter dislodgement occurred in three patients spontaneously, and two of them were managed by reinsertion of the catheter.

**CONCLUSION:** As an alternative to surgery, percutaneous cholecystostomy seems to be a safe method in critically ill patients with acute cholecystitis and can be performed with low mortality and morbidity. Delayed cholecystectomy and ERCP, if needed, can be performed after the acute period has been resolved by percutaneous cholecystostomy.



**Key words**: Percutaneous cholecystostomy; Acute cholecystitis; Ultrasound; High risk; Elderly

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## INTRODUCTION

Cholecystectomy is the appropriate treatment of acute calculous and acalculous cholecystitis, and it has a mortality rate of 0%-0.8%[1,2]. However, the mortality rate of surgical treatment may be as high as 14%-30% in elderly or critically ill patients with comorbid diseases[3,4].

Percutaneous cholecystostomy (PC) has been introduced as an alternative method to treat acute cholecystitis in patients with significant comorbid diseases[5-8]. PC can be achieved with the guidance of either computed tomography (CT) or ultrasonography (USG). PC, which was initially described by Radder in 1980, has proved to be an effective treatment for acute cholecystitis in critically ill patients, and it has low morbidity and mortality rates[9].

In this study, we have retrospectively evaluated the clinical data of patients treated with PC for acute cholecystitis. The aims of the study were to investigate the efficiency of PC in the treatment of acute cholecystitis in critically ill patients, the ratio of delayed cholecystectomy, and the clinical outcome of patients who had no further interventions following PC.

## MATERIALS AND METHODS

Between January 1999 and June 2003, percutaneous transhepatic cholecystostomy was performed on 27 elderly patients with acute cholecystitis and comorbid diseases. All patients received physical examination, whole blood count, biochemical analysis, and ultrasonography. Age, gender, comorbid diseases, etiology of cholecystitis, clinical and laboratory findings, morbidity associated with PC, and the clinical outcome were recorded. The diagnostic criteria of acute cholecystitis included clinical signs and symptoms (right upper quadrant pain or tenderness), leukocytosis or positive C - reactive protein (CRP) (> 5 mg/L) value, and at least one of the ultrasonographic criteria including gallbladder stones (for calculous cholecystitis), ultrasonographic Murphy's sign, gallbladder wall thickening (> 3 mm), pericholecystic fluid, and gallbladder distension. All patients were given empiric intravenous Ampicillin-sulbactam (1 g/d four times) treatment starting immediately after the diagnosis, and subsequently antibiotic therapy was managed according to the culture results of bile samples. All of the procedures were performed in the Department of Radiology. None of the patients were premedicated and the procedure was performed under local anesthesia.

### Risk classification

The American Society of Anesthesiologists (ASA) physical status classification was used to define the risk for cholecystectomy. Twenty (74.1%) patients were classified as ASA Ⅲ and 7 (25.9%) as ASA Ⅳ.

### Procedure

Under aseptic conditions, a USG guided transhepatic approach through the right lobe was used to access the gallbladder in all patients. After puncture of the bile duct with an 18 GA Seldinger needle, a guide-wire (Amplatz, 0.035 inch, 75 mm) was inserted into the gallbladder via the lumen of the Seldinger needle. The route was then dilated using 8F and 10F dilatators. Finally a 10F locking pigtail catheter was inserted into and locked within the gallbladder. Catheter localization was controlled by fluoroscopy (by injecting a small amount of contrast) and USG. Bile samples were obtained for cultures. The catheter was flushed with 10 mL of a saline-gentamycin (40 mg) mixture every 6-8 h to avoid obstruction, and the catheter was left for gravity drainage.

On the second day of the PC, USG was performed to check the site of the catheter, look for bile or blood leakage, and to observe the ultrasonographic findings following PC. Positive clinical response was defined as the normalization of at least two of the three clinical parameters of acute cholecystitis (abdominal pain, fever, and leukocytosis) and a decrease in CRP value within 72 h.

### Statistical analysis

Demographic data are given as medians (range), whereas the other data are expressed as means ± SE. Differences were evaluated using the Wilcoxon signed ranks test. $P < 0.05$ was accepted as significant. Data were analyzed using statistical software (SPSS for windows 10.0; SPSS, Chicago, IL, USA).

**Table 1 Comorbid diseases of the patients**

| Comorbid diseases | *n* |
|---|---|
| Cardiac disorders[1] | 19 |
| Haematological diseases[2] | 2 |
| Chronic obstructive pulmonary failure | 1 |
| Polytrauma | 1 |
| Diabetes mellitus and chronic renal failure | 4 |
| Total | 27 |

[1]Congestive heart failure, hypertension, ischemic heart diseases, valve replacement; [2]Chronic myeloid leukemia, thrombotic thrombocytopenic purpura.

**Table 2 White blood cell (WBC), C-reactive protein (CRP), and axillary temperature of the patients before and after PC**

| | Before PC | 24 h after PC | 72 h after PC |
|---|---|---|---|
| WBC × $10^3/\mu L$ | 13.7 ± 1.3 | 13 ± 1 | 8.3 ± 1.2 |
| CRP mg/L | 51.2 ± 18.5 | 27.3 ± 10.4 | 5.4 ± 1.5 |
| Body temp. (℃) | 38 ± 0.35 | 37.3 ± 0.32 | 36.9 ± 0.15 |
| | | $P < 0.05$ | $P < 0.0001$ |

## RESULTS

The median age of the patients was 71.4 years (range 64-93 years) and the female/male ratio was 4.4/1 (22/5). Two (7.4%) patients had acalculous and 25 (92.5%) had calculous cholecystitis. Comorbid diseases included congestive heart failure or severe ischemic heart disease in 19 (70.3%) patients, haematologic diseases [chronic myeloid leukemia (CML) and thrombotic thrombocytopenic purpura (TTP)] in 2 (7.4%) patients, renal failure in 4 (14.8%), chronic obstructive respiratory failure in one (3.7%), and polytrauma in one (3.7%) patient (Table 1). The majority (96.3%, $n = 26/27$) of the patients had pain and tenderness in the right upper quadrant.

The mean white blood cell counts (WBC) upon admission ($13.7 \times 10^3 \pm 1.3 \times 10^3/\mu L$), axillary temperatures (38 ± 0.35℃), and CRP (51.2 ± 18.5 mg/L) values were significantly decreased in the 72 h following PC [$8.3 \times 10^3 \pm 1.2 \times 10^3/\mu L$ ($P < 0.0001$), 36.9 ± 0.15℃ ($P < 0.0001$), 5.4 ± 1.5 mg/L ($P < 0.0001$), respectively ] (Table 2).

The clinical and ultrasonographic findings of acute cholecystitis decreased in all patients. Bile cultures were negative in 5 (18.5%) patients. *E.coli* was the only bacteria in 17 (63%) patients and *E.coli, Enterobacter,* and *Enterococci* were identified in the remaining 5 (18.5%) patients.

The median hospitalization time was 8 d (1-20 d) and the median catheterization period was 23 d (14-32 d). Catheter dislodgement occurred in three patients on the $6^{th}$, $8^{th}$, and $14^{th}$ d. Catheters were reinserted in two patients due to prompt recurrent cholecystitis but the other patient did not need recatheterization. Unexpected bleeding from the liver parenchyma occurred in only one (3.7%) patient following catheter removal. USG guided percutaneous drainage of a hematoma in the subhepatic region was performed in this patient and no further complications occurred.

Cholecystocholangiography was performed prior to the removal of the catheter to visualize the bile tree and gallbladder in 24 (88.8%) patients. This procedure could not be performed in 2 patients due to noncompliance and difficult mobilization (polytrauma) in one patient with acalculous cholecystitis. The cystic duct was patent in 16 (66.7%) and occluded in 8 (33.3%) patients. Common bile duct stones were detected in 3 (11.1%) patients.

These 3 patients underwent sphincterotomy and stone extraction from the common bile duct with ERCP. Delayed cholecystectomy was performed in 5 (18.5%) patients within a mean period of 2 mo following PC. The postoperative outcome was uneventful in these patients. The remaining 22 patients were followed up by physical examination and USG monthly for a median of 11 (6-18) mo after the removal of the catheter. Three of these 22 patients experienced recurrent cholecystitis and recovered with medical therapy (Table 3).

The morbidity rate of PC was 25.9% [catheter dislodgement ($n$ = 3), recurrent cholecystitis ($n$ = 3), and bleeding ($n$ = 1)] and there was no mortality associated with PC.

**Table 3 Follow-up of the patients after PC**

| | Calculous cholecystitis ($n$ = 25) | Acalculous cholecystitis ($n$ = 2) |
|---|---|---|
| No further treatment | 18 (72%) | 2 |
| Recurrence | 3 (12%) | 0 |
| Reinsertion of the catheter | 2 (8%) | 0 |
| Medical treatment | 1 (4%) | 0 |
| ERCP | 3 (12%) | 0 |
| Delayed surgery | 5 (20%) | 0 |

## DISCUSSION

PC was defined as an alternative treatment method in patients with acute cholecystitis who were at high risk during surgery due to comorbid diseases. The morbidity and mortality associated with emergent cholecystectomy is considerably higher in such patients, 55%-66% and 14%-30% respectively[3,4]. The morbidity and mortality rates of PC were found to be much lower, less than 10% and 2% respectively[10,11]. PC resulted in successful treatment in 56%-100% of patients with acute cholecystitis and significant comorbid diseases[12-17]. The therapeutic rate of PC was significantly higher in patients with clinical signs and symptoms referred to the right upper abdomen compared to patients without clinical signs and symptoms (intensive care patients)[12]. Although unexplained sepsis regressed dramatically after PC in approximately 60% of intensive care patients, the therapeutic response to PC is lower in patients with symptoms and signs in the right upper abdomen who received open cholecystectomy[11,12]. In this study, morbidities were seen in 7 (25.9%) patients, but 3 of these complications were due to catheter squeezing as a result of patient carelessness. There was no procedure-related mortality.

PC can be easily performed under local anesthesia with USG or CT guidance. Two ways of accessing the gall bladder were defined for the procedure. Either a transhepatic or transperitoneal approach can be used. The complications related to the procedure include bile duct injury, bile leakage and peritonitis, portal or parenchymal vessel injury and bleeding, catheter dislodgement, colon injury, and vagal reactions. The transhepatic approach decreases the risk of bile leak, portal vessel injury, and colon injury but it carries a risk of pneumothorax and bleeding from the liver parenchyma[18-20]. Although the transperitoneal approach decreases the risk of bleeding and secondary liver contamination by infected bile, it increases the risk of bile peritonitis, colon perforation, portal vessel injury, and displacement of the catheter after decompression of the gall bladder[21-23]. In addition to other complications, van Sonnenberg *et al* also reported vagal reactions in the transperitoneal approach due to vagal innervation of the gall bladder wall[24]. Therefore, the transhepatic approach seems to be the best approach for PC except in the presence of severe liver disease and coagulopathy[25-27]. In this study the transhepatic approach was preferred to access the gall bladder. All procedures were completed under local anesthesia without any complications related to the procedure.

We performed cholecystocholangiography in 24 patients. The cystic duct was patent in 16 (66.7%) patients. Common bile duct stones which could not be visualized by USG, were detected in three (11.1%) of these 16 patients. These three patients underwent sphincterotomy and stone extraction with ERCP. Bleeding from the liver parenchyma was detected unexpectedly in only one (3.7%) patient following removal of the catheter. No bile leakage was detected in any patient.

The management of patients after the acute cholecystitis period is still controversial. Lebigot *et al* followed up 90% of patients for 12 mo after PC and reported only one (6.25%) endoscopic sphincterotomy and one (6.25%) delayed cholecystectomy[28]. No additional therapy was needed in the remaining 87.5% of the patients. The risk of recurrent cholecystitis was reported as 12% at 1 year in a study by Welch *et al*, and the authors mentioned PC as a possible definitive treatment in patients with acalculous cholecystitis[29]. In a recent study, the authors were able to apply delayed surgery in 56.4% of patients after the acute period[6]. In this study, we reevaluated the patients after regular medical treatment of the comorbid diseases. Five (18.5%) patients were then selected for delayed elective surgery and the remaining 22 were followed. Nineteen (86.4%) of these 22 patients required no additional therapy. Recurrent cholecystitis was detected in 3 (13.6%) patients and the symptoms regressed with medical therapy.

In conclusion, PC is a fast, easy, effective, and safe treatment method for the acute phase of cholecystitis in elderly and critically ill patients. Procedure-related morbidity and mortality is very low compared to surgery. Patient management after the acute phase of cholecystitis is still controversial. Conservative treatment for patients who are not suitable for surgery is acceptable. Delayed elective cholecystectomy is another option in patients who respond well to medical treatment of comorbid diseases.

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*RAPID COMMUNICATION*

# Efficacy of leukocyte esterase dipstick test as a rapid test in diagnosis of spontaneous bacterial peritonitis

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## Abstract

**AIM:** To evaluate the efficacy of dipstick test in diagnosis of spontaneous bacterial peritonitis (SBP) in cirrhotic patients who underwent abdominal paracentesis based on the locally available dipstick test.

**METHODS:** There were 200 consecutive samples from cirrhotic patients who underwent abdominal paracentesis. Urine dipstick (Combur$^{10}$ Test®M, Roche, Mannheim, Germany) was used as a screening test. A manual cell count with differential study was done in all samples by experienced technicians. The polymorphonuclear (PMN) cell count more than 250 cells/mm$^3$ was used as a diagnostic cut off level. One to three plus dipstick results were used as cut off levels for a positive result. The dipstick test results had to be agreed by three experienced readers. The sensitivity, specificity, positive and negative predictive values and accuracy of two different colorimetric cut off scales (1+ and 2+) were calculated and compared.

**RESULTS:** The prevalence of SBP diagnosed by manual cell count was 21.0%. There were 128 specimens that had a true negative result by dipstick. The sensitivity, specificity, positive and negative predictive values and accuracy of 1+ and 2+ cut off scale to diagnose SBP were 88%, 81%, 55%, 96% and 83% respectively, and 63%, 96%, 82%, 81% and 89% respectively.

**CONCLUSION:** Dipstick test can be used as a rapid test for screening of SBP. The higher cut off colorimetric scale has a better specificity and positive predictive value but a lower sensitivity.



**Key words:** Spontaneous bacterial peritonitis; Diagnosis; Dipstick; Screening

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## INTRODUCTION

Apart from variceal bleeding, spontaneous bacterial peritonitis (SBP) is another serious complication that can develop in cirrhotic patients. Prompt diagnosis and treatment are essential for the survival of patients with SBP[1,2]. Unfortunately, symptoms of SBP including fever, abdominal pain, nausea, and vomiting are not presented in all cirrhotic patients who develop SBP[3,4]. In addition, many hospitalized cirrhotic patients develop SBP during their non-SBP related admissions especially those with gastrointestinal bleeding[5]. Therefore, routine diagnostic paracentesis is the recommended practice for patients with ascites who develop signs or risk factors for SBP. The standard criteria for diagnosis of SBP are an ascitic fluid polymorphoneuclear (PMN) cell count of $\geqslant$ 250/mm$^3$ and/or a positive ascitic fluid bacterial culture[6-8]. Due to the nature of bacterial culture, the result is not available within a day. Therefore, decision making for SBP treatment is mainly based on PMN cell count. However, manual ascitic fluid PMN cell count is not always available in some small patient care units especially in the out patient offices and sometimes it cannot be done as in emergency basis.

Recently, leukocyte esterase activity testing by dipstick has been used for a rapid diagnosis of infection in many body fluids such as urine, pleural fluid, and cerebrospinal fluid[9-11]. The leukocyte esterase released from PMN cells reacts with an esterified chemical compound in the reagent strip yielding a violet azo dye, the intensity of which correlates to leukocyte count[12]. Recently, many studies have shown the efficacy of dipstick in diagnosing SBP[13-19]. However, strip tests used in these studies are not the same and have different colorimetric scales. Therefore, the sensitivity and accuracy of different dipsticks may vary. To date, there has been no recommended calorimetric scale for each dipstick to diagnose SBP.

The aim of this study was to evaluate the usefulness of dipstick in rapid diagnosis of SBP in cirrhotic patients who underwent abdominal paracentesis based on the locally available dipstick test and to define the validity scores from 2 different thresholds of colorimetric scales.

## MATERIALS AND METHODS

During the period between July 2004 and November 2005 at King Chulalongkorn Memorial Hospital, 200 consecutive ascitic fluid specimens (including 21 from out patient unit) were collected from 127 cirrhotic patients (Table 1). The clinical indications for paracentesis were routine paracentesis ($n$ = 95), relief of patient's discomfort ($n$ = 54), suspected SBP ($n$ = 13), miscellaneous ($n$ = 38). The diagnosis of cirrhosis was established according to the histologic criteria or analytical, clinical, and ultrasonographic findings of the liver. Informed consent was obtained from all patients, and the study was approved by the Ethics Committee of the University.

Immediately after paracentesis, fresh ascitic fluid specimen was collected in a clean dry container and tested using a dipstick (Combur$^{10}$ Test®M, Roche, Mannheim, Germany) for granulocyte esterase designed for urine analysis. The strip has a colorimetric 4-grade scale (negative, 1+ to 3+) and is read at 90 s for granulocyte esterase activity. The procedure was the same as what the manufacturer described for urine.

Each of the colorimetric scale readings was graded by 2 well trained on-duty internists (the reading processes were scored separately). All the readings were confirmed by WR who is a house staff covering for this project. WR made the final decision for all discrepancy results. A correlation between PMN cell count and the 4-grade scale was suggested by the manufacturer as follows: negative, 0 PMN cell/mm$^3$; 1+, 10-25 PMN cells/mm$^3$; 2+, 75 PMN cells/mm$^3$; 3+, 500 PMN cells/mm$^3$.

Ascitic fluid was conventionally processed including cytology, PMN cell count and lymphocyte count, and appropriate biochemical tests (glucose, protein, albumin, lactic dehydrogenase, and sugar). The sample for PMN and total leukocyte count was collected into a heparin anticoagulant tube and analyzed within 4 h of extraction. Differential cell count and cytology were examined with a conventional optical microscope. A manual cell count with differential study was done in all samples by experienced technicians.

Ascitic fluid cultures were performed by inoculation of at least 10 mL fluid in VersaTrek® REDOX 1® (TREK Diagnostic Systems, Cleveland, OH). All bottles were processed in a standard fashion according to the manufacturer's guideline.

SBP was defined as PMN cell count in ascitic fluid $\geqslant$ 250/mm$^3$. The 1+ or 2+ result of the leukocyte esterase from dipstick was considered as a positive test. The negative result from dipstick was considered as a negative test. We also defined bacterascites as the combination of a positive ascitic fluid culture, PMN cell count in ascitic fluid < 250/mm$^3$, and no evident intra-abdominal surgically treatable source of infection. In patients with hemorrhagic ascites (i.e. ascites red blood cell count > 10000/mm$^3$), a subtraction of one PMN leukocyte per 250 red blood cells was made[6]. Secondary peritonitis was suspected when there was an abdominal source of infection, acute pancreatitis, or more than one organism in the ascitic fluid culture.

**Table 1 Characteristics of 127 patients and 200 ascitic fluid specimens**

| Characteristics | $n$ (%) |
|---|---|
| Male/Female | 75/52 |
| Age (mean + SD) | 57.4 ± 13.3 |
| Child-Pugh classification A/B/C | 3/34/90 |
| **Etiology of cirrhosis** | |
| Hepatitis B | 34 (26.8) |
| Hepatitis C | 8 (6.3) |
| Alcohol | 25 (19.7) |
| Alcohol and hepatitis B | 9 (7.1) |
| Alcohol and hepatitis C | 9 (7.1) |
| Hepatitis B and hepatitis C | 1 (0.8) |
| Autoimmune | 2 (1.6) |
| Hemochromatosis | 2 (1.6) |
| Cryptogenic | 10 (7.9) |
| Others | 27 (21.3) |

### *Statistical analyses*

Results of dipstick test were compared to PMN cell count, ascitic fluid culture and clinical data in all patients. The sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) and accuracy of dipstick in the diagnosis of SBP from two different colorimetric scales (1+ and 2+) were calculated and compared (Figure 1).

## RESULTS

We diagnosed 42 episodes of SBP by PMN cell count, of which 37 (88.1%) was diagnosed using 1+ cut off scale and 27 (64.3%) 2+ cut off scale. Of the 158 specimens with PMN cell count < 250/mm$^3$, 128 had a true negative result by dipstick test (Table 2). Five patients were diagnosed as SBP by manual cell count but had a negative dipstick test result.

Sixteen specimens had a positive culture for bacteria, of which four were compatible with bacterascites. The dipstick test results in these bacterascites specimens were all negative. No secondary bacterial peritonitis was diagnosed in this series. The most common organism growing from these specimens was *E. coli* (Table 3).

The sensitivity, specificity, PPV, NPV and accuracy of 1+ and 2+ cut off scales to diagnose SBP were 88%, 81%, 55%, 96% and 83% respectively, and 63%, 96%, 82%, 91% and 89% respectively (Table 4).

## DISCUSSION

Due to the high rates of morbidity and mortality from delayed treatment in cirrhotic patients who suffered from SBP, diagnostic tests for early diagnosis are very important[20,21]. Currently, ascitic fluid PMN count is the standard tool for SBP treatment decision making. However, several-hour delay in reporting the result may arise from the laboratory department. In addition, this test may not be available in a small out patient setting.

Leukocyte esterase has been shown as an important marker for PMN cell activity[22]. Originally, the purpose

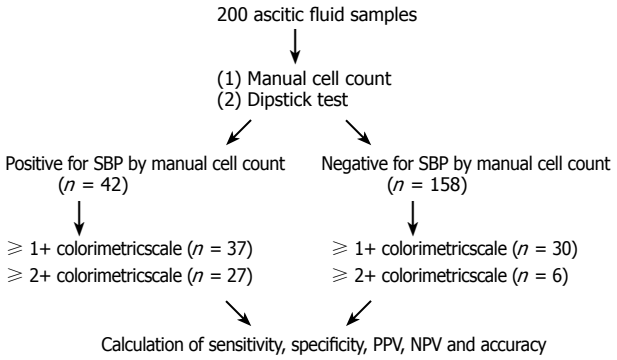


**Figure 1** Flow diagram for SBP evaluation.

**Table 2 Dipstick test results in SBP and non-SBP groups**

| Colorimetric scales | PMN cell count ⩾ 250/mm$^3$ | PMN cell count < 250/mm$^3$ | Total |
|---|---|---|---|
| 1+ | 10 | 24 | 34 |
| 2+ | 19 | 6 | 25 |
| 3+ | 8 | 0 | 8 |
| 0 | 5 | 128 | 133 |
| Total | 42 | 158 | 200 |

of leukocyte esterase test was limited to urine analysis[23]. Recently, it has been found to be useful across the wide range of body fluid infections[9-11]. The efficacy of this test in detecting infection in the ascitic fluid has been confirmed from many centers[14-19]. The overall sensitivity, specificity, PPV and NPV of this test are approaching 90% (Table 5)[14-19]. However, the different commercial dipsticks used in previous studies had different colorimetric scales for PMN cell count. In addition, the cut off colorimetric scale for each dipstick has not been standardized yet.

To date, there has been no dipstick that was specifically designed to use in ascitic fluid. Moreover, many dipsticks do not have a precise colorimetric scale for 250 PMN cells/mm$^3$. In our study, we used the Combur$^{10}$ Test®M that is widely available in Thailand as a screening test for SBP. According to the manufacturer's guideline, the 1+, 2+ and 3+ scales are equivalent to 10-25 PMN cells/mm$^3$, 75 PMN cells/mm$^3$ and ⩾ 500 PMN cells/mm$^3$ respectively. We chose the lower scales (1+ and 2+) as cut off levels for SBP diagnosis. The limitation of the Combur$^{10}$ Test®M is an absence of precise calorimetric scale for the cut off level of PMN cell count at ⩾ 250/mm$^3$. Because of this limitation, we speculated that our specificity might be lower than others[17-19]. Although our sensitivity (88%) from 1+ cut off level was comparable to others, the specificity and PPV were lower (81% and 55% respectively) compared to other studies[17-19]. By using 2+ scale as a cut off level for SBP, our specificity and PPV increased (96% and 82% respectively), unfortunately the sensitivity decreased to only 63%. Anyhow, NPV and accuracy from these two cut off levels were comparable to other series[17-19].

Aution stick (A. Menarini Diagnostics, Firenze, Italy) is another dipstick that can be read at 90 s. The benefit of the Aution stick over the Combur$^{10}$ Test®M is the precise colorimetric scale that correlates with ⩾ 250 PMN cells/mm$^3$. The correlation between PMN cell count and colorimetric scale suggested by the manufacturer are as follow: grade 0, 0 PMN cell/ mm$^3$; grade 1, 25 PMN cells/ mm$^3$; grade 2, 75 PMN cells/ mm$^3$; grade 3, 250 PMN cells/mm$^3$; and grade 4, 500 PMN cells/mm$^3$. Catellote *et al*[15] studied the use of Aution sticks for diagnosis of SBP in cirrhotic patients with ascites who underwent abdominal paracentesis at a university based hospital, and found that the sensitivity, specificity and PPV are 89%, 99% and 98%, respectively[15].

**Table 3 Results of dipstick tests and PMN cell counts in positive culture specimens**

| Patient | PMN cells/mL | Calorimetric scale | Culture result |
|---|---|---|---|
| 1 | 14 | 0 | *A.Baumanii* |
| 2 | 20 | 0 | *S.agalactae* |
| 3 | 21 | 0 | *P.aeruginosa* |
| 4 | 100 | 0 | *K.pneumonia* |
| 5 | 254 | 0 | *E. coli* |
| 6 | 271 | 0 | *S.bovis* |
| 7 | 257 | 0 | *K.pneumoniae* |
| 8 | 672 | 1 | *E. coli* |
| 9 | 3640 | 1 | *P.aeruginosa* |
| 10 | 13312 | 1 | *E. coli* |
| 11 | 2129 | 2 | *Streptococcus gr.D* |
| 12 | 3494 | 2 | *Streptococcus gr.D* |
| 13 | 12840 | 2 | *P.aeruginosa* |
| 14 | 25805 | 2 | *E. coli* |
| 15 | 41990 | 2 | *E. coli* |
| 16 | 3398 | 3 | *E. coli* |

**Table 4 Validity scores of the Combur$^{10}$ Test®M to diagnose SBP considering a positive test > 1 or > 2 in the 4-grade colorimetric scale**

| Variables | ⩾ 1 grade, % | ⩾ 2 grade, % |
|---|---|---|
| Sensitivity | 88 | 63 |
| Specificity | 81 | 96 |
| Positive predictive value | 55 | 82 |
| Negative predictive value | 96 | 91 |
| Accuracy | 83 | 89 |

Multistix®10SG (Bayer Diagnostics Corporation, Puteaux, France) has colorimetric scales as follow: grade 0, 0 PMN cell/ mm$^3$; grade 1, 15 PMN cells/mm$^3$; grade 2, 70 PMN cells/ mm$^3$; grade 3, 125 PMN cells/ mm$^3$; and grade 4, 500 PMN cells/mm$^3$. Butani *et al*[24] used the Multistix®10SG to diagnose SBP in 136 specimens by using grade 2 as a cut off scale, and found the sensitivity, specificity, PPV and NPV of the Multistix®10SG are 83%, 99%, 91% and 98% respectively. Although, the Multistix®10SG has no precise colorimetric scale for 250 PMN cells/ mm$^3$, its specificity was still better than that of the Combur$^{10}$ Test®M in our series. This may be due to a closer 250 PMN/mm$^3$ colorimetric scale of Multistix® 10SG (grade 3+ ⩾ 125 PMN cells/mm$^3$).

There are many factors that influence the accuracy of the dipsticks. False positive result can result from alteration of pH, osmolality and temperature of the specimens[25].

**Table 5 Validity scores reported in previous studies**

| Authors | Castellote *et al* | Kim *et al* | Butani *et al* | Sapey *et al* | Thévenot *et al* |
|---|---|---|---|---|---|
| Dipsticks | Aution sticks | UriSCAN/Mutistix10SG | Multistix®10SG | Nephur test/ Multistix10SG | Multistix 8SG/Combur²test LN (combined result) |
| Inclusion scale | $\geqslant$ 3 (PMN $\geqslant$ 250/mm$^3$) | $\geqslant$ 3 (PMN $\geqslant$ 500/mm$^3$) /$\geqslant$ 3 (PMN $\geqslant$ 75/mm$^3$) | $\geqslant$ 2 (PMN $\geqslant$ 70/mL mm$^3$) | N/A | $\geqslant$ 3 (PMN $\geqslant$ 125/mm$^3$) /$\geqslant$ 2 (PMN $\geqslant$ 75/mm$^3$) |
| Sensitivity (%) | 89 | 67/50 | 89 | 88/65 | 89 |
| Specificity( %) | 99 | 100/100 | 99 | 100/100 | 100 |
| PPV (%) | 98 | 100/100 | 89 | 94/92 | 100 |
| NPV (%) | 97 | 89/87 | 99 | 99/97 | 99 |

In addition, non-leukocyte cells can release esterase and produce a false positive result[26]. Moreover, antibiotics can produce both false positive and negative results[27]. The false negative result in the present series was found in 5 patients (12%), which is similar to the series presented by Sapey *et al*[19] None of our patients with false negative result received antibiotics prior to abdominal paracentesis. Interestingly, 3 specimens had a manual PMN cell count between 250/mm$^3$ and 300/mm$^3$ (data not presented), suggesting that the smaller number of PMN cells in these specimens may lead to a false negative result. Campillo *et al*[28] demonstrated that the sensitivity of dipsticks remains low with PMN cell count $\leqslant$ 1000/mm$^3$ when they used Multistix 8 SG and Combur 2 LN as dipstick tests.The false positive rate in our series was 19% which is higher than that of Sapey *et al*[19], who found that only one patient form each test (Nephur-test® and MutistixSG®) had a false positive result. The possible explanation for a better result from their series is a much closer 250 PMN cells/mm$^3$ of their dipstick scales. Again this information helps to confirm that a precise colorimetric scale for 250 PMN/mm$^3$ is essential for an ideal dipstick to detect SBP.

Currently, there is no specific dipstick test for bacterial count in the ascitic fluid. In our series, it is not surprised that the correlation between the culture and dipstick results was very poor. There were 4 specimens that were compatible with a condition generally called "monomicrobial non-neutrocytic bacterascites (MNB)". All the MNB specimens had a negative dipstick result. Generally, the clinical outcome of asymptomatic MNB is much better than that of classic SBP[29]. Our 4 patients with MNB did not receive any treatment and were asymptomatic during 3-6 mo follow-up. In addition, repeat ascitic fluid analysis and culture were performed and reported unremarkable results in these 3 patients.

Patients with $\geqslant$ 250 PMN cells/mm$^3$ in the absence of bacterial growth from the culture are classified as culture-negative neutrocytic ascites (CNNA). In the present series, only 12 patients (28.6%) had a classic SBP (positive culture and cell count $\geqslant$ 250 PMN cells/mm$^3$). Of these culture negative SBP patients, at least 25 (83.3%) had CNNA (no history of prior antibiotics use), demonstrating that CNNA has the same clinical course as classic SBP[6]. Therefore, dipstick that determines mainly for PMN cell activity can be used for SBP screening.

In conclusion, dipstick test in ascitic fluid from cirrhotic patients is a good screening tool for SBP. However, not all dipsticks have a precise colorimetric scale for a measurement of $\geqslant$ 250 PMN cells/mm$^3$, therefore the sensitivity of different dipsticks may not be similar. With a good NPV the decision making not to treat SBP based on the results of Combur$^{10}$ Test®M can be made in the majority of cases. Further study is needed to improve the efficacy of the test in detecting PMN cells and bacteria in ascitic fluid.

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## COMMENTS

### Background

The decision making for SBP treatment is mainly based on PMN count. However, manual ascitic fluid PMN count is not always available in every patient care unit.

### Research frontiers

Leukocyte esterase activity testing by dipstick has been used for a rapid diagnosis of infection in a variety of body fluids such as urine, pleural fluid, and cerebrospinal fluid. The efficacy of this test in detecting infection in the ascitic fluid has been confirmed from many centers. The overall sensitivity, specificity, PPV and NPV of these tests are approaching 90%.

### Innovations and breakthroughs

The sensitivity and NPV of dipstick test is excellent in all different types of stick. However, some have a slightly better value due to their approximity to the 250 cell/mm$^3$ cut-off level.

### Applications

The dipstick may be used as a screening tool for SBP. A better designed test is awaited.

### Peer review

The study used Combur as a screening test. The sensitivity of this test is slightly poorer than others. However, the other validity scores are comparable. The author may need to continue to explore other dipsticks and compare their results using manual cell count as a gold standard.

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*RAPID COMMUNICATION*

# Expression of altered retinoblastoma protein inversely correlates with tumor invasion in gastric carcinoma

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## Abstract

**AIM:** To investigate the clinical and pathological significance of altered retinoblastoma (Rb) encoding protein (pRb) in gastric carcinoma.

**METHODS:** Expression of altered pRb was analyzed in 91 patients with gastric adenocarcinoma by immunohistochemistry.

**RESULTS:** Sixty-five percent (59/91) of the tumors were positively stained and the staining in tumor nuclei of gastric carcinoma ranged 0%-90%. Moreover, strong expression of altered pRb was found in 35% (6/17), 24% (5/21), 17% (8/46) and 0% (0/7) of T1, T2, T3 and T4 gastric carcinomas, respectively. Altered pRb expression was inversely correlated with the depth of tumor invasion ($P$ = 0.047). Degree of immunoreactivity had no significant correlation with tumor grade, node metastasis and distant metastasis. In terms of prognostic significance, univariate analysis showed that poor differentiation [41 (66.1%) *vs* 34 (42.5%) $P$ = 0.051], advanced tumor stage ($P$ < 0.001) and weakly altered pRb expression [17 (80.5%) *vs* 58 (49.6%) $P$ = 0.044] were associated with worse prognosis in these patients. However, multivariate analysis revealed that advanced tumor stage was the only independent poor prognostic factor ($P$ < 0.001).

**CONCLUSION:** The mutation of Rb gene is frequent in gastric carcinoma. The expression of altered pRb inversely correlates with tumor invasion and is not an independent prognostic marker in gastric adenocarcinoma.



**Key words:** Gastric adenocarcinoma; Altered retinoblastoma protein; Immunohistochemistry

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## INTRODUCTION

Cellular proliferation normally follows an orderly progression through the cell cycle, which is governed by protein complexes encoding various genes. Malignant transformation results from a series of genetic alterations that lead to aberrant regulation of cell division (cell-cycle control), cell death (apoptosis), and maintenance of genomic integrity (DNA repair).

The retinoblastoma gene (Rb) encoding the 105kd nuclear phosphoprotein, maps to chromosome 13, band 13q14, and is a prototypical tumor-suppressor gene[1,2]. The retinoblastoma protein plays an important role in regulating the ability of cells to enter S phase, during which DNA is synthesized[3]. Although the alteration of the Rb gene has been shown to be critical for the development of retinoblastoma[4-6], dysregulation of the Rb gene is important in a wide array of human cancers, such as sarcoma[7], hepatoma[8], leukemia[9], and esophagus[10], breast[11], bladder[12], prostate[13] and lung cancers[14]. But studies concerning Rb gene change in gastric carcinoma are limited.

This study was to examine the correlation of altered pRb expression with the clinicopathological status and prognostic value of altered protein expression in patients with gastric carcinoma.

## MATERIALS AND METHODS

### Patients

The study population consisted of 91 patients (80 men, 11 women; mean age: 68 years; range: 32-86 years) who underwent surgery for primary gastric adenocarcinoma at our institute between 1992 and 2000. Eighteen (19.8%) tumors were located in the upper third, 19 (20.9%) in the middle third, and 54 (59.3%) in the lower third of the stomach, respectively. Following the international tumor node metastasis (TNM) staging system, 23 (25.3%)

tumors were considered stage Ⅰ, 20 (22.0%) stage Ⅱ, 28 (30.8%) stage Ⅲ, and 20 (22.0%) stage Ⅳ, respectively. Histological grade was assessed following the World Health Organization (WHO) criteria. Forty-nine (53.8%) tumors were well- or moderately- differentiated, and forty-two (46.2%) poorly- or undifferentiated.

Sixteen of the 20 stage Ⅳ patients had distant metastasis (5 peritoneal, 5 hepatic, 1 lung, 3 peritoneal and T4 lesion, 1 peritoneal and hepatic, 1 peritoneal and N3 lymph node status), while the other cases were classified as stage Ⅳ because of N3 lymph node status ($n$ = 3) and/or T4 lesions ($n$ = 1). Sixteen patients underwent palliative surgery (16 distant metastases). The other 75 patients underwent radical D2 resection. Of the patients 12 were treated with postoperative systemic chemotherapy (5 for 6 cycles of FEM, and 7 for 6 cycles of 5-FU). To rule out any confounding effect of adjuvant chemotherapy, this variable was entered in the multivariate analysis of survival. Follow-up evaluation consisted of physical examination, chest radiography, and tumor marker (CEA, and CA19.9) assay every 6 mo for the first 3 years and once a year thereafter, abdominal computed tomography (CT), and endoscopy once a year.

### *Immunohistochemistry*

Formalin-fixed and paraffin-embedded tumor samples taken during surgery were used for the study. pRb expression was detected immunochemically using the monoclonal antibody (M7131, DAKO, Denmark) generated from mouse anti-human Rb product. The 4-μm thick sections from their original histology blocks were eparaffinized in xylene and treated with 3% hydrogen peroxide in methanol to block endogenous peroxidase. For immunohistochemical detection of the pRb protein, the sections were boiled for 30 min in 10 mmol/L citrate buffer solution (pH6.0) using a microwave heater for antigen retrieval. Mouse monoclonal anti-Rb (Clone Rb1, DAKO, Glostrup, Denmark) was used as primary antibody. The clone Rb1, which can be used on (archival) paraffin-embedded material, is a mouse monoclonal antibody against recombinant human retinoblastoma gene product representing amino acid 330-612 and reacts with the retinoblastoma gene product as indicated by immunoblotting SV40 transformed human tumor cell lines[15]. The sections were first incubated for 60 min at room temperature with the primary antibody. Biotinylated anti-mouse IgG was then applied as secondary antibody, followed by peroxidase-cojugated streptavidin. Demonstration of binding sites with the peroxidase reaction was achieved with 3, 3'-diaminobenzidine tetrahydrochloride (0.25 mg dissolved in 1 mL 0.02% hydrogen peroxide). Phosphate-buffered saline was used for rinsing between each step. Faint nuclear staining, sufficient to aid in orientation but not to influence the judgement of positivity, was performed with Mayer hematoxylin solution.

### *Evaluation of pRb protein expression*

For each section, 10 high-power fields were chosen randomly, and a total of 1000 cells were evaluated by a pathologist (Tseng HH), who had no prior knowledge of the patients' outcomes or tumor characteristics. The pattern of immunoreactivity was scored on the basis of the percentage of tumor cell nuclei with positive staining. The parameter obtained was the mean relative nuclear positive area evaluated on at least 10 fields observed at a magnification of × 40.

Immunoreactivity was semiquantitatively graded. The grade scheme was: 0+ (no immunoreactive cells present), 1+ (1%-24%), 2+ (25%-49%), 3+ (50%-74%), 4+ (75%-100%) immunoreactivity cells, respectively.

In evaluation of the clinical significance of pRb expression in gastric carcinoma, tumors with grade "0+ - 2+" immunostaining were grouped as weak staining, and tumors with grade "3+ and 4+" immunostaining as strong staining.

### *Statistical analysis*

Associations between categorical variables were analyzed using Fisher's exact test, $\chi^2$ test, or Mann-Whitney $U$ test. Univariate analysis of survival was carried out with a Kaplan-Meier estimator, and the difference between curves of subgroups was determined using the log-rank test. Survival curves were calculated by the Kaplan-Meier method and compared by the log-rank test. Independent survival variables were studied by Cox regression analysis. All data were calculated with SPSS for windows with $P < 0.05$ as the level of significance.

## RESULTS

A total of 59 out of 91 (65%) tumors were positive for altered pRb, with 16 cases graded as "1+", 24 as "2+", 11 as "3+" and 8 as 4+". In 91 unselected primary gastric carcinomas, we compared the common prognostic markers, such as age, sex, tumor location, Lauren type, tumor grading, TNM system, and altered pRb expression. On examining the distribution of tumors with strong or weak altered pRb expressions (Table 1), no significant differences were found in sex, age, tumor site, Lauren type, and histologic grading. However, statistical analysis demonstrated a significant association between altered pRb status and tumor stage. The altered pRb protein-high tumors were more in earlier stage (stages Ⅰ and Ⅱ) than in more advanced stage (stages Ⅲ and Ⅳ) ($P$ = 0.010). Moreover, the altered pRb status significantly correlated with depth of invasion (T status), but not with lymph node metastasis (N status), and distant metastasis (M status). The altered pRb expression was weak in 11 of the 17 (65%) T1 tumors, 16 of the 21 (76%) T2 tumors, 38 of the 46 (83%) T3 tumors, and 7 of the 7 (100%) T4 tumors. The altered pRb was inversely correlated with the depth of tumor invasion ($P$ = 0.047).

Analysis of survival was carried out in 75 patients undergoing curative resection. The results of univariate analysis of survival in relation to clinicopathological findings are reported in Table 2. Tumor staging and altered pRb expression were correlated with 5-year survival. Kaplan-Meier curves showed that the 5-year survival was 80.5% in patients with strong altered pRb expression ($n$ = 17) compared with 49.6% in patients with weak altered pRb expression ($n$ = 58) ($P$ = 0.0436). Only TNM

**Table 1 Altered pRb expression and clinicopathological findings in 91 patients with gastric adenocarcinoma**

| Variables | *n* | Altered pRb expression, *n* (%) | | *P* |
|---|---|---|---|---|
| | | Strong | Weak | |
| Age | | | | |
| < 70 yr | 43 | 7 (16) | 36 (84) | 0.307 |
| ⩾ 70 yr | 48 | 12 (25) | 36 (75) | |
| Sex | | | | |
| Male | 80 | 17 (21) | 63 (79) | 1.000 |
| Female | 11 | 2 (18) | 9 (82) | |
| Tumor location | | | | |
| Upper third | 18 | 2 (11) | 16 (89) | 0.553 |
| Middle third | 19 | 5 (26) | 14 (74) | |
| Lower third | 54 | 12 (22) | 42 (78) | |
| Lauren type | | | | |
| Intestinal | 67 | 12 (18) | 55 (82) | 0.304 |
| Diffuse | 20 | 4 (20) | 16 (80) | |
| Mixed | 4 | 2 (50) | 2 (50) | |
| Differentiation | | | | |
| High or moderately | 49 | 10 (20) | 39 (80) | 0.905 |
| Low | 42 | 9 (21) | 33 (79) | |
| Tumor depth | | | | |
| T1 | 17 | 6 (35) | 11 (65) | 0.047 |
| T2 | 21 | 5 (24) | 16 (76) | |
| T3 | 46 | 8 (17) | 38 (83) | |
| T4 | 7 | 0 (0) | 7 (100) | |
| Lymph node metastasis | | | | |
| N0 | 28 | 9 (32) | 19 (68) | 0.109 |
| N1 | 35 | 6 (17) | 29 (83) | |
| N2 | 22 | 3 (14) | 19 (86) | |
| N3 | 6 | 1 (17) | 5 (83) | |
| Distant metastasis | | | | |
| No | 75 | 17 (23) | 58 (77) | 0.366 |
| Yes | 16 | 2 (13) | 14 (87) | |
| TNM stage | | | | |
| I and Ⅱ | 43 | 14 (33) | 29 (67) | 0.010 |
| Ⅲ and Ⅳ | 48 | 5 (10) | 43 (90) | |

**Table 2 Univariate analysis of survival in 75 patients with curative resection for gastric adenocarcinoma**

| Variables | 5-year survival, *n* (%) | *P* |
|---|---|---|
| Age | | |
| < 70 yr | 36 (54.0) | 0.8036 |
| ⩾ 70 yr | 39 (58.8) | |
| Sex | | |
| Male | 69 (57.2) | 0.5069 |
| Female | 6 (50.0) | |
| Tumor location | | |
| Upper third | 14 (51.9) | 0.4312 |
| Middle third | 14 (64.8) | |
| Lower third | 47 (54.6) | |
| Differentiation | | |
| Well + moderate | 41 (66.1) | 0.0509 |
| Low | 34 (42.5) | |
| TNM stage | | |
| I and Ⅱ | 43 (88.6) | < 0.0001 |
| Ⅲ and Ⅳ | 32 (14.7) | |
| Altered pRb expression | | |
| strong | 17 (80.5) | 0.0436 |
| weak | 58 (49.6) | |

**Table 3 Multivariate analysis of survival in 75 patients wirh curative resection for gastric adenocarcinoma**

| Variables | Category | Relative risk | 95% CI | *P* |
|---|---|---|---|---|
| Histologic grade | Poorly *vs* well and moderately-differentiated | 1.073 | 0.640-1.800 | 0.788 |
| Altered pRB expression | Weak *vs* strong | 0.891 | 0.443-1.791 | 0.746 |
| TNM stages | Ⅲ & Ⅳ *vs* I & Ⅱ | 5.067 | 2.764-9.289 | 0.000 |

stage was found to be an independent prognostic factor for survival (Table 3) on multivariate analysis by Cox regression.

## DISCUSSION

The half-life of mutant Rb protein is even longer than that of the wild Rb protein. This feature results in immunohistochemically detectable expression of the mutant Rb protein. It has been established that altered Rb protein expression pattern gives a fairly good estimate of the mutation frequency in the Rb gene[16,17].

In this study, 65% (59/91) of the tumors were positively stained and the staining in tumor nuclei of gastric carcinoma ranged 0%-90%. Constancia *et al*[18] reported that alterations affecting the Rb gene are rather infrequent in human gastric carcinomas, but our data indicate the altered pRb protein expression is frequent and widely variable in gastric carcinoma. We directly detected the altered Rb protein in tumor cells, which is different from the Constancia's study detecting the normal Rb protein[18]. The Rb gene mutation is frequent in various human malignancies including sarcoma[7], hepatoma[8], leukemia[9], and esophagus[10], breast[11], bladder[12], prostate[13] and lung cancers[14], being similar with that in gastric carcinoma in our study.

Rb mutations resulting in the loss of Rb function play a role in the initiation of retinoblastoma and other tumors that develop as second malignancies in individuals with the hereditary form of retinoblastoma[19]. The functional loss of the Rb gene has been implicated in a diverse group of human malignancies, including carcinoma of the breast, urinary bladder, liver, esophagus, prostate, and colon[8,10-12,20,21]. The significance of these mutations in other tumors is a potentially critical issue. A key issue is whether the changes reported within the Rb gene in other various cancers are causally related to the initiation of tumors or whether they are involved in tumor progression. Phillips *et al*[20] reported that loss of Rb gene function is an early event in prostatic tumorigenesis. Our findings demonstrate that the altered pRb protein expression is more frequent in gastric carcinomas with superficial invasion than in those with advanced invasion. This feature means that the Rb gene mutation is also an early event in the development of gastric carcinoma and then gradually gets lost during its progression. However, only 35% (6/17) of T1 lesions had strong staining in our study. The T1 lesions occurred earlier than T2, T3, and T4 lesions and might be a "late" event in the development of gastric carcinoma. Therefore, in-depth studies should be undertaken to clarify whether the Rb gene mutation is causally related to the initiation of gastric adenocarcinoma

as in the hereditary retinoblastoma.

The prognostic value of pRb protein expression in various carcinomas has been investigated. Some studies found that pRb protein expression is an important prognostic indicator of cancers, such as bladder cancer[22] and non-small-cell lung cancer[23]. Low pRb protein expression is associated with significantly poorer survival among them. In contrast, in colorectal cancer no prognostic significance of pRb protein expression has been reported[14]. Our findings demonstrate that there is a negative correlation between pRb protein expression and survival in gastric carcinoma at univariate analysis, but not significantly correlated between them at multivariate analysis. In the present study, however, the pRb protein-high tumors were more frequent in earlier stage (stages Ⅰ and Ⅱ) than in advanced stage (stages Ⅲ and Ⅳ), suggesting that the pRb protein expression is not an independent prognostic marker of gastric adenocarcinoma.

In conclusion, our data indicate that the mutation of Rb gene is frequent in gastric carcinoma. The expression of altered pRb protein inversely correlates with tumor invasion and is not an independent prognostic marker in gastric adenocarcinoma

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*RAPID COMMUNICATION*

# Comparison of amplicon-sequencing, pyrosequencing and real-time PCR for detection of YMDD mutants in patients with chronic hepatitis B

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## Abstract

**AIM:** To compare the sequencing of PCR products, pyrosequencing, and real-time PCR for detection of Tyrosine-methionine-aspartate-aspartate (YMDD) mutants in patients with chronic hepatitis B.

**METHODS:** Mixtures of plasmids and serum samples from 69 chronic hepatitis B patients treated with lamivudine were tested for YMDD mutations by sequencing of PCR products, pyrosequencing, and real-time PCR, respectively. Time required and reagent costs of the three assays were evaluated.

**RESULTS:** Real-time PCR detected 100%, 50%, 10%, 1% and 0.1% of YVDD plasmid in mixtures with $10^6$ copies/mL of YMDD plasmid, whereas sequencing and pyrosequencing only detected 100% and 50% of YVDD plasmid in aliquots of the corresponding mixtures. Completely concordant results were obtained from 60 (87%) out of the 69 clinical serum samples by the three assays. Mutants were detected by real-time PCR in less than 20% of the total virus population, but no mutant was detected by sequencing and pyrosequencing. In addition, real-time PCR required less time and was more cost-effective than the other two assays. However, throughput of pyrosequencing was the highest.

**CONCLUSION:** Among the three assays compared, real-time PCR is the most sensitive, cost-effective, and time saving for monitoring YMDD mutants in patients with chronic hepatitis B on lamivudine therapy.



**Key words:** Tyrosine-methionine-aspartate-aspartate mutant; Hepatitis B virus; Real-time PCR; Sequencing; Pyrosequencing

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## INTRODUCTION

It is estimated that 350 million individuals are chronically infected with hepatitis B virus (HBV) and that more than 1 million will die of liver cirrhosis and hepatocellular carcinoma (HCC) each year[1-3]. Lamivudine is an effective antiviral agent for patients with chronic hepatitis B and advanced liver diseases[4]. However, long-term lamivudine monotherapy induces emergence of lamivudine-resistant HBV mutants in some patients chronically infected with HBV[4,5]. Resistance is associated with mutations in the highly conserved tyrosine-methionine-aspartate-aspartate (YMDD) motif of the reverse transcriptase, which is part of the catalytic site of HBV polymerase[6]. Virological breakthrough and alanine transaminase (ALT) flare have been observed in 2 and 3 mo after the emergence of YMDD mutants, respectively[7]. Several technologies developed can detect lamivudine-resistant mutants[8], but the number of studies actually comparing these assays is limited.

Nucleotide sequencing of PCR products is routinely used to detect lamivudine resistance. However, this method is expensive and laborious and only can detect mutant virus when it comprises at least 25% of the total virus population[9]. Pyrosequencing has a high throughput and can detect minor sequence variants[10]. Real-time PCR is a rapid and highly sensitive method for detection of mutant HBV[11-14].

In the present study, we compared the sequencing of PCR products, pyrosequencing, and real-time PCR for detection of YMDD mutants in mixed plasmids and clinical samples from chronic hepatitis B patients treated with lamivudine. Time required and reagent costs were also analyzed to enable a comparison among these assays for their cost-effectiveness.

## MATERIALS AND METHODS

### Plasmids and controls

Plasmids and controls were prepared as previously described[14]. Briefly, three previously identified serum samples containing YMDD, YVDD and YIDD respectively were used as a template and amplified by PCR. PCR products were cloned using pGEM-T systems (Promega, Madison, WI, USA), and clones were sequenced using ABI 3100 sequencer (Applied Biosystems, Foster, CA, USA).

### Patients, samples and extraction of HBV DNA

Serum samples were collected from 69 patients with chronic HBV infection. Real-time PCR showed that all the patients after treatment with lamivudine for three months to three years were HBV-DNA positive. HBV DNA was extracted from serum samples using the QIAamp blood kit (Qiagen, Chatsworth, CA, USA) according to the manufacturer's instructions. HBV DNA was measured on Mx3000P real-time PCR system (Stratagene, La Jolla, CA, USA) with quantitative real-time PCR reagents (Fosun Diagnostics, Shanghai, China) approved by the State Food and Drug Administration of China for *in vitro* diagnostic use.

### Sequencing of PCR products

HBV DNA samples were prepared for sequencing by PCR amplification as described by Allen *et al*[6]. HBV DNA extracted from serum samples was amplified by PCR. PCR products were purified with QIAquick PCR purification kits (Qiagen, Chatsworth, CA, USA) and eluted from the column with 80 μL of distilled deionized water. The DNA quality and concentration of DNA were determined by absorbance measurements at 260 and 280 nm and by gel electrophoresis on 2.5% agarose gel. All sequencing reactions were performed on ABI 3130 DNA sequencer (Applied Biosystems, Foster, CA, USA).

### Pyrosequencing

Pyrosequencing was carried out as described by Lindstrom *et al*[10]. In brief, the primer (5'-AGT GGG CCT CAG TCC GTT TC-3') was designed to anneal the adjacent YMDD motif. HBV DNA was extracted from serum samples and amplified by nested-PCR. One of the inner PCR primers was biotinylated to prepare single-stranded DNA with PCR products on the streptavidin-coated beads for pyrosequencing. Pyrosequencing was performed on the automated 96-well PSQ pyrosequencer (Pyrosequencing AB, Uppsala, Sweden) with the PSQ SNP 96 reagent kit (Pyrosequencing AB, Uppsala, Sweden). The nucleotide dispensation order was ATGTATGATG, which was designed on sequences of the interested YMDD motif of the wild-type and mutant HBV.

### Real-time PCR

Real-time PCR for detection of YMDD mutants was performed as previously described[14]. In brief, parallel reactions C, V and I were used to detect total HBV, YVDD, and YIDD, respectively. The reactions differed only in the reverse primer they contained. The amplification was performed on Mx3000P PCR system (Stratagene, La Jolla, CA, USA) by incubating the reaction mixture (50 μL) at 50℃ for 2 min, at 95℃ for 5 min, followed by 40 cycles of PCR amplification at 94℃ for 20 s and at 53℃ for 30 s. The reaction system was provided and optimized by Fosun Diagnostics (Fosun Diagnostics, Shanghai, China). The threshold cycle (Ct) is the cycle at which a significant increase in fluorescence occurs. The percentage of mutants in total viruses was calculated by the equations of Bernard (ΔCt = Ct of control-Ct of mutants)[15] and Shi (ratio of mutants to total viruses = $2^{\Delta Ct}$)[14].

### Mixing experiments

Mixing experiments were used to evaluate the abilities of the sequencing, pyrosequencing and real-time PCR to accurately detect and quantify minor sequence variants. Mutant plasmid containing YVDD sequence and wild-type plasmid were mixed at a final concentration of $10^6$ copies/mL, and the percentage of YVDD plasmid in the mixture was 100%, 50%, 10%, 1%, 0.1% and 0.01% respectively. The mixtures were analyzed by the three methods, respectively. For real-time PCR, each mixture was analyzed five times, and the mean of the five runs was calculated.

### Time study

Time analysis was carried out as described by Krafft and Lichy[16]. Three skilled technologists were selected to perform the assays. Time required for each assay was calculated by direct observation during the procedures.

### Cost analysis

Costs for each assay were estimated based on the prices of reagents in China. The costs of instruments and labors were not included.

## RESULTS

### Detection of YVDD and YMDD mutants in plasmid mixture

YVDD and YMDD mutants in the plasmid mixture were detected at different ratios by sequencing, pyrosequencing and real-time PCR, respectively. YVDD, YVDD/YMDD mutants in the mixture containing 100% and 50% of YVDD plasmid and only YMDD in the mixture containing 10%, 1%, 0.1% and 0.01% YVDD plasmid were detected by sequencing and pyrosequencing. YVDD mutants in the mixture containing 100% YVDD plasmid, YVDD/YMDD mutants in the mixture containing 50%, 10%, 1% and 0.1% YVDD plasmid and only YMDD mutants in the mixture containing 0.01% YVDD plasmid were detected by real-time PCR. The ratio of YVDD to YMDD mutants in the real-time PCR assay was almost identical to the true ratio in the mixtures (Table 1).

### Comparison of sequencing, pyrosequencing and real-time PCR for detection of YMDD and YVDD mutants in clinical samples

We detected YMDD and YVDD mutants in clinical serum samples from 69 chronic hepatitis B patients treated with lamivudine. The results obtained by sequencing, pyrosequencing and real-time PCR were compared (Table 2). Completely concordant results were obtained from 60

**Table 1 Results of sequencing, pyrosequencing and real-time PCR for detection of mixed plasmids containing YVDD and YMDD at a final concentration of $10^6$ copies/mL**

| YVDD plasmid in the mixture | Sequencing | Pyrosequencing | Real-time PCR | ΔCt (mean ± SD) | Calculated percentage (mean ± SD) (%) |
|---|---|---|---|---|---|
| 100% | YVDD | YVDD | YVDD | -0.024 ± 0.06 | 98 ± 4.0 |
| 50% | YVDD | YVDD | YVDD | -0.98 ± 0.07 | 51 ± 2.5 |
| 10% | YMDD | YMDD | YVDD | -3.12 ± 0.09 | 11 ± 0.7 |
| 1% | YMDD | YMDD | YVDD | -6.60 ± 0.14 | 1 ± 0.1 |
| 0.1% | YMDD | YMDD | YVDD | -10.12 ± 0.20 | 0.09 ± 0.01 |
| 0.01% | YMDD | YMDD | YMDD | ND | 0 |

ND: Not detected.

(87%) samples by the three assays. YMDD mutants in 24 samples and YVDD mutants in one sample were detected by pyrosequencing, while YMDD mutants in 22 samples and YVDD mutants in three samples were detected by real-time PCR. The percentage of mutants in the virus population obtained by real-time PCR in the three YVDD samples was 20%, 7% and 6% respectively. Twenty-four types of YIDD mutant and two mixed types of YIDD and YVDD mutants were detected by pyrosequencing, while 21 types of YIDD mutants and 5 mixed types of YIDD and YVDD mutants were detected by real-time PCR. The results were identical in 18 YVDD samples obtained by pyrosequencing, but YVDD mutants were detected in one sample by sequencing and pyrosequencing while YMDD mutants were detected in one sample by real-time PCR.

Among the five samples containing mixed types of YIDD and YVDD mutants by real-time PCR, mixed mutants were detected by pyrosequencing whereas only dominant mutants were detected by sequencing in two samples.

### *Time required*

The time required for each assay of a certain number of samples was dependent on the throughput of the instruments used for detection. We measured the time required for each assay based on a 4-sample run because the highest throughput of ABI 3130 sequencer used in this study was 4 samples per run. The total assay time for sequencing, porysequencing and real-time PCR was 5, 4.5 and 2 h, respectively.

### *Costs*

The cost for each assay was calculated based on the prices of the reagents in China. The sequencing reagents were from Applied Biosystems (Applied System, Foster, CA, USA). Pyrosequencing reagents were from Pyrosequencing AB (Pyrosequencing AB, Uppsala, Sweden). Primers and probes were synthesized in TaKaRa Biotech (TaKaRa, Dalian, China). Real-time PCR mixtures were from Fosun Diagnostics (Fosun Diagnostics, Shanghai, China). The total reagent cost for each sequencing, pyrosequencing and real-time PCR was 120, 150 and 80 Yuan RMB, respectively.

**Table 2 Comparison of results obtained by sequencing, pyrosequencing and real-time PCR *n* (%)**

| Type | Sequencing | Pyrosequencing | Real-time PCR |
|---|---|---|---|
| rtM204 | 25 (36) | 24 (35) | 23 (33) |
| rtM204I | 26 (38) | 24 (35) | 21 (30) |
| rtM204V | 18 (26) | 19 (27) | 20 (29) |
| rtM204I + rtM204V | 0 (0) | 2 (3) | 5 (8) |

## DISCUSSION

Lamivudine is one of the first-line medicines for chronic hepatitis B. Mutations in the YMDD motif of polymerase gene have been detected in patients with chronic hepatitis B treated or untreated with lamivudine[14,17-19]. Clinical breakthrough can be observed 2 wk to 7 mo after the emergence of YMDD mutations[7,9,19-20]. Acute and severe exacerbation due to YMDD mutations can cause considerable morbidity and mortality[21-24]. Lamivudine therapy after the emergence of YMDD mutations has no effect on chronic hepatitis B patients[25]. Antiviral agents, such as adefovir and entecavir, are effective against lamivudine-resistant HBV[26-28]. Pegylated interferon is also one of the first-line therapeutic options for hepatitis B virus infection and can induce sustained responses in some of lamivudine-resistant patients[29-31]. Monitoring YMDD mutation during lamivudine therapy contributes to the clinical decision of treatment of chronic hepatitis B patients.

Many assays have been used for detection of lamivudine-resistant mutants in patients with hepatitis B[8]. However, they are different in sensitivity, specificity, cost, and time required. Sequencing of PCR products is a conventional assay and pyrosequencing is a high throughput method that can detect minor sequence variants. In the present study, we compared sequencing of PCR products, pyrosequencing, and real-time PCR for detection of YMDD mutations in chronic hepatitis B patients treated with lamivudine. The results obtained by sequencing, pyrosequencing and real-time PCR were completely concordant in 60 (87%) patients. YVDD mutants were detected by real-time PCR in three samples. The percentage of the mutants in total viruses in the three samples was 20%, 7%, and 6%, respectively, all of which were below the detection limit of sequencing[9]. YVDD mutants were detected by pyrosequencing in the sample with 20% of mutants. These results suggest that real-time PCR is more sensitive, cost-effective and time saving than sequencing and pyrosequencing. In one sample, YVDD mutants were detected by sequencing and pyrosequencing and YMDD mutants were detected by real-time PCR. Further study of the clinical data of the sample revealed that it contained 620 copies/mL of HBV DNA, which was below the limit of real-time PCR assay[14].

Pyrosequencing is a high throughput, non-gel-based DNA sequencing technique based on real-time detection of the released pyroghosphate during DNA synthesis[10]. Pyrosequencing is widely used in detection of single nucleotide polymorphisms (SNP) in the human genome[32].

However, pyrosequencing only can detect DNA sequence with a length of no more than 40 base-pair, while sequencing of PCR products can detect much longer DNA sequences. Real-time PCR detects specific point mutations recognized by the primers or probes.

The time required for each assay is dependent on the throughput of instruments used. In the present study, ABI 3130 was used for sequencing of PCR products. Its highest throughput was 4 samples per run. The instruments used for pyrosequencing and real-time PCR were 96-well plates and had the highest throughput of 96 samples and 32 samples, respectively. Although pyrosequencing has the highest throughput, relatively few laboratories have used pyrosequencing equipments[10]. Real-time PCR assay is well suited for routine screening of YMDD mutants because real-time PCR systems are routinely used in many laboratories.

For detection of mutants in virus population, real-time PCR can calculate the ratio of mutants to total viruses[14], while sequencing and pyrosequencing only can estimate the ratio by the signal intensity. The background noises affect the results. When serum HBV-DNA is below $10^8$ copies/mL, no false positive results have been observed[14]. However, the accuracy of the ratios calculated in real-time PCR can be affected by variations in the ΔCt value, especially in cases where mutants are above 50% in the virus population.

In conclusion, among the three assays studied, real-time PCR is the most sensitive, cost-effective, and time saving method for monitoring YMDD mutations in patients with chronic hepatitis B on lamivudine therapy.

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*CASE REPORT*

# Hypertriglyceridemia-induced pancreatitis: A case-based review

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## Abstract

Hypertriglyceridemia is an established cause of pancreatitis. In a case-based approach, we present a review of hypertriglyceridemia and how it can cause pancreatitis. We outline how to investigate and manage such patients. A 35 year old man presented to the emergency department with abdominal pain and biochemical evidence of acute pancreatitis. There was no history of alcohol consumption and biliary imaging was normal. The only relevant past medical history was that of mild hyperlipidemia, treated with diet alone. Physical exam revealed epigastric tenderness, right lateral rectus palsy, lipemia retinalis, bitemporal hemianopsia and a delay in the relaxation phase of his ankle reflexes. Subsequent laboratory investigation revealed marked hypertriglyceridemia and panhypopituarism. An enhanced CT scan of the head revealed a large suprasellar mass impinging on the optic chiasm and hypothalamus. The patient was treated supportively; thyroid replacement and lipid lowering agents were started. He underwent a successful resection of a craniopharyngioma. Post-operatively, the patient did well on hormone replacement therapy. He has had no further attacks of pancreatitis. This case highlights many of the factors involved in the regulation of triglyceride metabolism. We review the common causes of hypertriglyceridemia and the proposed mechanisms resulting in pancreatitis. The incidence and management of hypertriglyceridemia-induced pancreatitis are also discussed.



**Key words:** Hypertriglyceridemia; Pancreatitis; Hyperlipidemia

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## INTRODUCTION

Acute pancreatitis is a common condition with several possible etiologies. The mortality rate may be up to 20%. While alcohol and gallstones are the most common etiologies, metabolic, structural and iatrogenic causes are responsible for 20%-25% of cases in the United States. Although hyperlipidemia can be associated with acute pancreatitis as an epiphenomenon, hypertriglyceridemia or chylomicronemia is the underlying cause in up to 7% of all cases of pancreatitis. It is the most common cause of acute pancreatitis not due to gallstones or alcohol[1-4]. Hypertriglyceridemia-induced pancreatitis rarely occurs unless triglyceride levels exceed 20 mmol/L[3,5]. For triglyceride levels to exceed 20 mmol/L, most patients will have some form of primary or genetic defect in lipid metabolism. Genetic factors determine over 60% of the variability in serum lipids. As most patients can be effectively treated with existing drug therapy, it is critical that the gastroenterologist recognize these patients and manage them appropriately. Since not all hyperlipidemia is familial in nature, an awareness of the secondary causes of hypertriglyceridemia and factors that can alter triglyceride metabolism is imperative.

In a case-based approach, we present a review of the common causes of hypertriglyceridemia, the proposed mechanisms resulting in pancreatitis as well as further details on the incidence and management of hypertriglyceridemia-induced pancreatitis (Table 1). Although the case presented is an uncommon scenario, it clearly demonstrates how a sequence of metabolic and endocrine abnormalities can lead to a common disease process- acute pancreatitis. The key to investigating, diagnosing and managing this patient was taking a detailed history and performing a thorough physical exam.

## CASE PRPORT

A 35-year-old man presented to the emergency department with a 36-h history of severe epigastric pain and vomiting. He also complained of a four-month history of headaches, fatigue, cold intolerance, constipation, reduced libido, and erectile dysfunction. One year prior to presentation, he was

**Table 1 Causes of hypertriglyceridemia**

| |
|---|
| **Primary** |
| Familial combined hyperlipidemia |
| Familial hypertriglyceridemia |
| Type Ⅲ hyperlipoproteinemia |
| Chylomicronemia |
| Lipoprotein lipase deficiency |
| Apolipoprotein C-Ⅱ deficiency |
| **Secondary** |
| Insulin resistance |
| Diabetes mellitus |
| Obesity |
| Hypothyroidism |
| Alcohol |
| Diet (excessive carbohydrate intake) |
| Nephrotic syndrome |
| Chronic renal failure, uremia |
| Biliary obstruction/cholestasis |
| Acute hepatitis |
| Monoclonal gammopathy |
| Multiple myeloma |
| Systemic lupus erythematosus |
| Stress, Sepsis |
| Pregnancy |
| Ileal bypass surgery |
| Lipodystrophy |
| Glycogen storage disease |
| Drugs |
| Estrogen |
| Isotretinoin |
| B-blockers |
| Glucocorticoids |
| Cyclosporine |
| Bile acid-binding resins |
| Protease inhibitors |
| Thiazides |
| Tamoxifen |

Modified from Fung and Frohlich[44].

told that he had mildly elevated "cholesterol" and was told to modify his diet. His past medical history was otherwise negative. He did not smoke, rarely consumed alcohol (none in the last 14 d) and was not taking any medications. Physical exam found a somnolent but easily rousable male with marked epigastric tenderness. No masses or organomegaly was noted on examination of his abdomen. A right lateral rectus palsy, lipemia retinalis and bitemporal hemianopsia were also noted on exam but there were no signs of increased intracranial pressure. The thyroid exam was normal but the relaxation phase of his ankle reflexes was delayed.

Electrolytes and complete blood count were normal. Serum lipase was 754 U/L (normal 20-190 U/L) and his liver transaminases, alkaline phosphatase and lactate dehydrogenase were normal. Ultrasound detected inflammatory changes in the pancreas but the gallbladder and biliary tree were normal. Serum triglyceride was 38.5 mmol/L (normal 0-2), cholesterol; 12.4 mmol/L (normal 0-5.2), thyroid stimulating hormone; 0.17 (normal 0.4-4.0), free T4; 3.8 pmol/L (normal 10.8-23.8), random cortisol; 62 mmol/L (normal 138-690), serum testosterone < 0.7 nmol/L (normal 10-33), and luteinizing hormone; 0.1 IU/L (normal 1.3-6.3). An enhanced CT scan of the head revealed a large suprasellar mass impinging on the hypothalamus and optic chiasm.

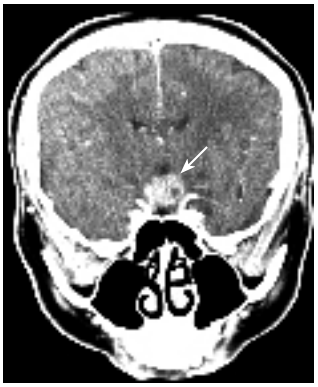

**Figure 1** Enhanced CT scan showing a large suprasellar craniopharyngioma (arrow).

A diagnosis of acute pancreatitis was made. Chylomicronemia caused by central hypothyroidism was the most likely etiology. The patient was treated with intravenous fluids and narcotics for pain control. His epigastric pain resolved over three days. He was given dexamethasone 4 mg three times daily preoperatively and L-thyroxine 50 micrograms daily. The fibric acid derivative gemfibrozil was started at 600 mg twice daily. At craniotomy, a suprasellar craniopharyngioma displacing the optic chiasm and encasing the pituitary stalk was completely removed (Figure 1). He was started on glucocorticoid replacement therapy, L-thyroxine, DDAVP and testosterone for panhypopituitarism. His postoperative course was uncomplicated; he has subsequently remained asymptomatic over a three-year follow-up period.

## DISCUSSION

### *Hypertriglyceridemia-induced pancreatitis*

**Epidemiology:** It is well known that hyperlipidemia is associated with acute pancreatitis, both as a precipitant and as an associated epiphenomenon[1,2]. Hypertriglyceridemia or chylomicronemia may be responsible for 1%-7% of all cases of pancreatitis[3,4]. Failure to consider and investigate chylomicronemia as a cause of pancreatitis may lead to an underestimate of the incidence. Pregnancy or medical conditions such as diabetes (both known to precipitate marked hypertriglyceridemia) should prompt further work-up[6,7]. Chylomicronemia may be the cause of 20% of episodes of acute pancreatitis in non-drinkers free of biliary tract disease. Chang *et al* found that hypertriglyceridemia was the cause of 56% cases of gestational pancreatitis[7]. In many settings, determining the exact etiology of pancreatitis may be complicated by the role of ethanol in precipitating severe hypertriglyceridemia. The proportion of "alcoholic pancreatitis" caused by direct as opposed to secondary hyperlipidemic effects is unknown.

Hypertriglyceridemia-induced pancreatitis rarely occurs unless triglyceride levels exceed 20 mmol/L[3,5]. In contrast, mild to moderate elevations in triglycerides (2-10 mmol/L) are extremely common in the early phase of acute pancreatitis of any etiology. One study noted such elevations in 47% of randomly selected patients presenting with acute pancreatitis[2]. In the same study, mild to moderate elevations of triglycerides were felt more

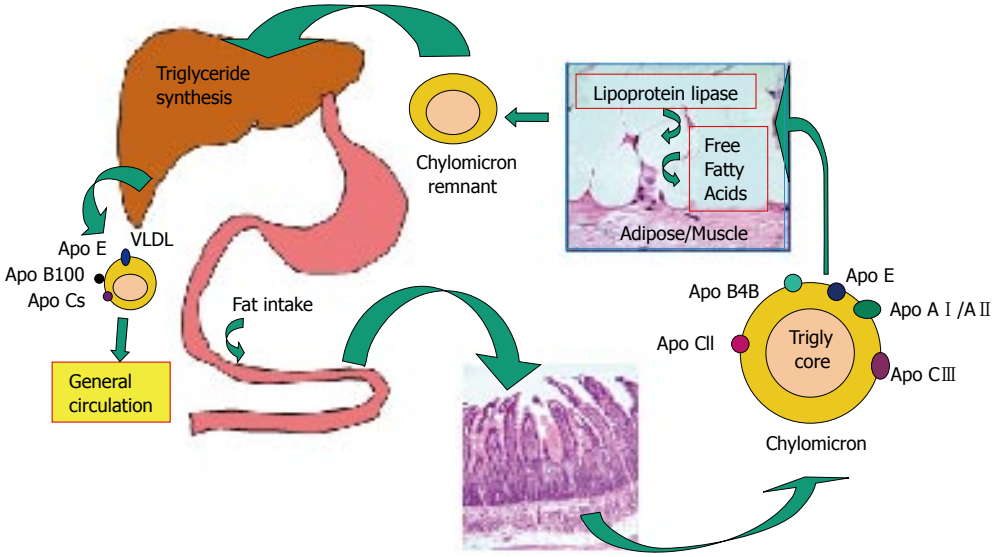


**Figure 2** Triglyceride metabolism.

likely to be an epiphenomenon of the acute pancreatitis rather than a true causal precipitant[2]. It is important to remember that chylomicrons are the product of dietary fat absorption. Enforced abstinence from eating after a diagnosis of pancreatitis may allow rapid metabolism of the triglyceride-rich chylomicrons. Dominguez-Munoz *et al* found severe elevations (> 20 mmol/L) in 10% of patients but levels rapidly fell in the majority of patients within 72 h of presentation; therefore, a delay in the presentation or consideration of diagnosis can lead to false conclusions about etiology. Triglyceride levels remained mildly elevated for up to 15 d, probably reflecting an underlying lipid disorder[2].

### *Pathogenesis of hypertriglyceride-induced pancreatitis*

The exact mechanisms involved in hypertriglyceridemia-induced pancreatitis are unclear. Chylomicrons are triglyceride-rich lipoprotein particles believed to be responsible for pancreatic inflammation. They usually present in the circulation when serum triglyceride levels exceed 10 mmol/L. These largest of lipoproteins might impair circulatory flow in capillary beds; if this occurs in the pancreas, the resulting ischemia might disturb the acinar structure and expose these triglyceride-rich particles to pancreatic lipase. The pro-inflammatory non-esterified free fatty acids generated from the enzymatic degradation of chylomicron-triglycerides may lead to further damage of pancreatic acinar cells and microvasculature. Subsequent amplification of the release of inflammatory mediators and free radicals may ultimately lead to necrosis, edema, and inflammation[8,9]. This hypothesized sequence of events was substantiated by studies showing both triglycerides and free fatty acids caused edema, hemorrhage and elevated amylase levels[9]. Hypertriglyceridemia has also been shown to exacerbate other experimental models of pancreatitis[10].

Studies using oral lipid-loading tolerance tests have documented elevated post-load plasma triglyceride levels in patients with previous pancreatitis as compared with controls[11,12]. To address whether hyperlipidemia is a pre-existing metabolic disorder or a consequence of acute pancreatitis, Dominguez-Munoz and co-workers addressed the hypothesis that mild to moderate elevation of serum triglyceride levels are likely to be an epiphenomenon of the pancreatic disease whereas the severe hyperchylomicronemia and hypertrygliceridemia required to trigger acute pancreatitis would require a relevant defect in the lipid catabolism and clearance[13]. They looked at a group of ten patients with a history of acute pancreatitis associated with severe elevations of serum triglycerides (> 20 mmol/L) and ten patients with acute pancreatitis and normal lipid values. Four to six months after recovery from the pancreatitis, they administered an infusion of intralipid of 20% and calculated the fractional removal rate (K2) and the maximal clearance capacity (K1) of exogenous triglycerides. All patients with a previous attack of "normo-lipidemic" acute pancreatitis had normal K2 and K1 values. However, five patients (50%) with previous hyperlipidemic acute pancreatitis had an abnormally low clearance capacity of exogenous triglycerides while the remaining five had normal removal values. They concluded that, even in the setting of a marked elevation of serum lipid levels, there may be other etiological factors involved[13]. These findings were supported by a similar earlier study[14].

Interestingly, mutations in the lipoprotein lipase (LPL) gene have been identified in patients with hypertriglyceridemia-induced pancreatitis[15-20]. Specifically, mutations in the LPL gene are thought to be a common cause of pregnancy-induced chylomicronemia. One group described 5 such patients all of whom had different mutations in the LPL gene leading to dramatic reductions in LPL activity[21]. Dysregulation or deficiency of key enzymes and substrates involved in triglyceride metabolism has also been described in patients with recurrent hypertriglyceridemia-induced pancreatitis (Figure 2). Lecithin-cholesterol acyltransferase (LCAT) deficiency is one example[22].

### *Hyperchylomicronemia and hypothyroidism*

All patients that develop significant chylomicronemia are thought to have an underlying genetic disorder of lipoprotein metabolism. A disturbance in lipoprotein

lipase activity, the rate-limiting enzyme of triglyceride-rich particle metabolism, is often the culprit. Clinical problems rarely develop unless there is another precipitating condition affecting metabolism such as diabetes, ethanol excess, pregnancy, certain drugs (estrogen[23-26], furosemide[27], isotretinoin[28,29], tamoxifen[30], beta-blockers[31]) or, as in this case, hypothyroidism. Physical findings can include eruptive xanthomas produced by macrophage uptake of chylomicrons which accumulate in the skin over extensor surfaces and lipemia retinalis that is a result of high concentrations of circulating chylomicrons in retinal vessels[32].

Hypothyroidism has a well-established association with hyperlipidemia. Elevations of triglyceride levels are observed in up to 35% of hypothyroid patients[33]. Impaired low-density lipoprotein (LDL) receptor activity may result in decreased clearance and thus an accumulation of LDL particles. A low circulating free thyroid hormone level may also impair lipoprotein lipase activity in adipose tissue. Replacement therapy with L-thyroxine treatment may reverse both of these defects.

### *Treatment*

In the acute setting, pancreatitis due to hyperlipidemia should be treated in much the same manner as other causes of pancreatitis. Currently there is no clear evidence that hyperlipidemia-induced pancreatitis differs from other types of pancreatitis in terms of frequency of necrosis[2], complications or outcomes[3]. A similar approach to medical and diagnostic management is thus indicated. Although chylomicron and triglyceride levels fall rapidly after oral fat intake ceases, efforts to accelerate the removal of the precipitating lipoproteins have been considered.

Direct removal of chylomicrons in the acute setting can be readily achieved by plasmapheresis and there are numerous documented reports[34-37]. Chronic plasmapheresis as prophylaxis has also been reported in patients with recurrent pancreatitis due to severe primary hypertriglyceridemia that was unresponsive to drug and dietary interventions[38]. Recognizing that decreased lipoprotein lipase (LPL) activity is a prominent cause of hypertriglyceridemia has fuelled attempts to enhance LPL activity. Although this is an interesting concept only one small study in the literature has addressed this in which intravenous insulin and heparin, both of which enhance LPL activity, were used to treat a small number of patients with hypertriglyceridemia-induced pancreatitis[39]. They found that the therapy reduced triglyceride levels and appeared to improve the pancreatitis[39]. Diabetic patients should be treated aggressively with intravenous insulin infusions to obtain and maintain euglycemia rapidly.

Patients with known rare defects in lipid metabolism have been treated with replacement therapies with some success. Purified apoC-Ⅱ infusion has yielded transient normalization of triglyceride levels and clinical improvement in pancreatitis patients with apoC-Ⅱ deficiency[40].

Therapeutic efforts following recovery from pancreatitis need to be directed at preventing recurrence by controlling triglyceride levels. As most cases of hyperlipidemia occur when a genetically-predisposed individual is exposed to a secondary condition, therapy should be targeted towards the concomitant disorder. Alcohol should be discontinued as should oral estrogen therapy or selective estrogen receptor modulators such as tamoxifen or raloxifene. Diabetes should be treated with oral hypoglycemics and/or insulin aiming for tight glycemic control. Obviously, as in our case, hypothyroidism should be treated with L-thyroxine.

Persistence of hyperlipidemia on a fat-reduced diet should prompt the institution of lipid-lowering agents. The fibric acid derivatives (fibrates), such as gemfibrozil, fenofibrate or bezafibrate, are the drugs of first choice. These agents are generally well tolerated and highly effective if taken regularly and diet restrictions are continued. Niacin is inexpensive and often effective alone or in combination with a fibrate. Side effects such as flushing, hyperuricemia and hepatic transaminasemia may limit the use of niacin as a first line agent. Controversy exists as to whether niacin may exacerbate glucose intolerance in patients with diabetes mellitus. Omega-3 fatty-acid products show promise as adjunctive agents in refractory cases. Medium-chain triglycerides have also been used to prevent acute hyperlipidemic pancreatitis during pregnancy[41] as their absorption doesn't require chylomicron formation.

Interestingly, in a study of 21 such patients with primary hypertriglyceridemia and a history of recurrent attacks of pancreatitis, lipid lowering toward normal was achieved in all patients with a program of combined dietary and drug therapy[42]. Five patients had recurrent episodes of pancreatitis during the treatment program all of whom were diagnosed subsequently with bulimia[42]. Anti-oxidant therapy (selenium, beta-carotene, vitamin C and $\alpha$-tocopherol) has been used with success in the reduction of recurrent pancreatitis episodes in patients with familial lipoprotein lipase deficiency who remained markedly hypertriglyceridemic after medical therapy[43]. It is postulated that the antioxidants might protect the pancreatic acinar cells from free radical damage brought on by the ischemia of chylomicron-induced changes in pancreatic capillary circulation[43].

## CONCLUSIONS

This case illustrates a sequence of clinical events, due to a combination of genetic and acquired disorders, which resulted in a common medical problem. The main clues to diagnosing this complex chain of events leading to pancreatitis became evident from a thorough history and physical exam. Identifying features of a possible intracranial lesion, hypothyroidism, hypogonadism and hypertriglyceridemia in the context of an understanding of triglyceride metabolism facilitated the diagnosis.

Hypertriglyceridemia is a common clinical problem that can be exacerbated by numerous medications and medical conditions. Markedly elevated triglyceride levels can lead to pancreatitis, a serious and potentially fatal complication. General and specific therapy is available to reduce triglyceride levels during the acute phase of pancreatitis, which may improve the outcome. Nutrition, pharmacologic therapy and avoiding agents that can

elevate triglycerides may be essential in preventing further attacks. There are numerous causes of pancreatitis. Various diagnostic tools can be very helpful in determining the cause of pancreatic inflammation; however, the most valuable approach remains a thorough history and physical exam.

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*CASE REPORT*

# Endoscopic naso-pancreatic stent-guided single-branch resection of the pancreas for multiple intraductal papillary mucinous adenomas

Tamotsu Kuroki, Yoshitsugu Tajima, Ryuji Tsutsumi, Noritsugu Tsuneoka, Amane Kitasato, Tomohiko Adachi, Takashi Kanematsu

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## Abstract

In benign or low-grade malignant pancreatic tumors, complete removal of the lesion is sufficient for a cure, and thus minimal resection techniques with preservation of the pancreatic functional reserve have advantages over more extended pancreatic resections. However, a high incidence of postoperative pancreatic fistula in such procedures has been reported. Moreover, branch-type intraductal papillary mucinous neoplasms of the pancreas tend to locate in the head of the pancreas, and show less malignant potential. We describe an endoscopic naso-pancreatic stent-guided single-branch resection of the pancreas for branch-type multiple intraductal papillary mucinous adenomas, along with a gastric wall-covering method for the prevention of pancreatic leakage.



**Key words:** Intraductal papillary mucinous neoplasm; Single-branch resection; Gastric wall-covering method

Kuroki T, Tajima Y, Tsutsumi R, Tsuneoka N, Kitasato A, Adachi T, Kanematsu T. Endoscopic naso-pancreatic stent-guided single-branch resection of the pancreas for multiple intraductal papillary mucinous adenomas. *World J Gastroenterol* 2006; 12(44): 7203-7205

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## INTRODUCTION

Intraductal papillary mucinous neoplasm (IPMN) of the pancreas is characterized by dilatation of the main and/or branch pancreatic ducts, mucin-secretion, and intraductal papillary growth. IPMNs are classified into three types, a main pancreatic duct type, branch type, and mixed type. The branch type IPMN is more frequently located in the head of the pancreas, and shows less malignant potential[1,2]. Therefore, several new surgical procedures for minimal resection of the pancreas with pancreatic functional reserve have been proposed, including duodenum-preserving pancreatic head resection, segmental pancreatectomy and single-branch resection of the pancreas[3-5]. Single-branch resection is a minimally invasive surgical procedure for the resection of the pancreas because it requires no reconstruction for the main pancreatic duct or the common bile duct. In addition, single-branch resection should avoid injury to the main pancreatic duct which could cause a postoperative pancreatic fistula. Although several surgical techniques and devices have been advocated to avoid breaking the main pancreatic duct, the incidence of pancreatic fistula is still relatively high[6]. We report herein a case of successful single-branch resection for two IPMNs within the head of the pancreas under the guidance of intraoperative pancreatography by using an endoscopic naso-pancreatic drainage (ENPD) tube along with a gastric wall-covering technique for the prevention of pancreatic leakage.

## CASE REPORT

A 59-year-old man was referred to our hospital for further evaluation of two cystic tumors in the head of the pancreas which were detected by routine ultrasonography (US). Upon admission, there were no abnormal findings in the laboratory data. The endocrine pancreatic function by a 75 g oral glucose tolerance test and the exocrine function by a pancreatic function diagnostic (PFD) test were within normal range. Abdominal US and computed tomography (CT) showed two multilobular cystic lesions, 15 mm and 40 mm in diameter, respectively, in the head of the pancreas. Endoscopic retrograde pancreatography (ERP) showed two cystic lesions communicating with the main pancreatic duct. Although mucous secretion was detected from the duodenal papilla, the main pancreatic duct showed no abnormality. Magnetic resonance cholangiopancreatography (MRCP) and ERP revealed a communicating branch to these two cystic lesions (Figure 1). No mural nodules were noted in the cysts. The cytology of

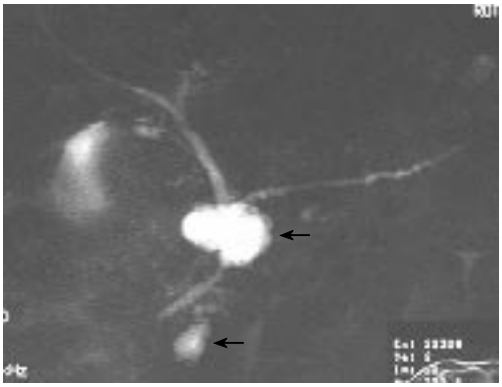

**Figure 1** MRCP showing two cystic lesions in the head of the pancreas (arrows).

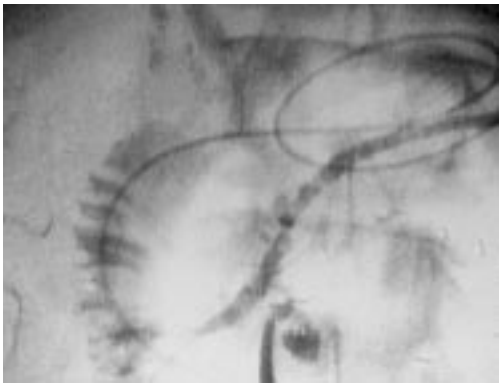

**Figure 2** Intraoperative pancreatography using an ENPD tube demonstrates a communicating duct between the main pancreatic duct and cystic lesion.

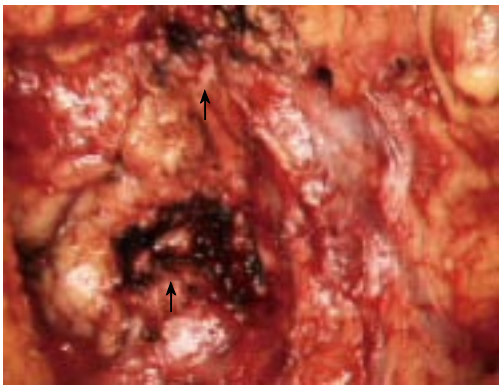

**Figure 3** Operative photograph shows the pancreas after completion of the single-duct resection of the pancreas for multiple IPMNs. Arrows indicate the cut surface after pancreatic resection.

the pancreatic juice was negative for malignant cells. K-ras point mutation at codon12 was detected in the pancreatic juice. Consequently, the cystic lesions were diagnosed as a branch-type intraductal papillary mucinous neoplasm (IPMN) and the patient was considered a candidate for surgery.

Under general anesthesia, we placed a 5F-size ENPD tube (Olympus, Tokyo Japan) for intraoperative pancreatography prior to laparotomy. The ENPD tube was inserted deep into the main pancreatic duct for approximately 10 cm through the orifice. After an upper abdominal midline incision, the gastrocolic ligament was divided and the pancreas exposed. The duodenum was mobilized to facilitate access to the head of the pancreas. The communicating branches between the cystic lesions and main pancreatic duct were carefully evaluated by using intraoperative US and pancreatography (Figure 2), and a single-branch resection for both cystic tumors was then performed. During the resectioning of the cystic tumors, the ENPD tube in the main pancreatic duct was clearly and easily detected by intraoperative US, and thus both the direction and position of the main pancreatic duct and the correlation between the cystic tumors and main pancreatic duct were correctly identified. The two cystic tumors were dissected along the border of the tumor and the surrounding normal pancreatic parenchyma, and then the confluences of the pancreatic branch ducts and main pancreatic duct were ligated using a 4-0 non-absorbable monofilament suture. Intraoperative frozen section histological examination of the dissected pancreatic ducts revealed a disease-free margin. After removal of the tumors, pancreatography through the ENPD tube was performed and revealed no injury to the main pancreatic duct or leakage from the transected pancreatic branch ducts. Because a mixture of indigocarmine and contrast material was used for the pancreatography, we were able to detect any minor pancreatic leakage as a blue spot at the cut surface of the pancreas and close the leaking points appropriately with 4-0 absorbable monofilament sutures. After pancreatic resection, the ENPD tube was used for the drainage of the pancreatic duct. It was not necessary to perform any reconstruction of the digestive tract (Figure 3). To prevent pancreatic leakage from the small pancreatic branches at the cut surface of the pancreas, the "gastric wall-covering method [7]" was performed as follows: each cut surface after resection of the two cystic tumors was fixed to the posterior wall of the gastric body by suturing between the pancreatic parenchyma and the seromuscular layer of the stomach with 4-0 absorbable monofilament. The operating time was 225 min, and blood loss was 240 mL. Histological diagnosis was intraductal papillary mucinous adenoma of the pancreas. The ENPD tube was removed on the 14th postoperative day. The postoperative course was uneventful, and the patient was discharged home on the 25th postoperative day with no endocrine or exocrine pancreatic insufficiency.

## DISCUSSION

The branch type of IPMN of the pancreas shows a more favorable prognosis than invasive ductal adenocarcinoma of the pancreas. In the benign or low-grade malignant IPMN, complete tumor resection is sufficient for a cure[1,2]. Therefore, several organ-preserving techniques, including duodenum-preserving pancreatic head resection, have been advocated in the surgical treatment for IPMN in the head of the pancreas[3]. The advantage of duodenum-preserving pancreatic head resection is the complete preservation of the duodenum, including the duodenal papilla sphincter function, compared with a pylorus-preserving pancreaticoduodenectomy. However, the

duodenum-preserving pancreatic head resection requires a reconstruction of the main pancreatic duct and/or common bile duct. In addition, another challenge when performing the duodenum-preserving pancreatic head resection is preserving the blood supply to the duodenum. Therefore, single-branch resection of the pancreas for multiple IPMNs has been performed as an ultimate organ-preserving surgery[5]. In the present case, we developed single-branch resection of the pancreas using an ENPD tube for multiple branch-type IPMNs as an ideal pancreas-preserving surgery. The most important advantage of the single-branch resection is both the complete preservation of the main pancreatic duct and the complete removal of the lesions. The insertion of an ENPD tube was useful for identifying the pancreatic duct in order to prevent injury to the main pancreatic duct. The ENPD tube in the main pancreatic duct is easily detectable using intraoperative US, and it can demonstrate a repeated intraoperative pancreatography. The first benefit of intraoperative pancreatography using an ENPD tube is that the identification of the anatomical relationship between the cystic lesions and main pancreatic duct can be confirmed not only by a radiological image, but also by a direct palpation. In addition, we used a mixture of a dye and contrast medium for the intraoperative pancreatography, which allowed us to identify the pancreatic leakage directly as a visible blue point by detecting the dye leakage. Pancreatic leakage is the most frequent complication and is still responsible for most mortality after pancreatic surgery[6]. In particular, the incidence of pancreatic leakage following partial resection of the pancreas is high. In the present case, a novel surgical procedure, a "gastric wall-covering method," was performed to prevent pancreatic leakage[7]. The concept of the gastric wall-covering method is that the opening small pancreatic ducts at the cut surface as a cause of pancreatic leakage were covered completely with the gastric wall. As shown in our patient, the multiple partial resections of the pancreas had a wide cut surface. Therefore, the gastric wall-covering method is useful for the prevention of pancreatic leakage from the opening of the small pancreatic branch ducts at the cut surface.

In conclusion, single-branch resection of the pancreas, without reconstruction of the pancreatobiliary tract, is the most ideal, least invasive pancreatic resection for a low-grade malignant neoplasm, including multiple IPMNs. ENPD tube-guided pancreatectomy is an improved technique that allows the surgeon to more accurately determine the precise location and correlation of tumors and pancreatic ducts.

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*CASE REPORT*

# Thrombotic microangiopathy involving the gallbladder as an unusual manifestation of systemic lupus erythematosus and antiphospholipid syndrome: Case report and review of the literature

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## Abstract

Gallbladder disease is no more common in patients with systemic lupus erythematosus (SLE) than in the general population. We describe a 17-year-old patient with SLE, who developed nephritis that was well controlled with medications. Initial treatment consisted of azathioprine, aspirin and prednisone with stable control of her symptoms. Two years later she developed a right quadrant abdominal pain, and an abdominal ultrasound revealed microlithiasic cholecystitis. Open cholecystectomy was performed and the histopathological findings revealed vasculitis with thrombotic microangiopathy in the gallbladder. This case presentation illustrates that calculous or acalculous cholecystitis should be considered as a manifestation of active SLE and APS.



**Key words:** Gallbladder; Cholecystitis; Systemic lupus erythematosus; Antiphospholipid syndrome

De-Leon-Bojorge B, Zaltzman-Girsevich S, Ortega-Salgado A, Prieto-Patron A, Córdoba-Córdoba R, Yamazaki-Nakashimada M. Thrombotic microangiopathy involving the gallbladder as an unusual manifestation of systemic lupus erythematosus and antiphospholipid syndrome: Case report and review of the literature. *World J Gastroenterol* 2006; 12(44): 7206-7209

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## INTRODUCTION

Gallbladder disease is no more common in patients with systemic lupus erythematosus (SLE) than in the general population[1]. Scarce reports on acalculous cholecystitis (ACC) in SLE or in antiphospholipid syndrome (APS) have been described in the literature[2-12]. We describe a patient with SLE, nephritis, pancreatitis and microlithiatic cholecystitis. Open cholecystectomy was performed and the histopathological findings revealed vasculitis and thrombotic microangiopathy in the gallbladder. She was diagnosed with secondary APS and started on anticoagulation therapy. Coincidentally, her lupus nephritis also improved. This case illustrates that calculous or acalculous cholecystitis should be considered as a manifestation of SLE and APS.

## CASE REPORT

In 2002, a 17-year-old female with arthritis and oral ulcers was referred to the Instituto Nacional de Pediatría in Mexico City. Laboratory results showed autoimmune hemolytic anemia, leucopenia, lymphopenia, hypocomplementemia. She also had positive anti-nuclear antibodies (ANA), anti-dsDNA, anti-$\beta_2$-glicoprotein-I, and anti-cardiolipin antibodies. Based on her clinical presentation and laboratory studies, she was diagnosed with SLE. Initial treatment consisted of azathioprine, aspirin and prednisone with stabilization of symptoms. In 2003 she presented with renal complication including proteinuria and hematuria. Oral cyclophosphamide was started with improvement in her renal function. In 2004 she developed right quadrant abdominal pain, and an abdominal ultrasound revealed microlithiasic cholecystitis. The abdominal pain spontaneously resolved and dietary modifications were initiated. She was clinically well until July 2005 when she presented with a flare consisting of hypertension, autoimmune hemolytic anemia and marked proteinuria.

On admission the physical examination was unremarkable. Laboratory tests showed proteinuria: 7.2

g/d, serum Cr: 1.6 g/dL, Coombs: +, Hb: 9.3 g/dL, leucocyte count: 3300/mm$^3$, total lymphocyte count: 369, platelets: 218000/mm$^3$. Immunological tests were positive for anti-dsDNA, anti-β2-glicoprotein- Ⅰ, and anti-cardiolipin antibodies, CH50: 49 IU/mL (normal 150-250 IU/mL), C3: 21 mg/dL (normal 86-184 mg/dL), C4: 5.3 mg/dL (normal 19-58 mg/dL). She was started on methylprednisolone and cyclophosphamide.

A renal biopsy showed a global, diffuse, proliferative and membranous glomerulonephritis (class Ⅳ-Ⅴ) without thrombosis. During her admission she developed acute abdominal pain, including a positive Murphy sign on exam. Liver and pancreatic function tests were elevated: gamma-glutamyltranspeptidase was 210 mU/mL (30-60 mU/mL), AST 339 mU/mL (11-33 mU/mL) ALT 154 mU/mL (4-30 mU/mL), total bilirubin 1.1 mg/dL (0.2-1.0 mg/dL) with direct bilirubin, 0.6 mg/dL,amylase 932 mU/mL (68-215 mU/mL) and lipase 649. Abdominal ultrasound revealed lithiasic cholecystitis and pancreatitis. The patient underwent open cholecystectomy and histopathological findings of the gallbladder showed segmental necrotizing vasculitis and vascular occlusion with fragmented erythrocytes (Figures 1A and 1B). Anticoagulation therapy was started with subsequent resolution of the proteinuria and normalization of the laboratory values including the creatinine level.

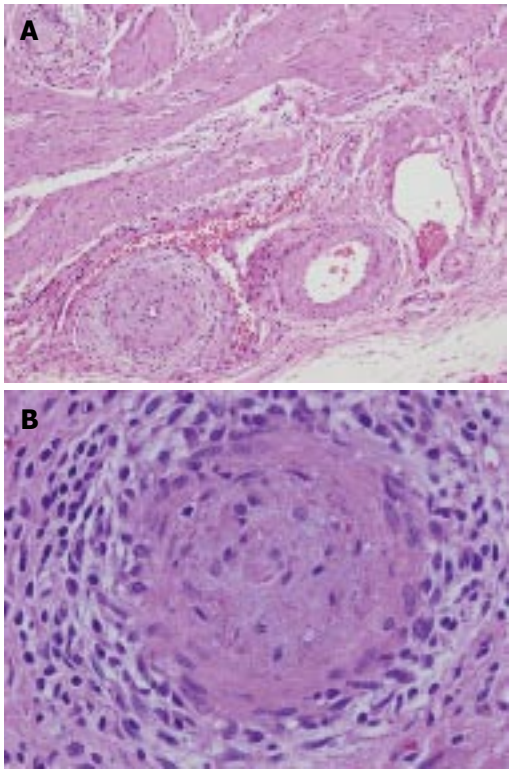


**Figure 1** Occlusion with fibrosis in a gallbladder artery (HE x 1) (**A**); Total artery occlusion, fragmented erythrocytes and perivascular inflammation with mononuclear infiltration (**B**).

## DISCUSSION

A variety of gastrointestinal complications have been reported in association with systemic lupus erythe-matosus (SLE), including mesenteric arteritis, intestinal perforation, and peritonitis[9]. Gallbladder disease is no more common in patients with systemic lupus erythematosus than in the general population[1]. Gallstone disease is a common condition in Western populations, and its etiology is multifactorial, including genetic and environmental factors. Hemolysis, obesity, aging, estrogen treatment, pregnancy and diabetes are consistently associated with a higher risk of developing gallstones[10].

Symptomatic gallbladder vasculitis requiring treatment by cholecystectomy is rare. Chen[13] has divided the gallbladder vasculitis into three groups: gallbladder vasculitis as a manifestation of polyarteritis nodosa (group 1), gallbladder vasculitis occurring in diseases known to be associated with vasculitis as in our SLE case (group 2) and isolated gallbladder vasculitis (group 3). In 1983, Swanepoel *et al*[5] described the first case of acute acalculous cholecystitis (ACC) in SLE. Since then, only a few SLE cases have been reported in the literature. Newbold *et al*[9] have reported two cases of gallbladder involvement in patients with SLE; histologically there was acute arteritis with periarterial fibrosis, changes similar to those found in polyarteritis nodosa. In fact, the development of gallbladder disease appears to be more common in polyarteritis nodosa than in SLE. Recently, Bando *et al*[12] have reported a case of acalculous cholecystitis induced by mesenteric inflammatory veno-oclusive disease in SLE. Although surgical removal of the gallbladder is the generally accepted treatment for SLE patients who develop acute cholecystitis successful treatment with corticosteroids alone has also been reported[11]. It is of note that, rarely, the symptoms of serositis can mimic those of acute cholecystitis[13,14].

Antiphospholipid syndrome (APS) was first described in patients who suffered from recurrent fetal loss and thromboses. Since then the spectrum of associated symptoms in APS has broadened considerably. The clinical criteria for diagnosis of APS include arterial, venous, or small vessel thrombosis in any tissue or organ. Thrombosis has to be confirmed by imaging, Doppler studies or histopathology. For laboratory diagnosis of APS, affected patients have antibodies to β2-glicoprotein- Ⅰ, cardiolipins or positive lupus anticoagulant in two separate occasions at least 6 wk apart. Gallbladder involvement secondary to APS has rarely been described in the literature. Dessailloud *et al*[2] reported a fatal case of a 29-year-old woman with acalculous cholecystitis secondary to catastrophic APS. The patient developed mesenteric ischemia, left limb ischemia and acute renal failure. In another report, Nolen *et al*[3] have described a 26-year-old female with primary APS who was treated successfully with medical management. Rhoton *et al*[8] described a 22-year-old woman with SLE who developed hemobilia, and also had high titers of anticardiolipin antibodies.

Our case presented with thrombotic and inflammatory complications involving the gallbladder, likely secondary to both SLE and APS. The vasculopathy showed thrombotic microangiopathy (TMA) and perivasculitis. Although microlithiasis could have contributed to the inflammatory process located in the gallbladder and pancreas, the presence of vasculitis reflects an autoimmune process.

**Table 1 SLE and APS cases reported in the literature with gallbladder involvement**

| Author | Gender | Age(yr) | Diagnosis | Gallbladder pathology | Serology |
|---|---|---|---|---|---|
| Present report (2006) | F | 17 | SLE/APS | TMA<br>Vasculitis | ANA<br>Anti-DNA<br>Anticardiolipin |
| Newbold *et al* (1987) | F | 28 | SLE | Acalculous<br>Cholecystitis<br>vasculitis and<br>thrombosis | ANA |
| Newbold *et al* (1987) | F | 38 | SLE | Small vessel<br>vasculitis | ANA<br>Anti-DNA |
| Kamimura *et al* (1998) | F | 27 | SLE | ACC | ANA<br>Anti-DNA<br>Anti-Sm |
| Rhoton *et al* (1993) | F | 22 | SLE | Hemobilia | Not specified |
| Raijman *et al* (1989) | F | 34 | SLE | Hemorrhagic<br>Acalculous<br>Cholecystitis | ANA<br>Anti-DNA<br>Anticardiolipin<br>Anti-platelet antibodies |
| Swanepoel *et al* (1983) | F | 22 | SLE | AAC | ANA |
| Bando *et al* (2003) | F | 43 | SLE | AAC | ANA<br>Anti-DNA |
| Shin *et al* (2002) | F | 39 | SLE<br>Sjogren<br>syndrome | AAC | ANA<br>Anti-dsDNA<br>Anti-Ro |
| Nolen *et al* (1999) | F | 26 | APS | AAC | Anticardiolipin<br>Lupus antcoagulant |
| Kara *et al* (2004) | M | 65 | APS | AAC | Not reported |
| Dessailloud *et al* (1998) | F | 29 | APS | Acalculous<br>Ischemic<br>Gallbladder<br>Necrosis | ANA<br>$\beta_2$-glycoprotein- I<br>Antiphosphatidylserine |

Eventually the histopathological findings helped us diagnose the APS and initiate anticoagulation therapy, which led to improvement of our patient's renal disease. Although presence of small gallstones is associated with increased risk of acute pancreatitis, our patient's pancreatitis could also be a manifestation of APS[15]. All the SLE and APS cases reported in the literature with gallbladder involvement are summarized in Table 1. Of note is that our patient is the youngest patient described to date, reflecting the rarity of this complication in the pediatric age group.

Renal thrombotic manifestations have been reported since the initial description of APS. TMA is the best known characteristic lesion of APS nephropathy, leading to hypertension, proteinuria and renal impairment. Recently, consideration of the APS nephropathy in every SLE case has been emphasized because successful treatment requires both anticoagulation in addition to immunosuppresion[16].

In summary, although rare, gallbladder involvement in patients with SLE should raise the suspicion of vasculitis or APS. We believe that both gallbladder vasculitis and thrombosis contributed in the clinical picture of our patients. Sometimes surgical cholecystectomy is needed for both therapeutic and diagnostic purposes. If thrombotic manifestations of APS are documented, anticoagulation therapy should be started.

## ACKNOWLEDGMENTS

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*CASE REPORT*

# Subcutaneous extension of a large diaphragmatic hydatid cyst

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## Abstract

A 53-year-old male patient with a large hydatid cyst of the left hemidiaphragm and smaller secondary cysts located in the left thoracic cavity and upper left abdominal quadrant presented with two progressively enlarging lipoma-like masses in the left hypochondrium and under the left scapulae respectively. Total excision of all the cysts was performed through a bilateral subcostal incision, with the left hemidiaphragm near totally excised and replaced by a synthetic bilayer mesh.



**Key words:** Hydatid cyst; Diaphragm; Mesh reconstruction; Intrathoracic cavity; Subcutaneous extension

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## INTRODUCTION

Hydatid disease is a parasitosis, known as hydatidosis or echinococcosis caused by the larval stage of the Echinococcus granulosus tapeworm. The disease was first described by Hippocrates as "cysts full of water" in a man's liver and by Aristotle in human lungs and liver[1]. The disease remains a considerable public health problem in endemic countries, including the Mediterranean and Middle East countries, New Zealand, Australia, South Africa and South American countries. The primary hosts are members of the Canidae family (dogs, wolves and coyotes); the intermediate hosts are sheep, cattle and deer[2]. Humans are accidental intermediates in the biologic cycle of taenia echinococcus granulosus, more frequently by direct contact with dogs than by ingestion of food or water contaminated with their feces[3]. The most commonly involved organs are the liver and lungs. Extrapulmonary intrathoracic location of a cyst involving the mediastinum, pleura, pericardium, chest wall and diaphragm is rare[4] and even rarer is the subcutaneous extension of echinococcus. Herein, we report a 53-year-old man with a primary large hydatid cyst of the diaphragm and smaller secondary hydatid cysts of the abdomen and thorax protruding subcutaneously.

## CASE REPORT

A 53-year-old farmer with a past medical history of two car accidents at 16 and 18 years of age, which resulted in a left hemothorax evacuated with a chest tube in the first one and a fracture of the left femur internally fixated in the second one, was admitted to our surgical department. The patient's main complaint was the presence of a small soft lump in the left hypochondrium 15 years ago that was gradually increasing in size. A similar lump protruding under the left scapulae 1 mo before his admission presented without any symptoms indicating absence of compression of adjacent organs. Diminished breath sounds in the lower left hemithorax, the presence of a small (3-5 cm) subcutaneous lump between the posterior segments of the 7th and 8th ribs and a larger (13-15 cm) soft-tissue subcutaneous mass in the left hypochondrium were the clinical findings. Chest radiograph showed a left-sided opacity with scarce microcalcifications (Figure 1A). Pulmonary functional tests were normal. Thoraco-abdominal CT scan (Figure 2) revealed a hydatid cyst (14 cm in diameter) located in the left hemidiaphragm extending simultaneously to the thoracic cavity and the upper abdomen (cyst a), a cystic lesion (6 cm in diameter) in contact with the left side of the pericardium and the anterior surface of pleura anteriorly to the previous cyst (cyst b), a cystic lesion (9 cm in diameter) in the postero-lateral thoracic wall (cyst c) protruding intercostally to a small subcutaneous cyst under the scapulae (solid arrow) and an abdominal cystic lesion (13 cm in diameter, cyst d) protruding through the left costal cartilage to the left hypochondrium subcutaneously (broken arrow). CT scan showed a sand-glass appearance of the last two cysts. Laboratory tests were normal and serologic tests were

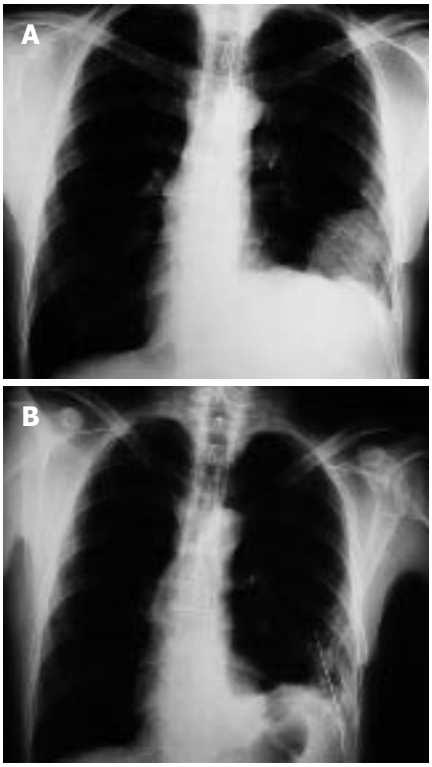


**Figure 1** (**A**) Preoperative chest radiograph showing the left-sided opacity and (**B**) postoperative chest radiograph showing the chest tube placed in the left thoracic cavity.

positive for echinococcus granulosus (1/2048 antibodies, using enzyme-linked immunosorbent assay).

A bilateral subcostal incision was performed. Precautions were taken in order to avoid any spillage of cyst contents in the operation field including intact excision of all hydatid cysts and placement of sponges impregnated with hypertonic saline surrounding the involved organs. The subcutaneous hydatid cyst of the left hypochondrium was removed with its intra-abdominal portion and a partial resection of the left costal cartilage. The large hydatid cyst of the left hemidiaphragm embedded in the muscular layer was free of adhesions to the adjacent abdominal and thoracic organs. A total excision of this cyst with a nearly total excision of the left hemidiaphragm was performed. The hydatid cyst adjacent to the pericardium was completely removed. The hydatid cyst on the posterolateral thoracic wall was removed with concomitant resection of the subscapular protrusion and the eroded posterior segment of the 9th rib. The left hemidiaphragm was replaced by a bilayer synthetic mesh (Bard Composix ® Mesh 15.2 cm × 20.3 cm) and a chest tube was placed in the left thoracic cavity (Figure 1B). The patient was discharged on the 7th postoperative day and albendazole therapy was started at a dose of 400 mg bid. The postoperative course of our patient during the follow-up period (14 mo) was uneventful, with a good respiratory function.

## DISCUSSION

Hydatid disease most frequently involves the liver (50%-70%) and lungs (20%-30%), which can be explained by a hypothesized novel dissemination theory via diaphragmatic lymphatic drainage proposed by Isitmangil *et al*[5]. However, other organs may be involved as well,

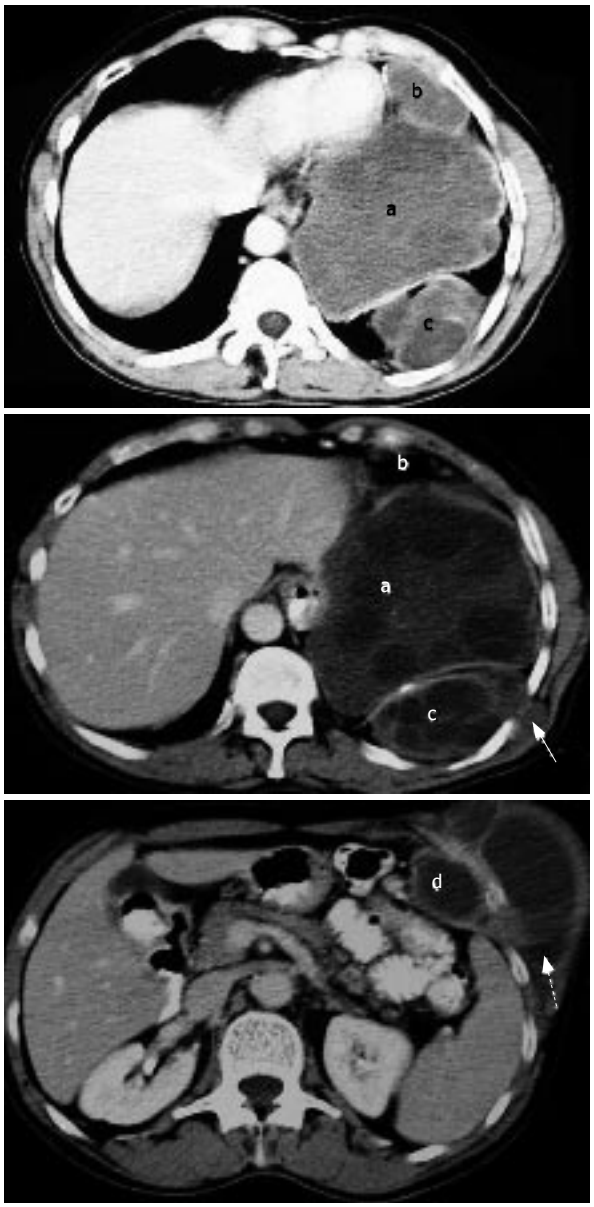


**Figure 2** Thoraco-abdominal CT scan showing four separate hydatid cysts located in the left hemidiaphragm (a), anteriorly in contact with the left side of the pericardium (b) and the posterolateral wall of the left hemithorax (c) and intra-abdominally in contact with the left hypochondrium (d). Subcutaneous extension of hydatid cyst c protruding intercostally to a small subcutaneous cyst under the scapulae (solid arrow) and abdominal cyst d protruding through the left costal cartilage to the left hypochondrium subcutaneously (broken arrow) are demonstrated.

such as the brain, heart, bone and muscle (10%)[6]. Subcutaneous location or extension of a hydatid cyst is even more rare[7-9]. This rare localization of echinococcosis presents as painless soft tissue tumors or lipomas and the patient usually does not seek surgical advice.

Total excision of hydatid cysts is the definitive therapy. A thorough preoperative study of the involved organs combining the findings of chest radiograph, ultrasonography, computed tomography and magnetic resonance imaging is necessary. The bilateral subcostal incision performed in this case has the advantage of a better approach to both the abdominal and thoracic lesions, without necessitating the use of standard thoracotomy which is preferred to treat intrathoracic extrapleural

hydatid disease. However, repair or reconstruction of the diaphragm poses a technical challenge. If a synthetic mesh is used to close the defect, it must be properly applied and sutured under tension to avoid paradoxical movement and respiratory distress postoperatively. Moreover, septic complications related to mesh reconstruction during these procedures are rare and have not been reported in the literature.

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*CASE REPORT*

# Esophageal perforation caused by fish vertebra ingestion in a seven-month-old infant demanded surgical intervention: A case report

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## Abstract

A seven-month-old infant was admitted to our hospital with a 1-wk history of shortness of breath, dysphagia, and fever. Diagnosis of esophageal perforation following fish vertebra ingestion was made by history review, pneumomediastinum and an irregular hyperdense lesion noted in initial chest radiogram. Neck computed tomography (CT) confirmed that the foreign body located at the cricopharyngeal level and a small esophageal tracheal fistula was shown by esophagogram. The initial response to treatment of fish bone removal guided by panendoscopy and antibiotics administration was poor since pneumothorax plus empyema developed. Fortunately, the patient's condition finally improved after decortication, mediastinotomy and perforated esophagus repair. To our knowledge, this is the first case report of esophageal perforation due to fish bone ingestion in infancy. In addition to particular caution that has to be taken when feeding the innocent, young victim, it may indicate the importance of surgical intervention for complicated esophageal perforation in infancy.



**Key words:** Fish bone; Esophageal perforation; Mediastinitis; Decortication; Mediastinotomy

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## INTRODUCTION

In East Asia (China, Japan, Korea), fish bone ingestion is a common cause of emergency room visits and usually needs no invasive management other than prompt removal of the fish bone[1,2]. However, fish bone impaction complicated with esophageal perforation and pneumomediastinum is life-threatening and needs emergency surgical management. Most of the patients are above one year old since self-feeding has not begun at infancy. The mortality is extremely high if diagnosis is delayed and the sequelae might exhaust the patient and cause a catastrophic result. The outcome depends on the size of the rupture, the time elapsed between rupture, and diagnosis, as well as the underlying health of the patient[1]. Successful treatment is very difficult. Here, we report such a case of 7-mo-old infant of fish bone ingestion, and review the related literature.

## CASE REPORT

A seven-mo -old male infant weighing 7.8 kg was brought to our pediatric emergency room for progressive shortness of breath and fever over the previous 1 wk. Associated symptoms included progressive irritability, poor sleep quality, fever off and on, drooling, and dysphagia. On review of his history, he was previously healthy and had no admission record. The patient's development and growth were within the normal range. He had been fed fish-rice soup as a nutrition supplement since the age of 5 mo. The initial chest radiogram showed pneumomediastinum (Figure 1A), and an irregular hyperdense lesion in the cricopharyngeal area was seen in the lateral view of the neck (Figure 1B). He was admitted to our pediatric Intensive Care Unit with unstable vital signs. Due to his feeding history, fish bone ingestion was strongly suspected. Emergency neck computed tomography (CT) and an esophagogram were arranged to confirm the diagnosis. The neck CT showed a hyperdense material at the cricopharyngeal level (Figure 2) and a small fistula tract at C3–C4 was noted on the esophagogram (Figure 3).

The day following admission, he was intubated via a bronchoscope to establish an accurate tube position. Panendoscopy was then performed after sufficient sedation

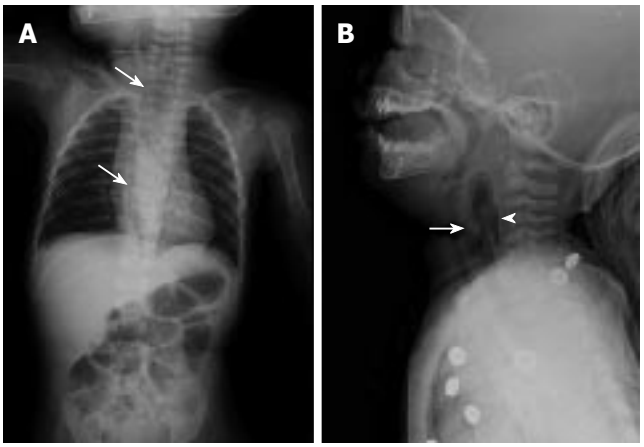


**Figure 1** **A**: Extensive pneumomediastinum and air collections in retropharyngeal area (arrows), **B**: Irregular hyperdense lesion (arrow head) around cricopharyngeal area, and intra-esophageal opacification (arrow) was noted at C3 - C4 level.

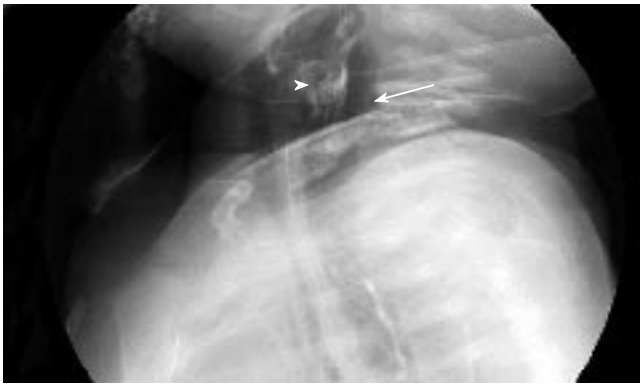

**Figure 3** Much air collection (arrow) was in the retropharyngeal space and mediastinum, contrast leakage (arrow head) into the retropharyngeal space with opacification of a small fistulous tract at C3-4 level (cricopharyngeal level), suggestive of esophageal perforation.

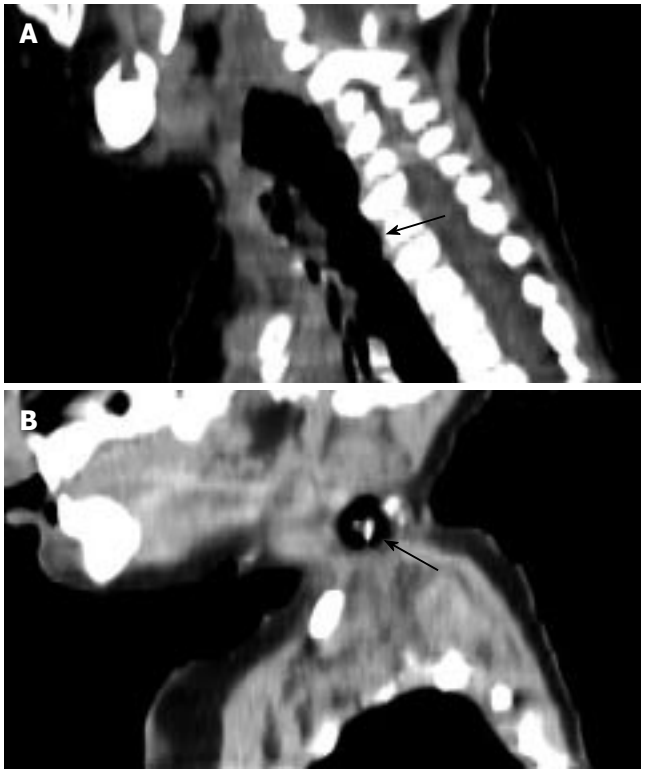


**Figure 2** **A**: Esophageal rupture with retropharyngeal air collection (arrow), and **B**: Irregular foreign body impacted at upper esophagus (arrow).

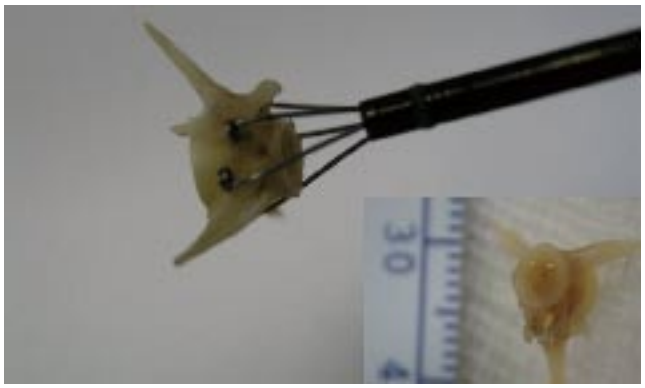

**Figure 4** The fish vertebra with three sharp spines, which is about 1 cm x 1 cm in size, was removed by grasping forceps.

of the patient. A fish bone (part of a vertebra) measuring 1 cm × 1 cm was found near the esophageal inlet, which was removed smoothly with grasping forceps (Figure 4).

Although the fish bone was removed, antibiotics (ceftriaxone + amikin + metronidazole) were administrated empirically to prevent esophageal wound infection. The patient continued to drool and suffer from dyspnea on the third day of admission, and a subsequent chest radiogram showed persistent retropharyngeal air collection. Bronchoscopy and panendoscopy were repeated, but did not improve the dyspnea. CT-guided retropharyngeal aspiration was performed on the fifth day of admission and 200 mL of air was aspirated. A pneumothorax with desaturation developed on the seventh day of admission, and empyema was detected after a chest tube was inserted. A pediatric surgeon was consulted, and surgery was performed. At surgery, the findings included multilocular empyema with trapped lung, mediastinitis (abscess formation), and esophageal perforation in the cervical region. Decortication and mediastinotomy were performed to remove the inflamed tissues, and the perforated esophagus was repaired with Dexon sutures. After surgical management, the medical treatment became more effective. Coagulase-negative Staphylococcus aureus was cultured in the pus, and the fever disappeared after combination treatment with antibiotics (vancomycin + ceftriaxone + metronidazole). Total parenteral nutrition was instituted, and a nasogastric (NG) tube was inserted via panendoscopy for decompression and to prevent esophageal stricture or synechia. The right Horner's syndrome that occurred postoperatively resolved within 6 mo. Although the patient's weight had decreased to 5.8 kg, he regained body weight steadily after beginning NG feeding (15 d after the operation). Complete oral feeding was achieved after 1 mo of NG feeding. The patient was discharged from our hospital after 4 mo, without developing the obvious symptoms of esophageal stricture. On follow-up, he weighed 7.7 kg at 8 mo ($20^{th}$ percentile),

9.4 kg at 13 mo (25th percentile), and 11.3 kg at 21 mo (30th percentile). No serious respiratory complications were noted.

## DISCUSSION

Foreign body ingestion is common in children and can be underestimated because there are no witnesses and 50% of cases are asymptomatic[3,4]. Less than 1% of foreign body ingestions result in serious morbidity; most cases need observation only. The peak age in children is from 6 mo to 3 years[5]. Based on previous experience with the pediatric population, fish bones are the second most commonly ingested foreign body (coins are the most common). In East Asia, fish bones are the most commonly ingested foreign body[2]. Nevertheless, fish bone ingestion is quite rare in infancy since self-feeding has not begun, and the caretaker screens the food before feeding.

The symptoms of foreign body ingestion are variable and depend on the size, shape, and material of the foreign body. Usually, large sharp objects cause greater morbidity[1]. The common signs and symptoms[6] in patients with a foreign body that has been retained for less than 24 h tend to be gastrointestinal and include dysphagia, drooling, vomiting, gagging, and anorexia. Major respiratory symptoms are more common weeks or months after ingestion, such as coughing, stridor, fever, chest pain, wheezing, chronic upper respiratory tract infections, pneumonia, and hemoptysis. Patients may also develop acute respiratory distress with choking and cyanosis. In our case, the patient's symptoms were compatible with typical foreign ingestion, and a lack of awareness delayed the diagnosis.

Only 32% of ingested fish bones can be identified radiographically[7], and most part of fish skeletons that cause impaction in esophagus is fish rib. Fish vertebra was seldom reported. Due to its prolonged course, pneumomediastinum was seen on the plain chest radiograph in our patient. The esophagogram confirmed the diagnosis of foreign body impaction and located the esophageal perforation. In fact, instrumental perforation and spontaneous perforation are the two major causes of esophageal injury in infants and children. Esophageal perforation due to fish bone ingestion in infancy has not been reported. Furthermore, the complications of mediastinitis, pneumothorax, and empyema were life-threatening in our patient. The following guidelines are suggested for selecting nonoperative treatment[8]: clinically stable patients; instrumental perforations detected before major mediastinal contamination has occurred, or perforations with such a long delay in diagnosis that the patient has already demonstrated tolerance for the perforation without the need for surgery; and esophageal disruptions well contained within the mediastinum or a pleural loculus. In a series of 12 children (3-7 years old) with esophageal perforation, Demirbag *et al*[9] reported that esophageal perforation could be treated safely by nonoperative means. Rivas *et al*[10] recommended conservative treatment as the best option for esophageal perforation in children. In contrast to adults, children have some important advantages in esophageal perforation with regard to complication severity, wound-healing rate, and mediastinal tissue resistance. In our case, however, the patient was younger (7 mo old), and the diagnosis delayed (about 1 wk). Although the fish bone was removed via panendoscopy soon after admission, the conservative management was ineffective, and surgery was required.

Total parenteral nutrition supplement played an important role in the postoperative therapy. It provided sufficient nutrition for the patient and allowed complete rest of the gastrointestinal tract. Treatment with broad-spectrum intravenous antibiotics was also essential for reversing the potentially fatal infection secondary to esophageal perforation. In this case, coagulase-negative S. aureus was identified on culturing of the pus. This organism is the predominant pathogen causing empyema in developing countries[5].

Two special techniques were applied in this case: bronchoscopy for intubation and CT-guided aspiration to reduce the retropharyngeal air collection. Both of these measures prevented deterioration in the patient's respiratory status. The excellent outcome in this patient was beyond our expectations. No major complications were noted one year later. We believe that the great support of his family contributed equally to the medical management.

To our knowledge, this is the first case report of esophageal perforation complicated by fish bone ingestion in infancy. Although current guidelines of therapy for fish bone ingestion in children remains conservative, this case illustrates the possibility of unpredictable and severe complications which demand surgical intervention.

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*CASE REPORT*

# Synchronous occurrence of carcinoid, signet-ring cell carcinoma and heterotopic pancreatic tissue in stomach: A case report and literature review

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## Abstract

We presented an unusual case with coexistence of carcinoid, signet-ring cell carcinoma (SRC) and heterotopic pancreatic tissue in stomach. Gastroscopic examination of this 63-year-old male patient showed multiple protrusions in gastric corpus near the greater curvature, identified by subsequent biopsy as carcinoid. Distal subtotal gastrectomy was performed. Histological and immunohistochemical examinations showed a carcinoid tumor in gastric corpus near the greater curvature, an intramucosal SRC at the lesser curvature of corpus and heterotopic pancreatic tissue in muscularis propria of the antrum at the lesser curvature with hyperplasia of peripheral endocrine cells producing multiple pancreatic hormones. We reviewed the literatures on clinicopathological characteristics and the differential diagnosis of the above three abnormalities, and concluded that the carcinoid in corpus near the greater curvature and SRC in the lesser curvature are independent lesions; the foci of endocrine cells in the muscularis propria and serosa are hyperplastic lesions from the heterotopic pancreatic tissue, rather than dissemination of carcinoid in corpus.



**Key words:** Heterotopic pancreas; Carcinoid; Signet-ring cell carcinoma; Gastric tumour; Endocrine cell hyperplasia

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## INTRODUCTION

Gastric carcinoid is rare, accounting for 0.4%-1.8% in tumors of the gastrointestinal tract[1], while signet-ring cell carcinoma (SRC) is frequent[2]. Both of them occur often in an occult way, with different behavior and prognosis. Pancreatic heterotopia in stomach is often incidentally encountered during surgery or autopsy, with its incidence ranging from 0.6% to 13.7%[3]. We presented a case with synchronous occurrence of these three lesions in stomach, and reviewed related literatures.

## CASE REPORT

A 63-year-old male patient visited this hospital with the complaint of "cauterized discomfort in the upper abdomen for more than 2 years, and aggravated for 2 mo". By gastroscopy, multiple flat nodules (Lesion 1) were found in the front wall near the greater curvature, ranging from 0.2-0.8 cm in diameter. Mucosal biopsy was taken, and the lesions were considered a malignant neuroendocrine cell tumor with the help of immunohistochemistry. Findings from physical examination were unremarkable except for deep tenderness under xiphoid. Chest X-ray, upper alimentary canal contrast, abdominal ultrasonography failed to detect any abnormalities, while a slight increase in thickness of the antrum wall was observed by computed tomography (CT) scanning. Levels of circulating CA199, CEA, CA724 and CA242 were in normal ranges. Serum antibody against parietal cell was positive, and serum gastrin was 93.2 mg/L. No family history of cancer was recorded. Distal subtotal gastrectomy specimen was routinely resected. The samples were fixed in 4% buffered formaldehyde, and embedded in paraffin. Sections of 5 μm in thickness were prepared and stained with hematoxylin and eosin (HE) and Alcin blue/periodic acid Schiff reaction (AB/PAS). Immunohistochemical staining was conducted using a polymer-peroxidase detection kit (PV-9000) following the instructions of the manufacturer. Polyclonal antibodies were used to detect glucagon, gastrin, somatostatin, vasoactive intestinal peptide (VIP), pancreatic polypeptide (PP), and monoclonal antibodies were used for demonstration of CK18, insulin, ACTH, synaptophysin (SY), chromogranin A (CgA), NSE, vimentin, p53 protein (DO-7) and Ki-67 antigen. All of the reagents were supplied by Zhongshan Golden Bridge Biotech Co. Ltd., Beijing. Samples of a gastric SRC and a

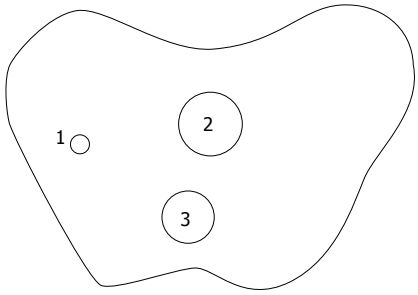


**Figure 1** Diagrammatic representation of three lesions. 1: Carcinoid nodule of 0.7 cm in diameter near the greater curvature of gastric corpus; 2: SRC sizing 3.0 cm × 2.0 cm within mucosa at the lesser curvature of gastric corpus; 3: Ectopic pancreas nodule of 2.5 cm in diameter between submucosa and serosa in gastric corpus-antrum.

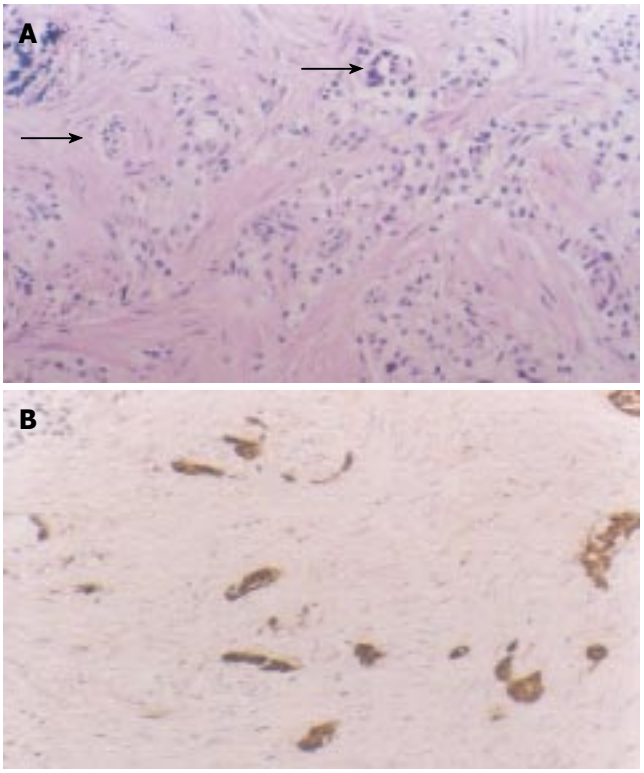


**Figure 2** Lesion 1, intramucosal carcinoid with the muscularis mucosa infiltrated (**A**) (arrows, HE × 20), positively expresses CgA (**B**) (S-P method, conterstained with hematoxylin, × 20).

pancreatic endocrine tumor were used as positive controls, and the immunoglobins from a preimmune rabbit and mouse were used to substitute for the primary antibodies as negative controls. Slides were scored semi-quantitatively according to the percentage of positive cells in all counted cells from 5 randomly selected representative fields based on staining distribution and intensity as below: positive rate < 5% was defined as negative (-); weakly positive (+): 5%-25%; moderately positive (++): 25%-50%; strongly positive (+++): > 50%.

Three non-continuous lesions (Figure 1) with distinct histochemical phenotypes (Table 1) were identified: Lesion 1 (Figure 2), multiple nodules of 0.2-0.8 cm in diameter was seen in the anterior wall near the greater curvature of

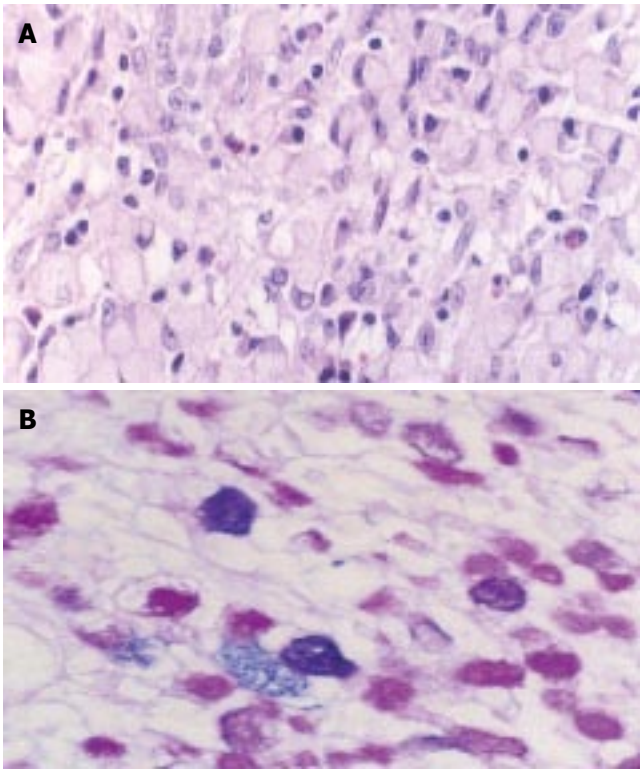


**Figure 3** Lesion 2, intramucosal signet-ring cell carcinoma (**A**) (HE × 40) with cytoplasmic mixed mucin (**B**) (AB/PAS reaction, × 40).

gastric corpus, and multiple foci of hyperplastic endocrine cells were seen in the deeper part of lamina propria, infiltrating the muscularis mucosa. Immunoreactivities were seen in the cells for CK18, NSE, SY and CgA. Therefore, Lesion 1 was regarded as intramucosal endocrine cell hyperplasia and formation of a type I carcinoid, with muscularis mucosae involved. Lesion 2 was identified beside the lesser curvature of corpus and sized 3.0 cm × 2.0 cm. The cancer cells contained mixed mucin, positive for both Alcin blue and PAS, but were negative for the neuroendocrine markers (Figure 3). Therefore, Lesion 2 was diagnosed as an intramucosal SRC (superficially flat type), rather than a signet-ring-like carcinoid. Lesion 3 (Figure 4) was a yellowish mural mass sizing 3.0 cm × 2.0 cm on cut surfaces, located between the submucosa and lamina muscularis of the antrum. Histologically, the mass was composed of well developed pancreatic acini and ducts, intermingled with hyperplastic smooth muscle tissue, giving rise to the pattern of an adenomyoma. At the peripheral area, multiple nodules of 0.1-0.4 mm in diameter were found. They were composed of well circumscribed small, bland epithelial cells which arranged in solid nests. The nodules resembled normal pancreatic islets in shape, scattered within the muscularis propria and serosa of the gastric wall. No stromal reaction was observed. The epithelial cells were shown to be diffusely positive for CK18, CgA, SY, insulin, glucagons, somatostatin and gastrin, the pattern being similar to that of pancreatic islets. Neither mitosis nor necrosis was identified. Therefore, the mass was diagnosed as pancreatic heterotopia and those peripheral nodules were hyperplastic

**Table 1 Immunohistochemical phenotypes of different mucosal lesions and various components of heterotopic pancreatic tissue in gastric wall**

| Markers | Mucosal lesions | | | Heterotopic pancreas (Lesion 3) | | | |
|---|---|---|---|---|---|---|---|
| | Carcinoid (Lesion 1) | NE cell hyperplasia | SRC (Lesion 2) | Endocrine cell hyperplasia | Islets | Acini | Ducts |
| CK18 | +++ | +++ | +++ | + | + | ++ | ++ |
| Vimentin | - | - | - | - | - | - | - |
| NSE | ++ | +-++ | - | +++ | +++ | - | + |
| CgA | +++ | +++ | - | +++ | +++ | - | + |
| Synaptophysin | +++ | +++ | - | +++ | +++ | - | + |
| Insulin | - | - | - | +++ | +++ | - | ± |
| Glucagon | - | - | - | ++ | ++ | - | + |
| Somatostatin | - | + | - | ++ | ++ | ± | ± |
| Gastrin | - | ++ | - | ++ | ++ | - | ± |
| VIP | ± | + | - | ± | ± | - | - |
| ACTH | - | - | - | - | - | - | - |
| PP | - | - | - | - | - | + | ± |
| p53 protein | 2% | - | - | - | - | - | - |
| Ki-67-LI | 2% | < 1% | 20% | 1% | < 1% | 3% | 5% |

ACTH: adrenocorticotropin; CK18: cytokeratin 18; CgA: chromogranin A; Ki-67-LI: Ki-67-lebelling index; PP: pancreatic peptide; NE: neuroendocrine; NSE: neuron-specific enolase; SRC: signet-ring cell carcinoma; VIP: vasoactive intestinal peptide.

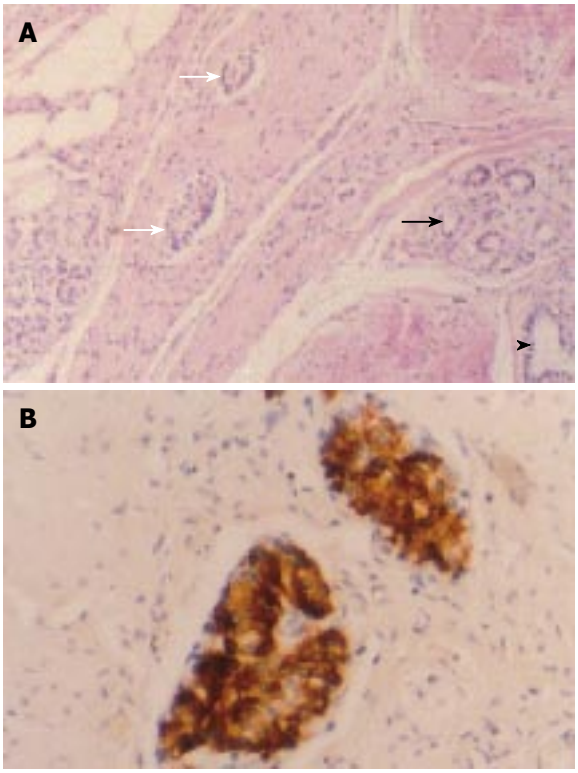


**Figure 4** Lesion 3, heterotopic pancreatic tissue in gastric wall, which was composed of well developed pancreatic acini (darker arrow), ducts (arrowhead), and islets (lighter arrow) intermingled with the hyperplastic smooth muscle tissue, giving rise to the pattern of an adenomyoma (**A**) (HE × 20); Small-cell nodules of 0.1-0.4 mm in diameter scattered within the muscularis propria and serosa of the gastric wall and strongly positive for insulin (**B**) (S-P method, conterstained with hematoxylin).

tissues from endocrine pancreatic heterotopia rather than dissemination of Lesion 1.

Standard lymph node dissection was conducted and no metastasis was found in all of the twelve dissected lymph nodes.

The patient remains well 17 mo after surgery.

## DISCUSSION

Gastric endocrine neoplasms are classified into carcinoid (well differentiated endocrine neoplasm), small cell carcinoma (poorly differentiated endocrine neoplasm) and tumor-like lesions (including hyperplasia and dysplasia of endocrine cells)[4]. Most endocrine tumors of the stomach are well differentiated, nonfunctioning enterochromaffin-like (ECL) cell carcinoids arising from lamina propria in the corpus or fundus[5]. Three distinct types have been recognized. Type I carcinoids are associated with autoimmune chronic atrophic gastritis. Type Ⅱ carcinoids are linked to multiple endocrine neoplasia type 1(MEN-I)) and Zollinger-Ellison syndrome (ZES). Type Ⅲ carcinoids are sporadic, without hypergastrinemia or autoimmune atrophic gastritis[6]. Types I and Ⅱ carcinoids develop through a sequence of "hyperplasia-dysplasia-neoplasia", and ECL-cell hyperplasia and dysplasia are identified as the precancerous lesions of ECL-cell carcinoid[7]. Lesions up to 0.5 cm in diameter or infiltrating submucosa are recognized as carcinoids[8]. In this case, mild to moderate atrophic gastritis was noted, coexisted with intestinal metaplasia and a high level of serum gastrin. A carcinoid of 0.7 cm in diameter was identified. All of the lesions listed above were in line with Type I carcinoid associated with endocrine cell hyperplasia.

In this case, superficial SRC (Lesion 2) was identified in corpus mucosa of the lesser curvature, which was failed to be detected by gastroscopy and biopsy before surgery. An unusual type of signet-ring-like carcinoid[9-11] was reported in literature, but the following three points in this case ruled out the possibility. First, cells in Lesions 1 and 2 were different from each other in morphology. Second, they occurred in two different sites, without anatomical continuity. Third, their histochemical and immunohistochemical phenotypes were different. Lesion 1 was negative for mucin, while positive for neuroendocrine markers (Table 1). Lesion 2, however, showed positive staining of mucin, while negative for neuroendocrine

markers. Therefore, the carcinoid and SRC in this case were two independent lesions. Up to date, 5 cases of gastric carcinoid coexisting with adenocarcinoma have been reported[12-16], and only one of them with SRC[16].

The incidence of gastric ectopic pancreas ranges from 0.6% to 13.7%, frequently in the antrum and prepyloric region on the greater curvature or posterior wall. It often locates in submucosa, lamina muscularis or subserosa. Histologically, heterotopic pancreas can be divided into 4 types: type 1, composed of all cell types, namely complete heterotopia; type 2, composed of ductal components only, the canalicular heterotopia; type 3, composed of acinar cells only, the exocrine heterotopia; and type 4, composed of islet cells only; the endocrine heterotopia[17]. In this case, the heterotopic pancreas belongs to the first type. Most of the patients with gastric ectopic pancreas are asymptomatic. When mucosa is involved, however, patients may complain of an upper abdominal pain, bleeding, and obstruction may occur. Malignant transformation has been encountered, albeit rarely, giving rise to an adenocarcinoma[18-25] or a neuroendocrine neoplasm[26]. Caution should be paid to establishing a diagnosis of a carcinoma from heterotopic pancreas. For this, 3 points were considered necessary. First, the carcinoma should be found within or close to the heterotopic pancreas. Second, the transitional area between pancreatic structures and carcinoma should be observed and a metastatic carcinoma or dissemination of carcinoma from the adjacent gastrointestinal tract must be excluded. In addition, the non-neoplastic heterotopic pancreatic tissue should contain at least fully developed acinar or ductal structures[3]. It is evident that SRC in this case is independent from Lesion 3. Besides, a functional pancreatic endocrine tumor is composed of neoplastic cells secreting the same hormone (insulin, glucagon or somatostatin), differing from the endocrine cell hyperplasia whose constituent cells produce multiple hormones. In this case, Lesion 3 belonged to the complete heterotopic pancreas, with endocrine cell proliferation, which scattered preferentially among bundles of smooth muscle fibers or along vascular structures, mimicking intramural infiltration or metastasis of the carcinoid. It is important to know whether the endocrine tissue is hyperplastic or neoplastic, in consideration of the presence of a carcinoid (Lesion 1) at the corpus of the stomach and their structural and morphologic similarities. The following data revealed that endocrine component in Lesion 3 is non-neoplastic. The endocrine tissue was accompanied by the exocrine components in most parts of the lesion. Functionally, the endocrine tissue was composed of cells separately producing insulin, glucagon or somatostatin, reflecting its polyclonal nature, the phenotypes being similar to that of islets and different from Lesion 1 (Table 1).

In summary, we presented a case with coexistence of carcinoid, SRC and heterotopic pancreas in stomach. Differential diagnosis of the three abnormalities was of great importance for prognosis evaluation of the patient.

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*CASE REPORT*

# Hyperinsulinemic hypoglycemia due to adult nesidioblastosis in insulin-dependent diabetes

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## Abstract

In neonates, persistent hyperinsulinemic hypoglycemia (PHH) is associated with nesidioblastosis. In adults, PHH is usually caused by solitary benign insulinomas. We report on an adult patient who suffered from insulin-dependent diabetes mellitus, and subsequently developed PHH caused by diffuse nesidioblastosis. Mutations of the MEN1 and Mody 2/3 genes were ruled out. Preoperative diagnostic procedures, the histopathological criteria and the surgical treatment options of adult nesidioblastosis are discussed. So far only one similar case of adult nesidioblastosis subsequent to diabetes mellitus Ⅱ has been reported in the literature. In case of conversion of diabetes into hyperinsulinemic hypoglycemia syndrome, nesidioblastosis in addition to insulinoma should be considered.



**Key words:** Hyperinsulinemic hypoglycaemia; Adult nesidioblastosis; Diabetes; Multiple Endocrine Neoplasia Type 1; Insulinoma

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## INTRODUCTION

Persistent hyperinsulinemic hypoglycemia (PHH) caused by functionally defectice β-cells in the setting of a nesidioblastosis is the most common pathological substrate in newborns, whereas in adults, PHH is usually caused by solitary insulinomas[1-3]. Several genetic abnormalities were identified as the causes of PHH in infancy. The most important mutations are in the β-cell sulfonylurea receptor (SUR1) gene and encoding proteins composing the ATP-sensitive potassium channel in the cell membrane of the β-cell (Kir6.2)[4,5]. Loss of function due to mutations of the glucokinase (GCK) and glutamat dehydrogenase (GLUD1) genes were also identified as possible causes of PHH of infancy[6-8]. The morphologic substrate of these molecular changes are either diffuse β-cell hypertrophy (diffuse nesidioblastosis) or focal β-cell hypertrophy and β-cell hyperplasia (focal nesidioblastosis)[4,9]. The term nesidioblastosis for describing the lesions of the endocrine pancreas in neonates was first used by Yakovac[10] in 1971, when he described 12 infants with PHH.

Establishing the diagnosis of nesidioblastosis in an adult is a great challenge in endocrinology. To date, it is not possible to diagnose diffuse nesidioblastosis in adults on clinical grounds, since imaging techniques or detection of a specific causative mutation are of no help. The fasten test for the diagnosis of nesidioblastosis has been controversially discussed. Up to now, the diagnosis is based on (1) exclusion of an insulinoma by all means of clinical diagnostic procedures and (2) pathological analysis of the pancreatic tissue specimens. A combination of various histopathological criteria, marked as major and minor criteria, has recently been published to establish the diagnosis of a diffuse adult nesidioblastosis[11].

Since the first reported case of an adult nesidioblastosis in 1975, less than 100 patients have been described in the literature. Most of them were reported in individual case studies. Reports of PHH with known diabetes mellitus are extremely rare[12-15]. In our hospital, diffuse nesidioblastosis has been diagnosed in 4 out of 137 patients suffering from PHH. A coincidence of PHH with diabetes was diagnosed in only one patient. The aim of our report is to describe this patient in detail concerning (1) clinical presentation, (2) mutational analysis of the MEN1 and Mody 2/3 genes, (3) pathology of the pancreatic specimens. The diagnostic procedures and the surgical treatment strategies are discussed on the basis of the presented case.

## CASE REPORT

### *Clinical presentation*

A 40-year-old man (170 cm in height; BMI: 24.9) diagnosed with organic hyperinsulinism was admitted to our

hospital. The previous medical history was marked by a primarily insulin-independent diabetes (type Ⅱ). Insulin treatment has been required over the past 6 years. At the age of 35, he developed an idiopathic facial nerve pareses and brainstem encepahlitis of unknown etiology. Family history showed no pathologic findings, except diabetes of a grandmother. On the onset of diabetes, his blood sugar rose to 3800 mg/L with elevated HbA1c (8.0%). The patient was started on insulin treatment. During three hospital admissions for hyperglycemia over a 5-year period, blood sugar levels were often higher than 4000 mg/L and he was treated with insulin. Starting in 2005, the insulin treatment began to reduce and two months later discontinued, because of normalized serum glucose levels. He was admitted to hospital for symptoms of hyperadrenergic reactions and neuroglycopenic symptoms of confusion and somnolence due to hypoglycemia with blood sugar levels of 250 mg/L.

Diagnosis with a pathological fasting test indicated hypoglycemia. Blood sugar levels were repeatedly reduced to 300 mg/L with typical neuroglycopenic symptoms. The insulin level was up to 93.3 mU/L with a raised insulin index of 2-3. Exogenous insulin intake could be excluded by C-peptide levels. Unfortunately, the real C-peptide level could not be specified because of insufficient terms of transport (external laboratory). Insulin antibodies were negative. Attempts to localize an insulinoma with computed tomography (CT), ultrasound (US) of the abdomen and endoscopic ultrasound (EUS) were unsuccessful.

Laparotomy was performed and the pancreas was completely exposed and investigated by bimanual palpation and intraoperative ultrasound. A spleen preserving pancreatic-tail resection was performed because of a suspected small tumor in the tail of the pancreas. The patient's postoperative course was uneventful. Postoperatively, he displayed no signs of recurrent hypoglycemia. The blood sugar levels measured rose up to 1660-2430 mg/L, therefore low dose insulin application was necessary. The patient was discharged on the seventh postoperative day.

The patient and his family doctor reported recurrent diabetes metabolism over the previous 5 mo during a nine month follow-up after surgery (blood sugar levels: 300-2420 mg/L depending on insulin intake). Unfortunately, a reassessment of the diabetes was declined by the patient at the time.

### *Mutational analysis*

Genetic sequence analysis of the exons 2-10 of the MEN1 gene on chromosome 11q13 as previously described[16,17], was performed for differential diagnosis. The genetic make-up ruled out a molecular germline mutation in the MEN1 gene. Additional genetic sequence analyses of the glukokinase-gene of exon 1A-10 on chromosome 7p15-p13 were performed. Mutations pointing to maturity diabetes of the young Typ 2 were excluded. In addition, genetic sequence analyses of exons 1-10 of the hepatic nuclear factor 1 alpha gene for MODY Typ 3 were negative.

### *Histopathological analysis*

Pathological examinations were done using serial sections from different regions of the specimens which were immunohistochemically stained, by the avidin-biotin-complex (ABC) method with a monoclonal antibody against insulin (BioGenex, San Ramon, Canada, primary dilution 1:40) and polyclonal antibodies against glucagon (BioGenex, dilution: 1:60), somatostatin (Dako, Glostrup, Denmark, dilution: 1:200) and pancreatic polypeptide (DAKO, dilution: 1:5000). Appropriate controls were performed in order to ascertain the staining specificity. Histological and immunostained pancreatic sections were analyzed by three different pathologists in a double-blind fashion. Histological criteria for nesidioblastosis in adults were used to evaluate the sections[11]. Age- and sex-matched control pancreatic specimens were used as references.

Gross examination and step sectioning of the pancreatic samples excluded the presence of an endocrine tumor. The lobular architecture of the exocrine parenchyma was preserved. The pancreatic duct was patent and of normal size. There was no evidence of amyloid deposits or a quantitative disturbance of beta-cells. A consistent finding was the presence of multiple enlarged islet cells, whose arrangement often produced a lobulated islet pattern. Cytologically, a considerable subpopulation of endocrine cells showed enlarged and hyperchromatic nuclei (Figure 1A). These cytological changes were seen in more than 2/3 of the islets. Using immunohistochemistry and subsequent staining with hematoxylin, the cytologically conspicuous endocrine cells were identified as beta-cells (Figure 1B). In contrast, glucagon, somatostatin and pancreatic polypeptide cells did not reveal any cytologic abnormalities (Figure 1C). The subcellular distribution of insulin was regular and apparently equal amounts of C-Peptide were seen. There was no evidence of an expression of the nuclear proliferation antigen ki-67 within the islets. According to this histopathological analysis the patient fulfilled the recently described "major criteria" of a diffuse nesidioblastosis in adults[11].

## DISCUSSION

Nesidioblastosis was detected and diagnosed in 4 patients out of a series of 137 patients treated for organic hyperinsulinism at our hospital. One of them was diagnosed by clinically reversing a highly insulin-dependant diabetes. Diagnosis of nesidioblastosis was established by (1) exclusion of an insulinoma by all means of clinical diagnostic procedures and (2) by pathological examination revealing the characteristic signs of a diffuse nesidioblastosis.

Preoperative localization by CT-scan, abdominal ultrasound and endoscopic ultrasound (EUS) failed to detect an endocrine tumor. An indication for surgery was based on hypoglycemia in the presence of elevated insulin levels and excluding exogene insulin intake by C-peptide levels and screening for sulfonylureas.

After completely exposing the pancreas and investigation by bimanual palpation and intraoperative

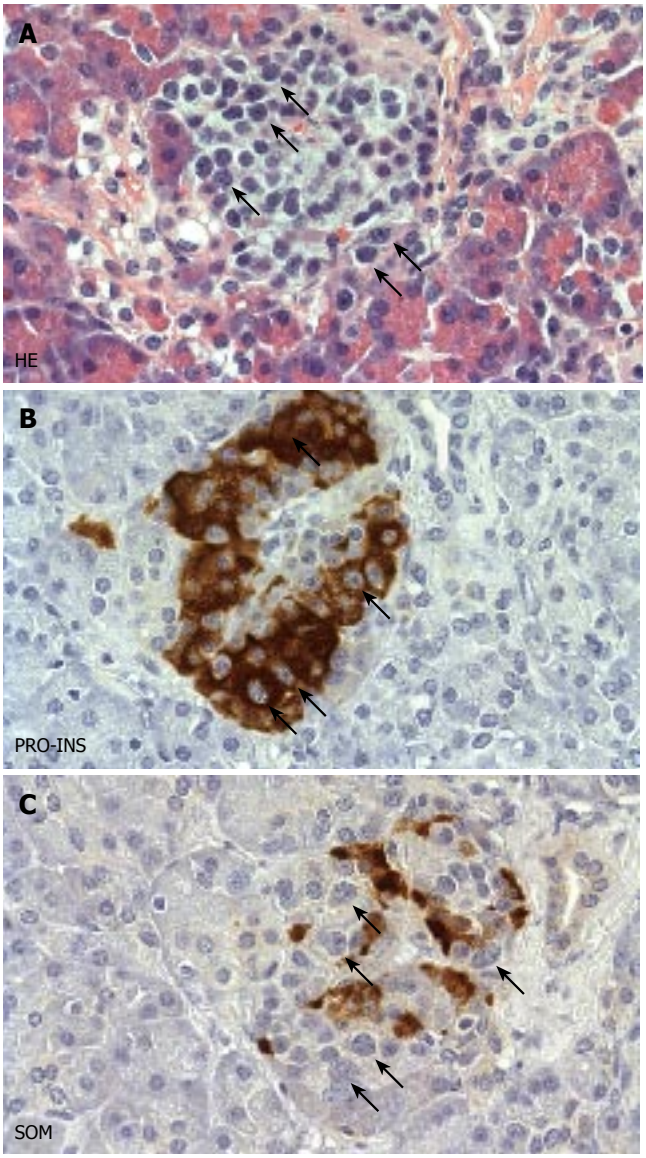


**Figure 1** Histopathological features of diffuse nesidioblastosis. **A:** HE stained sections demonstrating a prominent lobulation of an islet. Some of the endocrine cells showing hyperchromatic and enlarged nuclei are labelled with arrows. **B**, **C**: Adjacent section analysis demonstrating cytoplasmic positivity for proinsulin (PRO-INS) in those endocrine cells with hyperchromatic nuclei (arrows in **B**). In contrast, these cells are negative for somatostatin (SOM) (arrows in **C**).

ultrasound, a small tumor in the tail of the pancreas was suspected and a spleen preserving pancreatic-tail resection was performed. Nesidioblastosis was diagnosed postoperatively by the pathologist according to the recently published major and minor criteria for nesidioblastosis in adults[11].

Our knowledge of the pathogenesis of PHH in infants has expanded in recent years. Some genetic abnormalities underlie the disease. The most important genetic defects are inactivating mutations on chromosome 11 (SUR1 and Kir6.2)[4,5]. Gain-of-function mutations of the glucokinase gene (GCK) and glutamate dehydrogenase genes are infrequent (GLUD1)[11]. Because of the patient's young age, a subgroup of diabetes mellitus, a maturity onset diabetes of the youth (MODY), should be discussed for differential diagnosis. So far 6 different types of MODY have been diagnosed. Glucokinase plays a critical role in the insulin secretion in β-cells. Heterozygous mutations in the glucokinase-encoding gene (GCK) results in the reduction of encymatic activity (MODY 2)[18,19]. Loss of function mutations in GCK could be ruled out as a possible reason for hyperglycemia in our patient.

The diagnosis and treatment of an adult nesidioblastosis still remain an open issue. There are no imaging techniques to differentiate focal from nonfocal (diffuse) organic hyperinsulinism. Frequently, insulin-producing tumors are less than 10 mm in diameter and may therefore escape preoperative detection. Additionally, preoperative imaging as EUS can yield misleading findings in the pancreas[20]. An intra-arterial calcium stimulation test may be able to differentiate focal hyperactive β-cells from diffuse hypertophic β-cells[21-23]. Due to the failure of the modern imaging techniques in these patients, the localization of pathologic increased insulin secretion would be a diagnostic tool for the surgeon to help avoid blind pancreatic resection. The treatment of adult nesidioblastosis is surgical resection. However, the line between 'too much' and 'too less' resection, with the risk of endocrine and exocrine pancreatic insufficiency and recurrent hypoglycemia is very narrow. Nesiodioblastosis has to be considered in all cases without localized insulinoma because it has been recently shown that this disease can be present in up to 4% of patients with PHH[11]. In the case of recurrence, a secondary surgical intervention with further pancreatic resection is necessary. In our case, hypoglycemia may be life threatening, but the risk of lifetime diabetes should be considered as well. Nowadays, recurrent pancreatic resections can be performed with low risk in specialized centers.

The present case illustrates a very rare coincidence of diabetes and adult nesidioblastosis. Up to now, only four cases with organic hyperinsulinism and diabetes have been reported[12-15]. Our case is the second description of a concomitant adult nesidioblastosis and diabetes type Ⅱ. In summary, diabetes can be reversed by functionally defective hypertrophic β-cells. In the case of reversing diabetes, an organic hyperinsulinemic hypoglycemia should be considered as a differential diagnosis.

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# Targeting intestinal microflora in inflammatory bowel disease

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## TO THE EDITOR

In their recent review article[1], Andoh and Fujiyama examined the various therapeutic approaches targeting intestinal microflora in patients with inflammatory bowel disease (IBD). I would like to provide some additional data to complete and update their comments. First of all, when considering the role of probiotics in IBD treatment it must be emphasized that, in addition to *Bifidobacteria*, the *Nissle 1917 E. coli* strain and cocktails of microorganisms such as *VSL # 3* mentioned in the article, other probiotic agents have been tested in the short- and long-term treatment of either ulcerative colitis and Crohn's disease, the results of those studies being reported in major international scientific journals.

For instance, *Saccharomyces boulardii*, a non-pathogenic yeast, which is widely used in the treatment of diarrhea and is effective in preventing relapse of *Clostridium difficile* infection[2], has been employed in the maintenance treatment of patients with inactive Crohn's disease[3]. Thirty-two patients were randomly allocated to a six-month maintenance therapy either with mesalazine alone (500 mg of sustained-release microgranules, 3 times daily) or with the same mesalazine preparation 500 mg twice daily plus *Saccharomyces boulardii* 500 mg once daily. Clinical relapse, defined as CDAI > 150 with an increase of 100 points over the baseline values for more than 2 wk, was observed in 37.5% of patients receiving mesalazine alone and in only 6.25% of subjects in the group treated with mesalazine plus *Saccharomyces boulardii* ( $P = 0.04$ by Fisher's exact test).

Furthermore, in 25 patients with a history of poor tolerance to corticosteroids, who had a clinical flare-up of mild to moderate left-sided ulcerative colitis while on maintenance with mesalazine, *Saccharomyces boulardii* (250 mg, 3 times daily) was added to the ongoing mesalazine treatment for 4 wk[4]. Clinical evaluation was performed before and after the treatment with Rachmilewitz's activity index. Clinical remission, -Endoscopically confirmed clinical emission was achieved in 68% of cases on an intention-to-treat basis.

Although the effect of each probiotic agent is different, placebo-controlled clinical trials employing *Lactobacillus GG* in the maintenance treatment of Crohn's disease have failed to show any significant effect in preventing recurrences at 6-24 mo[5,6]. However, the probiotic is able to maintain clinical remission of ulcerative colitis[7].

Finally, when considering the possible role of antibiotics in IBD, the recent data on rifaximin, a poorly absorbable antibacterial agent, must be quoted. A double-blind, placebo-controlled trial showed that addition of 800 mg rifaximin twice a day for 12 wk is effective in inducing clinical remission of active Crohn's disease[8]. It was reported that 400 mg rifaximin twice daily for 4 wk can achieve clinical remission in 76% of ulcerative colitis patients who had relapse while on mesalazine maintenance[9].

The virtual absence of systemic side-effects and the encouraging results reported so far suggest rifaximin can effectively inhibit the intestinal flora in IBD patients without severe side effects.

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*AUTHOR'S FEEDBACK*

# Premature chomosome condensation technique: A very promising approach to radiotherapy for digestive system cancers

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Yang JS. Premature chomosome condensation technique: A very promising approach to radiotherapy for digestive system cancers. *World J Gastroenterol* 2006; 12(44): 7227

http://www.wjgnet.com/1007-9327/12/7227.asp

Dear Editior-in-Chief,

I am grateful to the renowned World Journal of Gastroenterology for publishing our manuscript about hepatoma radiosensitivity in volume 11 issue 26, page 4098-4101[1]. Since this paper appeared online first, it was widely considered. Up to date, it has been cited four times in various journals covered by Science Citation Index[2-4], and the click count and download count added up to 459 and 163[1], respectively. Some scientists from Australia and Germany frequently sent me letters to ask for original data to be cited, but a fatal mistake made them arduous. The correspondence author's e-mail address should be yangjs@impcas.ac.cn, but not tuyangjs@impcas.ac.cnit. I sincerely expected your editors could rephrase and upload it to your online data base.

## REFERENCES

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# Meetings

## MAJOR MEETINGS COMING UP

First Biennial Congress of the Asian-Pacific Hepato-Pancreato-Biliary Association
March, 2007
Fukuoka, Japan
http://www.congre.co.jp/1st-aphpba

American College of Gastroenterology Annual Scientific
20-25 October 2006
Las Vegas, NV

14th United European Gastroenterology Week, UEGW
21-25 October 2006
Berlin, Germany

APDW 2006: Asian Pacific Digestive Week 2006
26-29 November 2006
Lahug Cebu City, Philippines

## EVENTS AND MEETINGS IN THE UPCOMING 6 MONTHS

Falk Symposium 151: Emerging Issues in Inflammatory Bowel Diseases
24-25 March 2006
Sydney - NSW
Falk Foundation e.V.
symposia@falkfoundation.de

10th International Congress of Obesity
3-8 September 2006
Sydney
Event Planners Australia
enquiries@ico2006.com
www.ico2006.com

Easl 2006 - the 41st annual
26-30 April 2006
Vienna, Austria
Kenes International

Prague hepatology 2006
14-16 September 2006
Prague
Foundation of the Czech Society of Hepatology
veronika.revicka@congressprague.cz
www.czech-hepatology.cz/phm2006

12th International Symposium on Viral Hepatitis and Liver Disease
1-5 July 2006
Paris
MCI France
isvhld2006@mci-group.com
www.isvhld2006.com

Falk Symposium 152: Intestinal Disease Part I, Endoscopy 2006 - Update and Live Demonstration
4-5 May 2006
Berlin
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symposia@falkfoundation.de

Falk Symposium 153: Intestinal Disease Part II, Immunoregulation in Inflammatory Bowel Disease - Current Understanding and Innovation
6-7 May 2006
Berlin
Falk Foundation e.V.
symposia@falkfoundation.de

ILTS 12th Annual International Congress
3-6 May 2006
Milan
ILTS
www.ilts.org

Internal Medicine: Gastroenterology
22 July 2006-1 August 2006
Amsterdam
Continuing Education Inc
jbarnhart@continuingeducation.net

6th Annual Gastroenterology And Hepatology
15-18 March 2006
Rio Grande
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www.hopkinscme.net

World Congress on Gastrointestinal Cancer
28 June 2006-1 July 2006
Barcelona, Spain
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International Conference on Surgical Infections, ICSI2006
6-8 September 2006
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European Society of Clinical Microbiology and Infectious Diseases
icsi2006@stocon.se
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7th World Congress of the International Hepato-Pancreato-Biliary Association
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www.edinburgh.org/conference

Society of American Gastrointestinal Endoscopic Surgeons
26-29 April 2006
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www.sages.org

Digestive Disease Week 2006
20-25 May 2006
Los Angeles
www.ddw.org

Annual Postgraduate Course
25-26 May 2006
Los Angeles, CA
American Society of Gastrointestinal Endoscopy
www.asge.org/education

American Society of Colon and Rectal Surgeons
3-7 June 2006
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10th World Congress of the International Society for Diseases of the Esophagus
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Falk Symposium 151: Emerging Issues in Inflammatory Bowel Diseases
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*EDITORIAL*

# Colon cancer and the immune system: The role of tumor invading T cells

Maximilian Waldner, Carl C Schimanski, Markus F Neurath

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## Abstract

Colon cancer is still one of the leading causes of cancer death worldwide. Although the host immune system has been shown to react against tumor cells, mainly through tumor infiltrating lymphocytes and NK cells, tumor cells may utilize different ways to escape anti-tumor immune response. Tumor infiltration of CD8+ and CD4+ (T-bet+) effector T cells has been attributed to a beneficial outcome, and the enhancement of T cell activation through T cell receptor stimulation and co-stimulatory signals provides promising strategies for immunotherapy of colon cancer. Growing evidence supports a role for the Fas/FasL system in tumor immunology, although the mechanisms and consequences of FasL activation in colon cancer are not completely understood. In animal models, depletion of regulatory T cells (CD4+ CD25+ T cells) can enhance the anti-tumor immune response under certain conditions. Taken together, recent insights in the immune reaction against colon carcinoma have provided new approaches to immunotherapy, although much remains to be learned about the exact mechanisms.



**Key words:** CD4-positive T-lymphocytes; CD8-positive T-lymphocytes; Immunology; Colonic neoplasms therapy; Colorectal neoplasms; Humans; Lymphocytes; Tumor-infiltrating; Tumor escape

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## INTRODUCTION

Colon cancer is still one of the leading causes of cancer death worldwide. In the United States approximately 145 290 new cases of colorectal cancer are diagnosed every year. With more than 56 000 deaths in the United States in 2005, colorectal cancer is responsible for more than 10 percent of all cancer deaths[1]. However, the molecular pathogenesis of colorectal cancer is still poorly understood. Recent studies suggested that different mechanisms such as mutations in cell cycle-[2] and apoptotic-pathways[3], signal transduction[4-6], angiogenesis[7,8], invasion and metastasis[9] significantly contribute to cancer progression (Table 1). Another important mechanism consists of the ability of tumor cells to escape the host immune reaction, as outlined below.

Sir Macfarlane Burnet and Lewis Thomas first proposed the existence of an immunological response to tumors in the cancer immunosurveillance hypothesis in the 1950s[10,11]. However, strong evidence supporting this concept was lacking and the hypothesis was abandoned for many years[12]. In the past two decades, however, the identification of tumor specific antigens and immune modulation leading to tumor regression suggested the existence of cancer immunosurveillance[13-17]. The activation of the host immune system through tumor cells is a complex cascade involving both the innate and adaptive immune systems (Figure 1)[11]. The presence of tumor specific T cells has been correlated with improved clinical outcome in different human cancers[18-21], but does not necessarily result in anti-tumor immunity, since T cells can also promote the progression of tumors through different growth factors[22]. It has been shown that CD8+ T cells and CD4+ effector T cells may have anti-tumor properties, whereas regulatory T cells (CD4+ CD25+ Tregs) may be responsible for immunological hypo-responsiveness observed in cancer[23-26].

The human gastrointestinal tract contains several phenotypically and functionally distinct populations of T cells, which may play a role in anti-tumor immunity[27-29]. Interestingly, T cell activation has been shown in colorectal cancer and proposed as a prognostic factor[30]. The following editorial will discuss recent advantages in our understanding of T cell activation in colorectal cancer and possible therapeutic strategies.

## EVIDENCE FOR T LYMPHOCYTE ACTIVATION IN COLON CANCER

Tumor-infiltrating lymphocytes (TILs) have been isolated from a variety of solid human cancers. It has been widely accepted that one of the most promising T cell subsets

for an effective anti-tumor response consists of CD8+ T cells[26]. In a study on 131 patients with colorectal cancer, Naito *et al* showed a positive correlation between CD8+ T-cells within cancer cell nests and patient survival[30]. Another study using 959 specimens of resected colorectal cancer analyzed the correlation between tumor metastasis and T cell activation[31]. Elevated expression of genes, specific for cytotoxic T lymphocytes (CTLs), such as CD8α, granzyme B, or granulysin as well as Th1-associated genes as T-bet, or interferon-γ was significantly higher in patients without signs of early metastatic invasion as compared to those with early invasion (VELIPI+). In addition, the authors proposed the presence of CD45RO+ CCR7- memory cells as an independent, positive prognostic factor in colorectal cancer. The detected cells represented all subpopulations in the differentiation pathway and are characterized by long-term persistence *in vivo* and the ability to rapidly expand upon reencounter with antigen[32].

Interestingly, microsatellite instability in colon cancer results in significantly increased infiltration of tumors with larger numbers of CD8+ T cells and is associated with a favorable prognosis[33,34]. Microsatellite instability is characterized by defective DNA mismatch repair mechanisms, which result in higher rates of mutations and cell-to-cell variability in the length of DNA microsatellites. Tumors revealing extensive microsatellite instability, such as Hereditary Nonpolyposis Colorectal Carcinoma (HNPCC), which is associated with germ-line mutations of DNA mismatch repair genes, are designated as microsatellite instability (MSI)-high. In contrast, sporadic colorectal cancers display MSI-H in only 15%[35]. The better prognosis of MSI-H carcinomas is explained by frequent mutations in oncogenic genes and mutation-dependent abnormal peptides resulting in a cytotoxic immune response against cancer cells and hence increased infiltration by CTLs[36]. Several immunogenic antigens, such as mutated CDX2, TGFβIIR, or Caspase-5, have been identified for MSI-H associated colon cancers[37-39]. In MSI-H negative sporadic colorectal carcinoma, the identification of different tumor associated antigens (TAAs) has also been correlated with the activation of the host immune system to tumor cells[34,40-44].

In some patients, recurrent or persistent inflammation may promote carcinogenesis and trigger cancer growth. For instance, the risk of colorectal cancer in patients with chronic inflammatory bowel disease (ulcerative colitis and Crohn's disease) increases with longer duration of disease[45,46]. In particular, patients with ulcerative colitis have a significant risk for development of colitis associated colon cancer dependent on the extension of the disease, the presence of backwash ileitis and the number of flares suggesting that inflammation drives carcinogenesis. Whereas defined molecular changes in progression of sporadic colon cancer lead to stepwise changes in histology according to the adenoma-carcinoma sequence, carcinogenesis in ulcerative colitis starts with a hyperplastic lesion in the inflamed mucosa and develops through dysplasia into adenocarcinoma, ("inflammation-dysplasia-carcinoma" sequence)[47]. Different molecular mechanisms such as oxidative stress and NF-κB-activation have been attributed to inflammation dependent carcinogenesis[47-49]. Even T cell activation can lead to tumor growth through cytokine secretion. For instance Becker *et al* showed that IL-6 signaling through TILs results in tumor progression, as IL-6 serves as a growth and proliferation factor for tumor cells. This signaling was dependent on tumor derived soluble IL-6 receptor and could be inhibited through TGF-β[50,51].

**Table 1 Different pathways of colon cancer progression and possible therapeutic targets**

| Pathway | Targets |
|---|---|
| Cell cylce | p53, pRb, p16, p21, D-type cyclins |
| Apoptosis and survival | Bcl-2, Cox-2 |
| Signal transduction | EGF-R, Akt, AP-1, Her2/neu, NFκB |
| Angiogenesis, invasion, and metastasis | VEGF, TSP-1, CXCR-4 |
| Immunity | IL-6, TGF-β |

Taken together, the immune system may promote progression of colorectal cancer in cases of chronic inflammation, but can also lead to tumor regression through the innate immune system with tumor specific activation of CTLs and effector CD4+ T cells (Figure 2). However, the high incidence and death rate of colorectal cancer suggest an ineffective immune response in many cases. Although it has been shown that CTLs can recognize specific TAAs, many mechanisms of suppression or failure of such recognition do exist[18,21,52].

## ESCAPE MECHANISMS IN COLON CANCER

It has been proposed that tumor progression is a result of the natural selection of rapidly growing tumor cell variants leading to a growth advantage of such cells over other cells. This concept has also been applied to the immune escape of tumor cells. The loss or down-regulation of HLA class I antigens, the lack of co-stimulation, defective death receptor signaling, apoptosis of activated T cells, immunosuppressive cytokines, and activation of suppressor T cells are important examples for this phenomenon[53].

Norazmi *et al* described decreased HLA class I antigen in malignant colonic tissues as compared to non-malignant tissues from the same individuals[54], thereby enabling tumor cells to escape CTL-mediated lysis due to circumvention of MHC-restricted, antigen-specific triggering of the T-cell receptor (TCR) complex. On the other hand cross-priming of CTLs through antigen presenting cells (APCs) is ineffective, as powerful APC-activating stimuli are usually absent in tumors[53,55].

Antibody-guided targeting of antigenic MHC class I -peptide tetramers on colon cancer cells results in a destruction of tumor cells by cytotoxic T lymphocytes *in vitro* and *in vivo* and has been proposed as a new form of immunotherapy[56,57]. Otherwise, data from Luo *et al* show a protection of colon carcinoma cells from apoptosis and cytolysis induced by hepatic NK cells through higher MHC

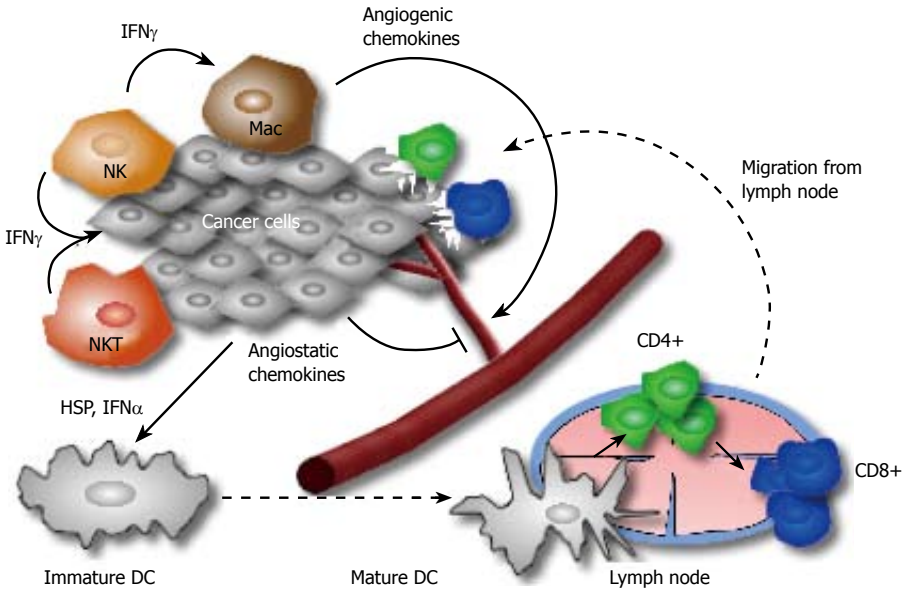


**Figure 1** A proposed model for the host immune reaction to cancer cells. At the initiation of the immune reaction lymphocytes and other cells participating in innate immunity (e.g. APCs, NK, NKT cells) recognize transformed tumor cells and produce IFN-γ. This starts a cascade of reactions with production of chemokines (for instance angiogenic or angiostatic chemokines like MIG, IP10 and I-TAC), IFN-γ (antiproliferative mediator for the developing tumor) and direct cytotoxicity of NK cells and macrophages on tumor cells. This cascade may result in partial tumor cell death and tumor cell debris is ingested by dendritic cells, which move to draining lymph nodes and activate CD4+ and CD8+ T cells. Activated and tumor specific T cells move to the tumor along a chemokine gradient and destroy tumor cells expressing a distinctive tumor antigen.

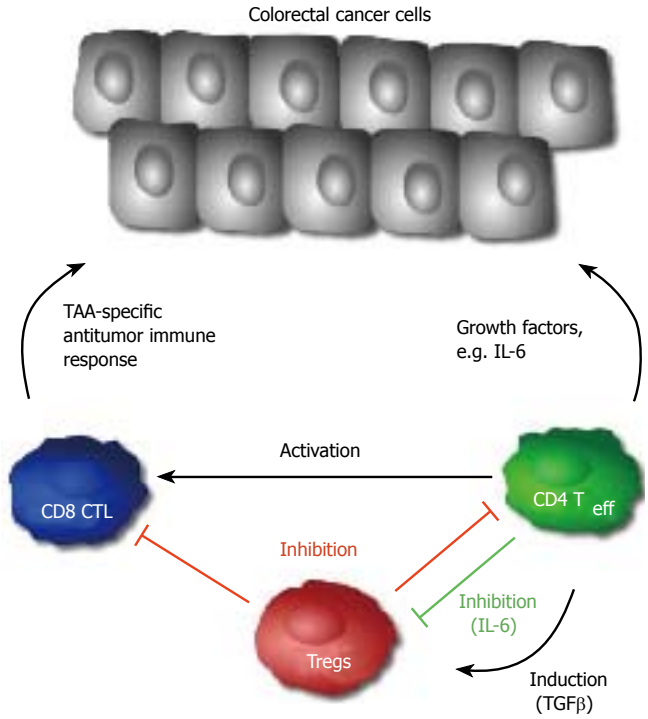


**Figure 2** The role of different T cell subsets in colorectal cancer. Activated CD4+ T cells can release growth factors and thereby lead to tumor progression or activate tumor specific CD8+ T cells. Secretion of TGFβ can induce adaptive Tregs, which may suppress the anti-tumor immune response. Once activated, Tregs can be suppressed by IL-6 derived from CD4+ T cells.

class I expression, most likely by blocking the perforin/granzyme pathway[58].

T cell activation is mediated not only by triggering of the T cell receptor complex, but also by antigen-independent mechanisms such as co-stimulation. Co-stimulation induces cytolysis, cytokine secretion, proliferation and protection from apoptosis in CTLs. The poor immunogenicity of tumor cells has been partly ascribed to the lack of expression of co-stimulatory ligands (see Abken *et al*[59] for review). In the past few years, the increasing knowledge about the mechanisms of T cell activation led to new approaches for immunotherapy. Concerning colon carcinoma, the therapeutic amplification of the expression of co-stimulatory molecules as B7.1[60] and CD40L[61], the induction of co-stimulatory molecules as OX40 and 4-1BB[62] on T cells, and the administration of soluble co-stimulator proteins as B7.1-Fc[63], a B7.1 fusion protein consisting of the extracellular domains of human B7.1 and the Fc portion of human IgG1, or Ig-4-1BBL[64], a soluble fusion protein of 4-1BB Ligand and IgG2a, have shown promising results in experimental settings.

Growing evidence supports a relevant role of Fas/Fas Ligand (FasL) interactions in the immune escape of tumors. Fas and FasL belong to the tumor necrosis factor receptor and ligand families and activation of Fas by anti-Fas antibodies results in apoptosis of Fas expressing cells. It has been shown that tumors may provide resistance to Fas-mediated cytotoxicity, and that FasL expression on tumor cells could counterattack the immune system by inducing apoptosis of immune effector cells[65,66].

Several studies gave evidence for a role of the Fas tumor counterattack in colon carcinoma[67-69]. However, in a study with two different Fas-expressing target cell lines and seven different human colon cancer lines Favre-Felix *et al* failed to detect an induction of apoptosis in Fas-expressing target cell lines, namely Jurkat T cells and murine leukemia cells[70]. Recent studies suggest different functions of FasL in the immune response, since it has been shown that FasL is also delivering costimulatory signals to T cells, inducing motility of tumor cells, contributing to liver regeneration and yielding growth stimulatory signals to neurons[65]. The role of FasL in tumor escape is far from being understood and further studies are mandatory to elucidate the mechanisms and consequences of FasL activation.

## CD25+CD4+ T CELLS IN COLON CANCER: REGULATORS OF IMMUNE ESCAPE?

While it is generally accepted that CD4+ T cells may

contribute to the host anti-tumor immune response, a small subset of CD4+ T cells, the CD4+ CD25+ regulatory T cells (Treg) have been shown to accumulate in the tumor environment and induce immune escape mechanisms[26,71]. Elevated expression of FOXP3, a transcription factor crucial in the development and function of Tregs, has been associated with a poor prognosis in different types of cancer[72,73].

Depletion of Tregs by specific antibodies has enhanced vaccine-induced anti-tumor immunity in colon cancer and other cancer subsets such as leukemia, plasmocytoma, melanoma, fibrosarcoma, or renal cell carcinoma[24,25,74-80]. On the other hand the addition of Tregs resulted in growth regression of inflammation associated intestinal tumors in two studies provided by Erdman *et al*[81,82]. This is also in agreement with the above-mentioned data about tumor progression through IL-6 signaling, since Tregs can suppress cytokine release. Interestingly enough, TGF-β seems to have a central role in these mechanisms, since the cytokine itself can inhibit IL-6 signaling and lead to FOXP3 expression in tumor infiltrating CD4+ T cells[83]. Accordingly, the role of Tregs in colon carcinoma may also depend on tumor pathogenesis, but the exact mechanisms of Tregs in the regulation of tumor immunology remain undefined.

## CONCLUSION

Colon cancer is still one of the leading causes of cancer death worldwide. Although the host immune system can initiate an immune response against colon cancer cells, tumor cells may utilize different ways to escape those defense mechanisms. Detection of tumor associated antigens, stimulation of the T cell receptor, enhancement of costimulatory signals and depletion of regulatory T cells have shown promising results to overcome tumor escape and provide new strategies for immunotherapy of colon cancer.

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*EDITORIAL*

# Hepatocellular carcinoma prevention: A worldwide emergence between the opulence of developed countries and the economic constraints of developing nations

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## Abstract

Hepatocellular carcinoma (HCC) is the fifth most common neoplasm, the major cause of death in patients with liver cirrhosis, and the third most common cause of cancer-related death in the world. The geographic distribution of HCC varies significantly and 80% of cases occur in developing countries (Far East and South Asia) where the prevalence of viral hepatitis is higher. The treatment of HCC is difficult because most patients are diagnosed when the tumour is in an advanced stage and is not amenable to potential curative therapy, thus prevention is the key to reducing HCC and its related morbidity and mortality. HCC is unique among cancers, occurring mostly in patients with a known risk factor. Ninety percent of HCCs develop in the context of chronic liver diseases and mainly in patients with cirrhosis. Viral hepatitis is the most common cause of HCC worldwide, followed by alcoholic liver disease (ALD) and other causes such as non-alcoholic fatty liver disease (NAFLD), genetic haemocromatosis (GH) and primary biliary cirrhosis in an advanced stage (Ⅲ-Ⅴ). In certain areas of the People's Republic of China, exposure to aflatoxin and HBV infection are thought to be responsible for the extraordinary high risk of HCC. Substantial progresses in the prevention of virusl-related hepatitis (screening of blood units, use of disposable sanitary tools, HBV vaccination) have been achieved in developed countries, but in the same areas, alcohol- and dysmetabolism-related HCCs are emerging problems which require specific interventions in terms of public health measures. In developing countries, economic constraints limit the development of any program for the prevention of viral hepatitis transmission (including health education campaigns, healthcare politics, primary prevention and the improvement of hygienic and sanitary conditions). When viral liver disease is established, only a minority of patients are treated worldwide and benefit a possible preventive effect of medical treatment on HCC development. Thus the real contribution of medical treatment to HCC prevention in patients with chronic viral hepatitis is small. Great efforts are needed to identify more effective medical measures for primary and secondary prevention of HCC.



**Key words:** Hepatocellular carcinoma; Viral hepatitis; Cirrhosis; Treatment; Prevention programs

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## INTRODUCTION

Hepatocellular carcinoma (HCC) is the fifth most common neoplasm, the major cause of death in patients with liver cirrhosis, and the third most common cause of cancer-related death in the world[1,2]. Every year, 560000 people in the world develop HCC and about the same number die of liver cancer. It is reported to be the leading cause of death in patients with cirrhosis in Europe[3] and Asia[4]. In Italy, its incidence varies from 2% to 4% each year in patients with cirrhosis[5-7].

The geographic distribution of HCC varies significantly and 80% of cases occur in developing countries (Far East and South Asia) where the prevalence of viral hepatitis is higher[8]. Epidemiological data show that its incidence is changing around the world according to the etiology since it is increasing in many developed countries, whereas HCC is declining in developing countries[9]. Studies on the epidemiology and natural history of chronic hepatitis C virus (HCV) infection suggest that HCC frequency has been increasing over the past 20-30 years in USA[10,11] and Europe[12]. HCC is one of the few types of cancer increasing in frequency and mortality in USA[11]. In Japan there has been a three-fold increase in HCC incidence since 1970[13]. Moreover, it has been shown that 80% of newly diagnosed HCCs in Japan are HCV-positive patients[14]. It has been hypothesized that HCC incidence and mortality will indeed increase over the next 10-20 years because

of the increased incidence of HCV-related cirrhosis[10]. With regard to the decline of hepatocellular cancer in developing countries, the introduction of mass hepatitis B virus (HBV) vaccination programs seems to play an important role. In Taiwan the carrier rate of HBsAg in children and adolescents has been significantly reduced[15], as has the incidence of childhood HCC[16]. Nevertheless, the decrease in the incidence of HCC among adults is still a long way off and is not expected for another 3 or 4 decades later[17]. Thus, the role of HBV vaccination in the current decline of HCC incidence, at least in Taiwan, is still to be clarified. In addition, other factors may have a role in changing the epidemiology of HCC incidence around the world: the migration of people from endemic areas and better detection of HCC in Western countries.

The treatment of HCC is difficult because most patients are diagnosed when the tumour is in an advanced stage and is not amenable to potential curative therapy[18], thus prevention is the key to reducing HCC and its related morbidity and mortality.

Notably, HCC is unique among cancers, occurring mostly in patients with a known risk factor. Ninety percent of HCCs develop in the context of chronic liver diseases and mainly in patients with cirrhosis[19,20]. Viral hepatitis is the most common cause of HCC worldwide, accounting for more than 80% of cases, followed by alcoholic liver disease (ALD) and other causes such as non-alcoholic fatty liver disease (NAFLD), genetic haemocromatosis (GH) and primary biliary cirrhosis in an advanced stage (Ⅲ-Ⅳ)[20-23]. In certain areas of the People's Republic of China where HCC is the leading cause of cancer death, exposure to aflatoxin, a potent hepatocarcinogen produced by food contaminant, and HBV infection are thought to be responsible for the extraordinary high risk of HCC[24].

Therefore, a prevention strategy should be addressed to each single risk factor ranging from food conservation to alcohol abuse, metabolic disease (NAFLD) and viral infection. Then, HCC prevention can focus on different levels: prevention of the underlying disease (primary prevention), prevention of liver disease progression to cirrhosis and cancer (secondary prevention), prevention of cancer relapse in successfully treated HCC (tertiary prevention).

## HCC RELATED TO VIRUSES AND OTHER FACTORS

### *HBV*

HBV-related chronic hepatitis is the most common cause of HCC in the world[25]. The incidence and ways of transmission of this virus vary substantially in different endemic areas throughout the world. HBV is usually acquired in adulthood and its incidence is low in Western countries, while its incidence is high in Asia and in most African countries and its transmission is vertical from mother to child[26].

The relationship between HBV and HCC development is not completely understood. Nevertheless, it seems that two main mechanisms play a role in the induction of liver cancer: one involves repeated liver damage causing necroinflammation and consequent regeneration of hepatocytes, the other is the direct oncogenic property of HBV which integrates itself into the hepatocyte DNA, producing cis- or transactivation of the cellular oncogenes[27,28]. It is important to note that HBV and HCV have been classified among human carcinogens by the International Agency for Research on Cancer[1].

There is a relationship between viral replication (defined as the presence of detectable HBV-DNA by non-PCR assay or HBeAg positivity) and HCC development. A recent study showed that the cumulative risk of HCC development is higher (87%) in patients at the age of 30-70 years persistently positive for HBsAg and HBeAg than in those (12%) who are positive for HBsAg only and in those (1%) who are HBsAg-negative[29]. HBeAg prevalence, a marker of infectivity and viral proliferation, decreases with increasing age. HBeAg seroconversion (both spontaneous and IFN-induced) usually confers a favourable outcome. However, the selection of the e minus mutant (HBV-DNA positive) leads to the development of cirrhosis (not only in the Mediterranean area but also worldwide) which is the prerequisite for HCC development[30].

Therefore, two strategies can be used to prevent HBV-related HCC: one is the prevention of HBV infection, the other which is more questionable is the treatment of HBV-related liver disease and cirrhosis. Only a minority of patients treated with interferon-α (IFN-α) achieve HBs clearance while the majority of HBsAg-positive and HBeAg-negative patients have a persistent viral replication. These patients are still at risk of developing cancer. About 35% of patients[31] who respond to IFN-α treatment achieve HBs clearance and therefore can be considered cured. In the remaining HBsAg-positive patients who can clear HBeAg, a persistent viral replication is still detectable[31]. These patients, especially those with liver cirrhosis remain at risk of developing cancer. On the other hand, the occult HBV infection (HBsAg-negative patients) is related to an increasing or a persistant risk of HCC development in both anti-HCV-related and unrelated cirrhosis[32].

Therefore, occult HBV infection should be investigated specially in patients with chronic HCV infection and considered as an additional factor related to HCC development.

### *HCV*

HCV-related carcinogenesis is still not clearly understood. Hepatitis C virus infection increases the risk of developing HCC and its prevalence among patients with liver cancer varies between different geographic areas from 20% to 90%[10,33]. HCC occurs mostly in patients with end stage liver disease and advanced fibrosis[34]. In this population, the risk of developing liver cancer is 2%-8% per year[35-37] based on clinical studies. The only prospective study evaluating HCC risk in HCV-positive patients, has attributed a 20-fold increased risk to viral infection, but the presence of cirrhosis in the study population was not evaluated[38]. Other studies have not evaluated the presence of co-factors, such as alcohol intake or HBV co-infection and the eventual role of antiviral therapy. Therefore, a bias may exist in the literature, which may explain the variation in the incidence estimates of HCC among patients with HCV infection[11].

HCV itself probably has a direct role in the induction of HCC. Some studies showed that the HCV replicon and both non-structural and core viral proteins are capable of inducing a complex series of intracellular events leading to apoptosis suppression and cellular growth through the down-regulation of PKR and the up-regulation of transcription factors including NF-κB, STAT-3 and ATF-6[39-41].

### *Other causes of HCC*

Alcohol consumption is a risk factor for the development of HCC[42,43]. HCC develops mostly in patients with cirrhosis although a direct carcinogenetic role of alcohol has been documented and is a known risk factor also for cancers other than HCC[44]. Ethanol is mainly metabolized in the liver, producing acetaldehyde and free radicals which produce liver injury and DNA damage through the increase of oxidant stress[45]. Moreover, alcohol abuse produces an accumulation of iron which in turn, contributes to oxidative stress[46] with an additive and possibly synergistic mechanism causing hepatocellular damage.

Hereditary hemochromatosis is a common genetic defect in the Caucasian population causing iron accumulation in different organs as well as in the liver[47]. As has already been stated, iron itself may have a direct role in the development of HCC. The risk of developing HCC in these patients is reported to be 20-200 times greater than that in healthy controls[48-51]. Male sex, older age, cirrhosis and the presence of co-factors such as alcohol, viral hepatitis and tobacco smoking are known risk factors[52,53].

Non-alcoholic fatty liver disease (NAFLD) has recently been described in cohorts of patients as a cause of HCC[54-56], but larger prospective studies are needed to define the real incidence and risk factors for HCC development in this setting of patients

Aflatoxins are hepatocarcinogens produced by *Aspergillus Flavus* and contaminate the food supply in South-east Asia and Africa[57,58], increasing the risk of HCC development, especially in patients with HBV infection, by causing a mutation of the p53 gene[59]. This kind of contamination occurs mostly in developing countries with a hot-humid climate where a variety of oilseeds and cereal crops are produced[60].

## PUBLIC HEALTH MEASURES FOR PRIMARY PREVENTION OF LIVER DISEASE

As already mentioned, HCC occurs in the context of known risk factors and its treatment is disappointing. Therefore the best way to prevent its development is preventing the onset of acquired liver diseases and applying surveillance programs for patients with estabilished liver diseases.

In developed countries, viral hepatitis infection has decreased since the mid-1980s when blood donor screening for viral hepatitis became available. Moreover, the use of medical disposables has contributed to this phenomenon[61] and nowadays, the risk of HBV infection is limited to sexual risk relations, intravenous drug users and in a few cases of patients undergoing dental therapy, acupuncture, piercing and tattooing[62-64]. In developing countries, this field is still a matter of public health because almost half of the blood units are not screened for viral hepatitis[65], sanitary conditions are poor and disposable sanitary tools are often not available.

**Table 1 Recommended doses and schedules for HBV vaccine**

| Vaccine | Patients | Dose (mcg) | Volume (mL) | Schedule (mo) |
|---|---|---|---|---|
| Engerix-B | < 11 yr | 10 | 0.5 | 0, 1, 6 |
| (GlaxoSmithKline, Research Triangle Park, NC) | 11-19 yr | 10 | 0.5 | 0, 1, 6 |
| | > 20 yr | 20 | 1.0 | 0, 1, 6 |
| | Dialysis | 40 | 2.0 | 0, 1, 2, 6 |
| Recombivax HB (Merk & Co. Inc., Whitehouse Station, NJ) | < 11 yr | 5 | 0.5 | 0, 1, 6 |
| | 11-19 yr | 5 | 0.5 | 0, 1, 6 |
| | > 20 yr | 10 | 1.0 | 0, 1, 6 |
| | Predialysis/ dialysis | 40 | 1.0 | 0, 1, 6 |

With regard to HBV, the development of a specific vaccine has dramatically changed the virus epidemiology. This is very important in developing countries where HBV transmission is mostly vertical. HBV vaccination has been shown to be able to prevent cancer[16]. Effective vaccines have been available since 1982 and the World Health Organization has recommended the implementation of mass immunization programs since 1991. This has led to a significant decrease in HCC incidence among children and adolescents in Taiwan and it is expected to occur in the next few decades, also in those countries where the vaccination program was started later[16,66-68]. Different HBV vaccines are available, with recombinant HBs antigen (Table 1). HBV vaccination is one of the fundamental and effective forms of public health prevention measures. Unfortunately, immunization programs have a cost and a large proportion of children do not receive basic vaccines because of economic problems in many developing countries[65]. A plant-derived HBV vaccination could be a cheaper approach, this vaccine is under study with the aim of incorporating the vaccine into the alimentary chain[69].

Especially in developing countries where HBV transmission is mainly vertical with a high prevalence of infection, the screening for HBV is advisable at least in late pregnancy. Mothers positive for HBV-DNA should be given lamivudine or continue lamivudine treatment in order to reduce the viral load and the risk of vertical transmission[70]. Passive immune prophylaxis and immediate vaccination of newborns are required immediately within the perinatal period[70].

Unfortunately, an effective vaccine against HCV is still unavailable. The lack of a small animal model, genomic HCV diversity and the difficulties in establishing an *in vitro* culture of large quantities of HCV are some of the problems encountered by researchers in this field[71]. Therefore, the uncertainty of obtaining an effective vaccine imposes the call for the hygienic strategies already applied in the Western countries in order to reduce the incidence of new cases from about $50 \times 10^5$[72,73] to $< 1 \times 10^5$ inhabitants[74].

The first step in the prevention of alcohol and

NAFLD-related HCC is the fight against chronic abuse and the education for a healthier lifestyle in the general population. Similarly, warnings against alcohol abuse or unsuitable diets and a campaign for the control of body weight through moderate physical activity could play a major role. These concepts are also good for the primary prevention of colon, breast and pancreatic cancer which recognizes a risky lifestyle as their primum movements.

Iron depletion is reported to have a protective effect[75] against the development of hepatocellular carcinoma in genetic hemochromatosis, therefore phlebotomy should be performed when indicated in patients with iron accumulation.

With regard to aflatoxin, the best preventive approach would be pre-harvesting crop management, avoiding infection of the crop with *Aspergillus*[60]. Irrigation and use of fungicides or pesticides may be helpful, but this approach is expensive, especially in poor countries. Post-harvesting technologies are available to limit fungus growth and crop contamination. There are drugs capable of modulating the aflatoxin metabolism once ingested. Oltipraz, an antischistosomal drug, has been shown to be effective in detoxifying patients with serum aflatoxin-albumin adducts[76]. Chlorophyllin is a cheaper drug which has been tested in a perspective, randomised study in China with good results[77].

## PATIENTS WITH ESTABLISHED LIVER DISEASE

### *HBV*

Although it varies according to different geographic areas, HCC incidence among subjects chronically infected with HBV is higher in cirrhotics than in patients with chronic viral hepatitis[6,7,18,30,78-100] (Table 2, adapted from[101]), suggesting that other factors play a relevant role in cancerogenesis. In general, endemic East Asian countries as compared to Western countries where HBV prevalence is low or intermediate have a higher incidence of HCC among all groups considered: asymptomatic carriers, inactive carriers, chronic hepatitis and cirrhosis. It was reported that the five-year cumulative incidence in cirrhotic patients is 15% in East Asian studies and 10% in European studies with a 3-fold higher risk of developing liver cancer in endemic areas than in those with a lower prevalence[101].

This phenomenon is due to several factors associated with an increased risk of HCC development such as patient's age at onset of infection, core promoter variants, the presence of HBe antigen, and probably HBV genotypes. However, the role of HBe antigen and HBV DNA replication in the development of HCC is still being debated. A large study from Taiwan on 11 893 men with chronic hepatitis B, found that the risk of developing HCC is 10-fold higher in patients with HBsAg alone at diagnosis and 60-fold higher in those with both HBs and HBeAg than in HBsAg negative patients[102]. The EUROHEP cohort, with a smaller sample size and a low incidence of HCC showed different results, suggesting that probably in different geographical areas, the same co-factors may produce different results[78]. However, two additional European cohort studies showed that cirrhotic patients, who cleared HBeAg and HBV-DNA (and eventually HBsAg), and achieved ALT normalization, are at low risk of developing HCC[3,103]. Therefore, suppressing HBV replication which represents "per se" a major risk factor may prevent HCC development. Only one randomised controlled trial has reported a decreased incidence of HCC in 67 Taiwanese men treated with interferon as compared to 34 untreated men followed up for 1-12 years. HCC occurred in 1.5% of patients in the treated group as compared to 12% in the untreated group ($P = 0.04$). It is important to note that the only patient who developed HCC in the treated group initially cleared HBeAg, but relapsed later, again becoming HBeAg-positive with elevated transaminases[96]. A meta-analysis of 7 studies comparing treated patients *versus* untreated controls with HBV-related compensated cirrhosis has shown a very weak protective effect of interferon with a 6.4% difference in risk (95% CI: 2.8% to 10%)[104].

In conclusion, interferon treatment may have a beneficial effect on the development of HCC in HBV-infected patients, particularly when a virological response is achieved.

Nowadays, different nucleotide and nucleoside analogues are available for the treatment of HBV. The oldest in this family is lamivudine, a cytidine analogue, which is able to inhibit HBV replication, improve liver enzymes and inflammatory score and arrest progression to fibrosis[105]. A retrospective study evaluated the efficacy of lamivudine in terms of HCC prevention in 377 Japanese patients, compared to the same number of untreated HBV infected controls. HCC occurred in 1.1% of patients with an annual incidence of 0.4% (patient/year) in the treated group and in 13.3% of patients with an annual incidence of 2.5% (patient/year) in the untreated group ($P < 0.001$)[106]. Only one prospective randomized controlled

**Table 2 HCC incidence rates according to clinical setting and geographic areas**

| Clinical setting | Geographic area | Studies (*n*) | Patients (*n*) | Mean follow-up (yr) | HCC incidence | 95% CI |
|---|---|---|---|---|---|---|
| Asymptomatic carrier | North America | 2 | 1804 | 16 | 0.1 | 0.07-0.14 |
| | Taiwan and China | 4 | 18869 | 8 | 0.7 | 0.61-0.70 |
| | Japan | 1 | 513 | 7.3 | 0.2 | 0.08-0.39 |
| Inactive carrier | Europe | 3 | 410 | 16 | 0.02 | 0-0.04 |
| | Taiwan | 1 | 189 | 8 | 0.2 | 0-0.42 |
| Chronic hepatitis | Europe | 6 | 471 | 5.9 | 0.1 | 0-0.27 |
| | Taiwan | 2 | 461 | 4.0 | 1.0 | 0.36-1.56 |
| | Japan | 2 | 737 | 5.1 | 0.8 | 0.46-1.06 |
| Compensated cirrhosis | Europe | 6 | 401 | 5.8 | 2.2 | 1.62-2.80 |
| | Taiwan/ Singapore | 3 | 278 | 4.3 | 3.2 | 1.94-4.55 |
| | Japan | 2 | 306 | 5.8 | 4.3 | 3.40-5.25 |

Adapted from ref. 101 with permission from American Gastroenterological Association.

study has been conducted using HCC development as an endpoint. In this study 651 patients with HBV-related cirrhosis or advanced fibrosis were randomly assigned with a 2:1 ratio, to receive lamivudine or a placebo. The study was stopped after a median duration of treatment of 32.4 mo based on the recommendations of an independent data and safety monitoring board because of a significant difference between the treated groups in the number of endpoints reached. HCC occurred in 3.9% in the lamivudine group and 7.4% in the placebo group (hazard ratio, 0.49; $P = 0.047$)[25]. This study showed that lamivudine plays a role in preventing HCC but a longer follow-up is needed to confirm this result in consideration of the high rate of lamivudine resistance which could reactivate HBV DNA replication.

A large Italian retrospective analysis of 656 patients with chronic HBV infection, with or without cirrhosis, showed that the likelihood of developing HCC is significantly less in cirrhotic patients having a virological response than in those having a virological breakthrough[84]. Further studies are also needed to evaluate the effect of the newest antiviral drugs (adefovir, entecavir, tenofovir, emtricitabine, *etc*) in terms of HCC prevention.

In conclusion, different factors contribute to the risk of HCC development in patients with HBV infection: HBV replication, HBV direct oncogenetic effect through the integration in host genoma and cirrhosis itself. Until now, the only mechanism we could control pharmacologically is viral replication and consequently liver disease evolution to cirrhosis. Therefore, a strict imaging follow-up is recommended in all patients even those responding to therapy.

### HCV

HCC incidence is higher in patients with cirrhosis due to chronic hepatitis C infection with a variable incidence in cirrhotics of 2%-8% per year[35-37,107]. As for HBV its incidence varies between geographic areas. Studies from Japan [79,108-112] have documented a summary HCC incidence of 1.8 per 100 subjects per year in patients with chronic hepatitis as compared to 7.1 in patients with cirrhosis. Therefore there is a 4-fold risk of developing HCC when cirrhosis is present[101]. In Europe and the United States[5-7,23,36,37,78,80,113-117], the summary incidence is 3.7 in cirrhotic patients whereas it is impossible to calculate it in non-cirrhotic patients due to the lack of HCC in the only study available[36]. Analyzing these data showed that the 5-year cumulative risk of HCC development in cirrhotics is 17% in Europe and 30% in Japan[101]. Thus the best way of preventing HCC development in HCV-infected patients is preventing cirrhosis itself. This is a challenging issue but difficult to carry out, as a large proportion of patients are unaware of their status and the disease is mostly asymptomatic. Therapy is offered to a small proportion of patients worldwide, and moreover, the infection can be eradicated in only about half of the treatable patients[118-120].

IFN may prevent HCC by preventing liver damage evolution by the eradication of viral hepatitis, this is generally the case of sustained responders[121]. Eradicating HCV infection may prevent HCC development, at least in patients without cirrhosis. With regard to cirrhosis, a different incidence of HCC between treated and untreated cirrhotics (Table 3) and a decline in HCC incidence in patients achieving a sustained virological response have been reported[104,108]. The residual risk is related to the cirrhosis itself. Moreover, if some dysplastic or neoplastic cells are present, IFN alone is not capable of eradicating them. As a consequence, we should follow up patients by ultrasonography even after eradication of the infection. In fact, the cirrhotic background probably persists and HCC may develop years after clearance of the virus[122]. A study recently published by our group has shown the best results in terms of HCC prevention in cirrhotic patients treated with IFN plus ribavirin as compared to those treated with IFN alone, and the reduction in HCC incidence after re-treatment of non-responders[122]. The tight relationship between a high Ag-NOR proliferative index [silver stained (Ag)-nucleolar organizing region (NOR)] and HCC development in this study confirms that clinical utility of cellular proliferation markers can predict HCC development as reported in previous studies[5,122]. Since IFN is an anti-proliferative cytokine, it may have a beneficial role in preventing HCC development in cirrhotic paptiens through the reduction of hepatocyte proliferation,

**Table 3 Effect of IFN on HCC incidence among HCV cirrhotic patients**

| Author, year | *n* | Follow-up month (range) | HCC rate | |
|---|---|---|---|---|
| | | | Treated (*n*/*n*) | Controls (*n*/*n*) |
| Nishiguchi 1995[150] | 90 | 54 (24-86) | 2/45 | 17/45 |
| Mazzella 1996[7] | 284 | 32 (12-71) | 5/193 | 9/91 |
| Bruno 1997[5] | 163 | 68 (60-84) | 6/82 | 14/81 |
| Fattovich 1997[35] | 329 | 60 (1-153) | 7/193 | 16/136 |
| IIHCSG 1998[151] | 491 | n.r. | 21/232 | 48/259 |
| Imai 1998[111] | 52 | 48 (3-65) | 8/32 | 7/20 |
| Gramenzi 1998[115] | 144 | 72 (n.r.) | 6/72 | 19/72 |
| Serfaty 1998[116] | 103 | 40 (6-42) | 2/59 | 9/44 |
| Sofia 1998[152] | 162 | 43 (n.r.) | 11/103 | 4/59 |
| Benvegnù 1999[6] | 152 | 72 (n.r.) | 4/75 | 20/77 |
| Mura 1999[153] | 57 | 76 (n.r.) | 0/28 | 5/29 |
| Shioda 1999[154] | 646 | 55 (n.r.) | 22/588 | 18/58 |
| Yoshida 1999[108] | 337 | 52 (n.r.) | 33/230 | 29/107 |
| Valla 1999[117] | 99 | 37 (37-53) | 5/47 | 9/52 |
| Overall | 3109 | | 132/1979 | 224/1130 |

Adapted from ref. 104 with permission from EASL. Meta-analysis of the three randomised controlled trials and 11 non randomised trials. Risk difference: -12.8; 95% CI: -8.3-17.2; $P \leqslant 0.0001$ (chi square test for heterogeneity).

even when a virological response is not achieved[122]. Moreover, a recent retrospective study has shown a lower incidence of HCC in patients with HCV chronic hepatitis who were non-responders to long-term IFN treatment as compared to those treated for less than 24 mo[123].

Data on non-cirrhotic patients derive from retrospective analysis. The largest from Japan, included 2890 patients, 490 of whom were untreated. HCC incidence was higher in the untreated group (3.1% per year) than in the treated group (1.1% per year), but the difference reached a statistical significance only among patients with stage 2 or 3 fibrosis. Patients were also stratified according to virological response showing the greater benefit in responder patients with fibrosis F2-F3[108]. These data have been confirmed by a recent update of the study[124].

This study has also confirmed that HCC development seems related to the transaminase level, which is basically the rationale of treatment, the aim of which is to reduce liver inflammation. The long term use of glycyrrhizin, an aqueous extract from licorice root capable of reducing transaminases when administered for a short period of time, can significantly reduce HCC incidence in HCV patients[125]. Long-term glycyrrhizin injections have recently been shown to reduce the incidence of HCC in patients with HCV-related chronic hepatitis C with or without cirrhosis and in non-responders to IFN-based treatment[126]. Similarly, ursodeoxycholic acid (UDCA) administration has been shown to reduce transaminases in patients with HCV-related chronic hepatitis although the data are insufficient to support this effect on viral markers, mortality, incidence of cirrhosis, or liver histology[127,128]. The mechanisms involved in ALT normalisation are unknown. However, the reduction of HLA antigen expression and cholestasis, changes in membrane plasticity and bile acid pool hydrophobicity may reduce liver inflammation[129]. All these mechanisms are probably involved in the ability of UDCA to lower the incidence of HCC both in rats and in humans[130,131]. Vitamin $K_2$ may also have a protective effect against HCC development in women with cirrhosis[132].

The preventive role of these drugs needs to be further investigated and their use in the treatment of HCV is not recommended as the only effective drug is interferon.

Finally, epidemiological studies have shown that coffee consumption lowers the incidence of HCC in patients with HCV-, HBV- or alcohol-related cirrhosis[133-136], and that the preventive effect is cup-dependent (3 cups decrease the risk of HCC by 75%).

## HCC RECURRENCE PREVENTION (TERTIARY PREVENTION)

HCC recurrence is about 80% after 5 years of curative treatment[137,138] with a local tumour progression of 10% after 5 years of radiofrequency ablation[138]. Recurrence may originate in intrahepatic metastasis from the treated tumour or from multicentric occurrence.

Several approaches to the prevention of HCC recurrence have been attempted. The most convincing seems to be polyprenoic acid administration. Polyprenoic acid is an acyclic derivate of retinoic acid which has been shown to have chemopreventive activity[139-142] by interfering with cellular regulation and differentiation. It was reported that oral administration of polyprenoic acid could significantly reduce the incidence of new HCCs after a median follow-up of 38 mo[143,144]. Its effect does not seem to be mediated by the reduction of liver inflammation (the transaminase level did not differ between the treatment and control groups)[145], but is related to the clearance of dysplastic/HCC clones by apoptotic mechanisms. The mechanism of polyprenoic acid seems to be different from that of interferon which seems to be related to multiple mechanisms such as increased immune-surveillance, S0 arrest of the cell cycle and restoration of apoptosis. A randomised controlled study on patients with HCV-related HCC showed that administration of IFN-$\alpha$ for 48 wk after the effective ethanol injection treatment of HCC, could improve the prognosis by reduction of the second and third recurrence with an improvement of survival in treated patients[146]. Thus, IFN seems to play a more significant role in cirrhosis as a pre-cancer condition than in liver cancer itself. IFN-$\alpha$ lymphoblastoids also seem to be capable of reducing HCC recurrence and improving survival in HBsAg-positive patients with their HCC resected[147]. Another small randomised controlled study showed that the recurrence rate is lower in patients treated with IFN-$\beta$ after surgery or curative alcohol injection than in untreated patients[148]. The problem in these studies is that they included a small number of patients who were treated with different kinds of IFN and different treatment schedules.

Another approach is irradiation of the liver using intra-arterial iodine$^{131}$-labeled lipiodol after HCC resection with the aim of eradicating neoplastic foci. This strategy has been used in a small randomised controlled trial, showing a reduction in the recurrence rate and an increase in disease-free survival when compared to controls[149].

There is evidence that IFN and polyprenoic acid have a modest effects at least in small studies. In practice, when HCC develops in a cirrhotic liver, medical treatment has a very limited efficacy on the prevention of recurrence.

## CONCLUSION

HCC prevention is a public health challenge. Since HCC mostly develops in a sick liver and the secondary prevention is of little effect, the best strategy is to prevent the onset of liver diseases.

Substantial progresses have been achieved in developed countries in the prevention of virusl-related hepatitis (screening of blood units, use of disposable sanitary tools, HBV vaccination) , but alcohol- and dysmetabolism-related HCCs are emerging problems in the same areas, which require specific interventions in terms of public health measures.

In developing countries, there is still a long way to go. In fact, economic constraints limit the development of any program for the prevention of viral hepatitis transmission (including health education campaigns, healthcare politicies, primary prevention and improvement of hygienic and sanitary conditions). In this setting, the

first step should be a political approach aimed to increase people awareness, extensive vaccination programs, blood units screening, sanitary conditions and use of disposables in poor countries. It is obvious that pharmacological approach should follow. In Western countries our efforts should be directed towards educational approach and counceling activities in order to reduce alcohol abuse and obesity.

As a rule, when the diagnosis of viral liver disease is established, only a minority of patients are treated worldwide and benefit from a possible preventive effect of medical treatment for HCC. Once again economic support in developed countries should be given to treat those patients who could benefit from antiviral treatment.

With regard to HCC recurrence, the real contribution of medical treatment to prevention of chronic viral hepatitis is small. However, more effective medical measures against secondary and tertiary prevention of HCC should be taken.

Finally, the role of co-factors in the development of HCC is still unknown and should be further investigated. Co-factors may also clarify the large differences in the geographic incidence of HCC and give further targets of intervention.

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*EDITORIAL*

# Concept on the pathogenesis and treatment of primary biliary cirrhosis

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## Abstract

Primary biliary cirrhosis (PBC) is an organ-specific autoimmune disease that predominantly affects women and is characterized by chronic, progressive destruction of small intrahepatic bile ducts with portal inflammation and ultimately fibrosis, leading to liver failure in the absence of treatment. Little is known about the etiology of PBC. PBC is characterized by anti-mitochondrial antibodies and destruction of intrahepatic bile ducts. The serologic hallmark of PBC is the presence of auto-antibodies to mitochondria, especially to the E2 component of the pyruvate dehydrogenase complex (PDC). Current theories on the pathogenesis of PBC favor the hypothesis that the disease develops as a result of an inappropriate immune response following stimulation by an environmental or infectious agent. Some reports suggest that xenobiotics and viral infections may induce PBC. The pathogenetic mechanism is believed to be caused by a defect in immunologic tolerance, resulting in the activation and expansion of self-antigen specific T and B lymphocyte clones and the production of circulating autoantibodies in addition to a myriad of cytokines and other inflammatory mediators. This leads to ductulopenia and persistent cholestasis, by developing end-stage hepatic-cell failure. In this review are given our own and literary data about mechanisms of development of intrahepatic cholestasis and possible ways of its correction.



**Key words:** Primary biliary cirrhosis; Pathogenesis; Treatment

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## ETIOLOGY OF PRIMARY BILIARY CIRRHOSIS

Primary biliary cirrhosis (PBC) is a chronic cholestatic, granulomatous, and destructive inflammatory lesion of the interlobular and septal bile ducts, which is likely to be caused by an autoimmune mechanism with a potential tendency to progress to cirrhosis[1,2].

Primary biliary cirrhosis is characterized by a T-cell-mediated destruction of bile duct epithelial cells that line the small intrahepatic bile ducts. This leads to ductulopenia and persistent cholestasis, by developing end-stage hepatic-cell failure.

The etiology of the disease is still unknown[3]. Many authors regard the disease as impaired immunoregulation with a loss of tolerance of histocompatibility antigen-enriched tissues. How and why the bile ducts are involved in this process remains unknown. Viruses[4,5], bacteria, xenobiotics, and human immunoregulatory defect may be possible PBC triggers that initiate the immunopathological cascade[6-8].

The development of PBC is preceded by a long asymptomatic period[9-11]. The wide use of computer-aided screening biochemical and immunological studies has significantly increased the detection of asymptomatic patients. In this period, there are generally no physical signs of PBC, at the same time anti-mitochondrial autoantibodies (AMA) are detectable in the serum of virtually all patients (95%)[12-15]. The fact that AMA are detectable many years before PBC manifests itself is indicative of their primary immunopathological role rather than a secondary phenomenon that occurs in the presence of cholestasis. The production of AMA is not an epiphenomenon, and an understanding of the mechanism of AMA induction will shed light on the etiology of PBC[3].

The activity of antibodies to antigens of various specificity (exogenous and autologous genesis) is associated with different classes of immunoglobulins. In PBC, there is an elevated concentration of the immunoglobulin M class (IgM)[16]. The increase in serum IgM is the result of chronic B-cell activation induced *via* the toll-like receptor (TLR) signaling pathway[17]. The activity of tissue, bacterial, and viral antibodies are assumed to be associated with the biological properties of IgM. Testing for AMA and IgM are the most useful laboratory procedure in the diagnosis of PBC[18].

Active searches are recently under way for autoantigens

whose expression induces an immune response that results in destruction of the biliary epithelium. The antigens reacting with AMA are located on the internal mitochondrial membrane. The targets of activated T-lymphocytes are the dihydrolipoamide acetyltransferase components of the 2-oxoacid dehydrogenase, enzyme complexes that are important in oxidative energy metabolism. Pyruvate dehydrogenase complex (PDC) is the best known of these. Among the events demonstrated to induce an antibody response cross-reactive with PDC are exposures to bacterial PDC or retroviral proteins or xenobiotics or microchimerism[2]. Its dihydrolipoamide acetyltransferase component is referred to as PDC-E2[6]. PDC-E1 and PDC-E2 antigens are sensitive (98%) and specific (96%) for the diagnosis of PBC[19,20]. A major question in understanding the pathogenesis of PBC is why PBC patients lose their tolerance to antigens that are found in virtually every cell in the body. The identification of anti-PDC responses (present in over 95% of PBC patients) has given rise to important questions pertinent to our understanding of the pathogenesis of PBC. How and why does immune tolerance break down to as highly conserved and ubiquitously expressed self-antigen as PDC? Why does the body stop recognizing individual components of the pyruvate dehydrogenase complex as proper proteins? Why does breakdown in tolerance to an antigen present in all nucleated cells result in damage restricted to the intra-hepatic bile ducts? How does the internal mitochondrial membrane antigen initiate the production of autoantibodies?

Noteworthy is the assumption that infectious agents are involved in the etiopathogenesis of PBC. Based on the proposed role of microorganisms in the pathogenesis of the disease, Mao TK *et al* and Amano K *et al* hypothesize that patients with PBC possess a hyperresponsive innate immune system to pathogen-associated stimuli that may facilitate the loss of tolerance[21,22]. In PBC patients, AMA shows a cross-reaction with the subcellular components of gram-negative and gram-positive microorganisms[23].

Recent studies have suggested that the induction of PBC is multifactorial, in which the primary player involves the xenobiotics modification of mitochondrial proteins or exposure to xenobiotics-modified bacterial mitochondrial protein homologs, leading to breaking of tolerance to the human mitochondrial autoantigens and eventually liver pathology in genetic susceptible individuals[3,24,25]. A possible cause is molecular mimicry between microbial agents and self-antigens[26-28]. Infection with or exposure to a microorganism whose PDC-E2 bears a close homology with human PDC-E2 could act as an immunological trigger that initiates the development of PBC. It is suggested that the mutant forms of *E. coli*[26,29,30] and mycobacteria[31-33] trigger an immunopathological process in PBC. Anti-PDC-E2 antibodies cross-react specifically with mycobacterial hsp65[34]. Immunization of rabbits with *E. coli* rough (R) mutants gives rise to PBC-specific AMA. The PBC patients' feces contain more or less counts of *E. coli* R-forms that specifically react with AMA[29]. Whether the intestinal R-forms are etiologically important for the development of PBC remains still unclear.

There has been recent evidence for the etiological role of *Novosphingobium aromaticivorans* in the development of PBC[35-37]. *N. aromaticivorans* is a gram negative strictly aerobic bacterium that is found worldwide in soil, water, and coastal plain sediments. Its PDC-E2-like proteins have a higher degree of homology with the immunodominant region of human PDC-E2 than any microorganism thus far studied (100-1000 times greater than that of *Escherichia coli*)[35,37]. *N. aromaticivorans* can metabolize xenobiotics that are similar to the chemical compounds that react with sera from PBC patients. Some of these xenobiotics are immunologically related to lipoic acid, the cofactor that is at the active center of PDC-E2. Thus, *N. aromaticivorans* can theoretically break down self-tolerance in two ways: by molecular mimicry due to subclinical infection and by the metabolism of xenobiotics that are present in the environment.

## PATHOGENESIS OF PRIMARY BILIARY CIRRHOSIS

It was deduced that antibodies against *N. aromaticivorans* were found in 77 of 77 PBC patients from Milan, Italy, who had antibodies to PDC-E2 and that the titers to *N. aromaticivorans* proteins were similar to those to human PDC-E2[35,37]. Thirteen of 14 Icelandic PBC patients who were AMA positive reacted against at least one of the 2-oxoacid dehydrogenase-E2 complexes[36]. These observations provide additional evidence that exposure to *N. aromaticivorans* may trigger the development of PBC.

The epithelium of the bile ducts whose cell surface expresses antigens of the histocompatibility complex is considered to be the major target for AMA. The cross-interaction of AMA with the epitheliocytic antigens of bile cholangioles may damage the epithelium of ductules, by resulting in their obliteration. This leads to cholestasis in the minor canaliculi and bile capillaries with impaired bile excretion processes.

A morphologic study of liver biopsy specimens from asymptomatic patients with anti-mitochondrial autoantibodies detected during a screening makes it possible to diagnose one of the early stages of PBC. In stages Ⅰ-Ⅱ PBC, the biopsy specimens show different phases of lesion of bile canaliculi. Early disorders develop in the interlobular ducts, 45-75 μm in diameter[38]. Dystrophy of ductal epitheliocytes should be considered to be the earliest disorder. Their cytoplasm becomes granular or homogeneous eosinophilic, swollen, and vacuolized, the nuclei get pycnotic. Later on, there is necrosis of a small segment of a canaliculus, but its outlines still remain and, finally, the wall is destroyed, that is a pattern of destructive cholangitis forms[39].

The generation of immune responsiveness to self-antigen can result in pathogenic autoimmune damage of the intrahepatic biliary epithelial cells mediated by both humoral and cellular immune responses[2,40]. In PBC osteopontin is an important immune molecule in portal tracts, and contributes to the recruitment of mononuclear cells into epithelioid granuloma and also participates in bile duct injury *via* B-cell differentiation and plasma cell expansion[41]. PBC is characterized by chronic destructive cholangitis with a Th1-predominant cytokine milieu[42-44].

CD8+ and CD69+ T cells were predominant in inflammatory infiltrates around damaged cholangiocytes; $\beta_2$-microglobulin conformational epitope and intercellular adhesion molecule-1 expression were enhanced in bile ducts and hepatocytes[45,46]. Toll-like receptor-3 and type I interferon signaling pathways are active in both the portal tract and liver parenchyma of early-stage PBC, and form the basis for hypothesis that these signaling pathways are involved in the pathophysiology of PBC[47]. Oxidative stress- and nitric oxide-mediated cellular senescence may be involved in bile duct lesions, which are followed by progressive bile duct loss in primary biliary cirrhosis[48-51].

The canals of Hering (CoH) are destroyed in PBC in concert with the destruction of small bile ducts. This destruction appears to be an early event, because CoH numbers are lowest around stage 0 portal tracts, which still contain normal bile ducts[52]. The canals of Hering (CoH), converging from the hepatic lobule onto the portal tract, connect bile canaliculi to the interlobular bile ducts, and represent the most proximal portion of the bile drainage pathway with a cholangiocyte lining. *De novo* expression of intercellular adhesion molecule-1 (ICAM-1) both on mature cholangiocytes in CoH and epithelial cells in bile ductules in PBC implies that lymphocyte-induced destruction through adhesion by ICAM-1 and binding of lymphocyte function-associated antigen-1 expressing activated lymphocytes takes place not only in the bile ductules, but also in the CoH[53,54].

## PATHOGENESIS OF INTRAHEPATIC CHOLESTASIS

Thus, autoimmune pathological processes gradually leading to ductulopenia develop in asymptomatic PBC[55-57]. The increased number of desolating bile ductules gradually result in impairment of bile excretion and in deficient entry of bile acids into the duodenum. In response to this, the hepatocyte increases the synthesis of bile acids. But ductulopenia does not diminish and bile secretion fails to restore. The reduction in the intestinal level of bile acids through the feedback system induces a compensatory hepatocytic increase in the biosynthesis of bile acids and cholesterol, the major substrate for their biosynthesis. The progressively increased bile acid biosynthesis occurring with the participation of cytochrome $P_{450}$ of the smooth endoplasmic reticulum and mitochondria leads, on the one hand, to hypertrophy of the endoplasmic reticulum and then to its hyperplasia and vacuolization, and, on the other, to the biosynthetic involvement of primary bile acids, other than specific $P_{450}$-dependent monooxygenase and non-specific $P_{450}$-dependent monooxygenase[58] that can oxidize cholesterol by the peroxide mechanism[59] to "atypical" metabolites. The latter may be further oxidized to "nonphysiological", "atypical" bile acids in the mitochondria. Moreover, the hepatocytic concentration of bile acids gradually elevates and their entry into the intestinal lumen remains insufficient, giving rise to a closed vicious circle that results in the accumulation of bile acids in the liver cell. Due to the elevated hepatocytic level of bile acids, their reabsorption from the portal venous bed decreases, leading to the entry and progressive accumulation of bile acids in systemic circulation. This triggers the development of intrahepatic cholestasis that shows up as immunological, morphological, and biochemical signs when the disease is asymptomatic.

## MECHANISMS OF DEVELOPMENT OF BIOCHEMICAL AND MORPHOLOGICAL SIGNS

To inactivate the detergent effect of excess bile acids on the cell membrane apparatus, their sulfation and glucuronidization occur and the hepatocytic biosynthesis of cholesterol and phospholipids that are able to generate micellar-lamellar structures wherein bile acids are inserted diminishes. The biosynthesis of phospholipids occurs with the use of orthophosphate formed by hydrolysis of organic phosphorus compounds under the action of phosphatases: alkaline phosphatase (AP), and 5'-nucleotidase. The activity of these enzymes increases in patients with PBC just in the asymptomatic stage of the disease[16,60,61]. The increased activity of AP and 5'-nucleotidase is presumed to be due to their accelerated biosynthesis in the hepatocytes[62-64]. To enhance the synthesis of these enzymes, it is necessary to increase delivery of amino acids to the cells with the participation of γ-glutamyltransferase (GTP). A.S. Loginov[60] has noted that the change in blood γ-glutamyltransferase activity in patients with PBC outstrips the enhancement of the activities of AP and 5'-nucleotidase.

It is theorized that AP and 5'-nucleotidase give rise to orthophosphate where the latter is required[65]. Based on this theory, the enhanced activity of AP and 5'-nucleotidase in PBC is indicative of the higher hepatocytic need for orthophosphate.

High-resolution $^{31}$P NMR spectroscopy has established the level of phosphate-containing compounds in the native bile of healthy individuals and PBC patients. There are lower levels of lecithin and orthophosphate in the patients' hepatic bile portion (Table 1).

The data given in the table show the average statistical integral intensity of the corresponding $^{31}$P NMR spectral signals in conditional units.

The change in the concentration of one of the native bile components-phosphatidylcholine (lecithin) suggests its altered acid-dependent (dependent on the secretion of bile acids) secretion by hepatocytes[66]. Biochemical studies have revealed that hepatic bile portions from PBC patients contain lower levels of not only lecithin, but also bile acids and cholesterol (Table 1). These findings suggest that hepatocytic bile secretion of the study components is decreased and that the relationship is retained between the secretion of bile lipid components (lecithin and cholesterol) and bile acids. At the same time there is an irregular reduction of bile acids and lipid components in the hepatic bile portion from patients with PBC. Thus, the levels of bile acids, lecithin, and cholesterol are decreased by 6, almost 5, and only 2.4 times, respectively, which is indicative of higher bile lithogenicity in these patients (Table 1).

The decreased quantity of orthophosphate in the hepatic bile portion from PBC patients points to its

**Table 1 Levels of bile acids, cholesterol, lecithin, and orthophosphate in the hepatic bile portion and serum of patients with PBC ($n$ = 16) and controls ($n$ = 14)**

| Parameters | | Values | | |
|---|---|---|---|---|
| | | Controls (mean$_1$ ± SE) | Patients with PBC (mean$_2$ ± SE) | mean$_1$/ mean$_2$ |
| Bile acids (g/L) | Bile | 3.9 ± 0.8 | 0.65 ± 0.02 | 6.0 |
| | Blood | 0.012 ± 0.008 | 0.054 ± 0.008 | -0.2 |
| Cholesterol (μmol/L) | Bile | 0.91 ± 0.06 | 0.38 ± 0.08 | 2.4 |
| | Blood | 4.2 ± 0.6 | 11.8 ± 1.6 | -0.4 |
| Lecithin[a] | Bile | 2.1 ± 0.3 | 0.5 ± 0.1 | -4.6 |
| Orthophosphate[a] | Bile | 1.2 ± 0.3 | 0.30 ± 0.07 | 4.0 |

[a] The data given in the table show the average statistical integral intensity of the corresponding $^{31}$P NMR spectral signals in conditional units.

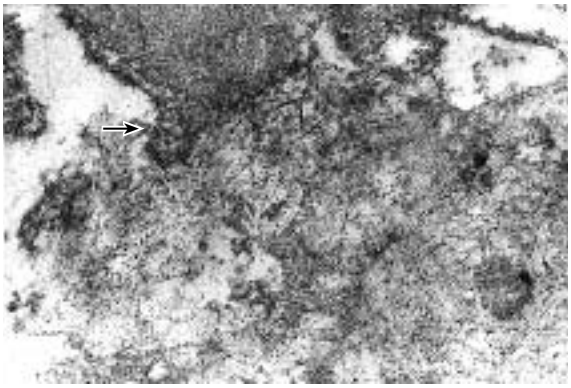

**Figure 1** Hepatocytic ultrastructure. Digitonin-treated biopsy specimen. The specimen is contrasted with aqueous uranyl acetate and lead citrate. Deposits of cholesterol-digitonin complexes (the view of "tender spoondrift clouds") are seen in the cytoplasm close to the mitochondria. The mitochondrial membrane forms pseudopodia here and there (indicated by arrow).

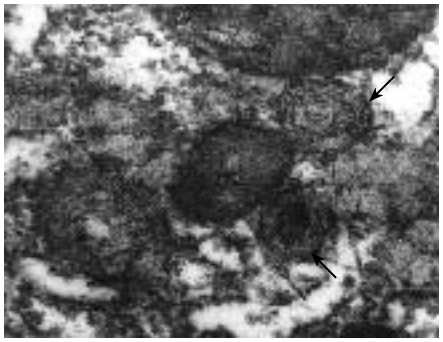

**Figure 2** Hepatocytic ultrastructure in PBC. Biopsy specimen. The specimen is contrasted with aqueous uranyl acetate and lead citrate. Arrows indicate myelin-like and multilamellar structures with increased osmiophilia.

hepatocytic secretion in bile excretion and its simultaneous reduction in its liver cell content since the secretion of orthophosphate, as other inorganic ions, is passively effected by the concentration gradient and does not depend on that of bile acids. The reduced hepatocytic concentration of orthophosphate (despite the enhanced activities of AP and 5'-nucleotidase) indicates the intensive uptake of a phosphorus group in the metabolic processes taking part in the liver cell in PBC.

Biosynthesis of phospholipids is one of the possible ways of using orthophosphate[67]. The hepatic biopsy specimens of PBC show a 1.5-fold increase in the total amount of phospholipids[68]. This increase occurs, by elevating the content of lecithin in the cell membranes. Moreover, the same membranes contain the lower levels of lysophosphatidylcholine, sphyngomyelin, phosphatidylserine, phosphatidylinositol, and phosphatidylethanolamine[68]. There is a 2-fold decrease in the ratio of free cholesterol to phospholipids in the cell membranes. Major alterations in protein status and lipid composition occur in the erythrocyte membrane of patients with PBC[69].

Along with this, segments of membrane cholesterolization and those of free cholesterol deposition in the cytoplasm (Figure 1) and Disse's space are detectable by electron histochemical studies of liver biopsy specimens from PBC patients, by using digitonin. All cholesterol-digitonin complexes show a more or less significant osmiophilia, which points to the presence of phospholipids and proteins in these complexes.

Yokomori H *et al* demonstrate the increased expression of caveolins in proliferating bile ductules in PBC, which may be related to the homeostasis of cholesterol transport in regenerating bile ductules in PBC liver[70]. Caveolins are cholesterol-binding proteins involved in the regulation of several intracellular processes including cholesterol transport.

Electron microscopic studies of liver biopsy specimens from patients with PBC, including those with its early stage, have revealed ultrastructural alterations: hypertrophy and vacuolization of the smooth and rough endoplasmic reticulum, formation of lipid drops and myelin-like structures (Figure 2); the mitochondrial shape and structural changes characterized by the reduced number of internal membrane cristae, the appearance of external membrane "pseudopodia" (Figure 1), as well as local discrepancies of hepatocytes due to their intercellular space.

In the PBC biopsies, the enzymatic activity is increased in the bile canaliculi and is also present in the lateral membranes of the hepatocyte. Transmission electron microscopy of the lateral surface of the hepatocyte in normal livers showed a smooth surface without microvilli but in PBC livers a large number of microvilli were seen in the lateral membranes[71]. The localization of the enzymatic reaction, microvilli and Golgi apparatus at the PBC hepatocyte lateral membranes may represent a compensatory mechanism for derivation of bile flow and other components from the hepatocyte to the intercellular space.

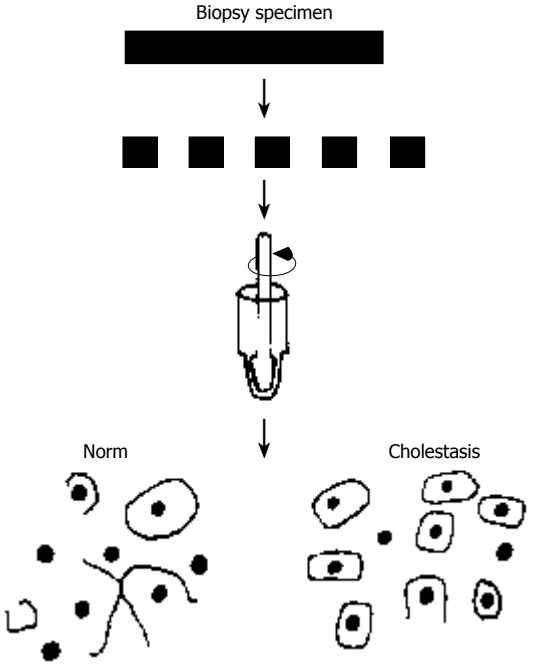


**Figure 3** The scheme of liver biopsy specimen homogenization for quantitative adhesiometric study.

The results of electron microscopic studies of intercellular contact impairments in cholestasis agree well with the estimates of cell adhesion, by using the quantitative method developed by the authors to evaluate adhesive interactions in hepatocytes[72]. The essence of the method is as follows: tight junction (TJ) ensures a mechanical strength of interhepatocytic interactions and when liver tissue disperses with strong intercellular contacts, the plasma membrane breaks and the cell nuclei release. Decreased intercellular contacts lead to that in mechanical dispersion of liver tissue, the hepatocytes move away from each other, without rupturing the plasma membrane. A great number of single cells and much fewer cell nuclei are detectable (Figure 3).

After homogenization of a liver tissue biopsy specimen, single hepatocytes and free cell nuclei are detectable by light microscopy. Then the dissociation coefficient ($C_d$) is the ratio of the number of single cells ($N_c$) to the amount of single cells and the cell nuclei ($N_n$) isolated after liver tissue dispersion is calculated by the formula: $C_d = N_c/(N_c + N_n)$.

A study of liver biopsy specimens indicated a statistically significant increase in $C_d$ in patients with PBC as compared with those with chronic liver diseases, which were similar (Table 2). There is a 1.5-2-fold decrease in the size of hepatocytes. In PBC, the increase in $C_d$ is associated with decreased intercellular contacts, particularly at the site of tight junction, probably due to cholestasis and its related elevated pressure in the bile capillaries. Substantial alteration in the TJ protein occurs predominantly in bile ducts and in hepatocytes in PBC, suggesting increased paracellular permeability along different paracellular routes for bile regurgitation in these chronic cholestatic liver diseases[73]. Melero S *et al* provide functional evidence that PBC cholangiocytes exhibit a widespread failure in the regulation of carriers involved in transepithelial $H^+/HCO^{3-}$ transport, thus, providing a molecular basis for the impaired bicarbonate secretion in this cholestatic disease[74].

**Table 2 Arithmetic means of the dissociation coefficient in different nosological forms of chronic diffuse hepatic lesions**

| Liver lesion | $C_d$ (mean ± SD) | $C_d$ ranges |
|---|---|---|
| PBC ($n$ = 11) | 0.61 ± 0.03[b] | 0.41 ± 0.84 |
| CH ($n$ = 20) | 0.14 ± 0.04 | 0.02-0.24 |
| LC ($n$ = 3) | 0.18 ± 0.03 | 0.14-0.24 |
| UH ($n$ = 8) | 0.11 ± 0.02 | 0.00-0.16 |
| FH ($n$ = 11) | 0.07 ± 0.02 | 0.00-0.14 |
| Controls ($n$ = 16) | 0.09 ± 0.02 | 0.00-0.18 |

CH: Chronic hepatitis of various etiology; PBC: Primary biliary cirrhosis; UH: Gilbert's unconjugated hyperbilirubinemia; FH: Fatty hepatosis; LC: Liver cirrhosis; [b]$P < 0.001$ *vs* both the controls and other groups of the disease.

There is no direct evidence for elevated hepatocytic levels of bile acids since methods for their determination within the liver cell are lacking. However, many indirect experimental data lead to the conclusion that liver cell lesion observed in chronic cholestasis is at least partially caused by the hepatocytic accumulation of excessive cellular toxic bile acids[75-77].

Osmiophilic myelin-like and cholesterol-digitonin structures detectable in the liver biopsy specimens by electron microscopic study appear to be intracellular located complexes of cholesterol, phospholipids, and bile acids.

The blood of patients with early-stage PBC contains higher levels of phospholipids, cholesterol[78,79], and bile acids[80-82] (Table 1). The change in the content of cholesterol, phospholipids, and bile acids in the blood of patients with PBC is associated with their increased formation in the liver and regurgitation into blood flow. Down-regulation of basolateral uptake systems and maintenance/up-regulation of canalicular and basolateral efflux pumps may represent adaptive mechanisms limiting the accumulation of toxic biliary constituents[83]. The elevated levels of phospholipids and cholesterol in the serum of patients with PBC seem to be determined by the necessity of neutralizing the detergent effect of excess bile acids entering the systemic circulation. Cholesterol and phospholipids are able to bind bile acids, by inactivating their solubilizing effect. This gives rise to the so-called micellar-lamellar structures that are water soluble.

## MECHANISM OF CLINICAL SIGN DEVELOPMENT

Protracted and steady-state elevations in the concentration of cholesterol in the blood of patients with PBC give rise to xanthelasmas. There is a relationship between the development of cutaneous xanthelasmas and the elevated serum levels of cholesterol. According to the data obtained by HG Kunkel & EH Ahrens[84], cutaneous xanthelasmas appear when the blood concentration of cholesterol is more than 450 mg/dL. Moreover, this cholesterol level should persist for at least 3 mo. Hyperlipidemia in PBC does not seem to be associated with an increased risk of atherogenesis[85].

The normal (or slightly enhanced) activity of aminotransferases for several years[86] suggests the preserved integrity and normal permeability of the cytoplasmic membrane of hepatocytes in most patients with PBC.

Skin hyperpigmentation is due to the excessive biosynthesis of melanin, as shown by S Sherlock[87]. The initial reaction of melanin biosynthesis is catalyzed by tyrosinase, the copper-containing enzyme. The liver is known to play an important role in the metabolism and regulation of copper homeostasis due to the formation of hepatocytic complexes of protein and copper and its bile excretion[39]. In normalcy, about 80% of the dietary copper is excreted into bile and eliminated with feces. Disturbance bile formation processes in PBC lead to the accumulation of copper in the body. Excess serum copper levels in patients with PBC may cause increases in the activity of tyrosinase and in the biosynthesis of melatonins that deposit in the skin, inducing hyperpigmentation. Simultaneous deposition of copper in the skin imparts a bronzed tint. Since copper is in ceruplasmin-bound state, its elevated content exerts no toxic effect on the body, as in the Wilson-Konovalov disease.

Further progression of intrahepatic cholestasis results in the occurrence other of clinical signs of the disease.

Local or diffuse, moderate or severe skin itching is the earliest and permanent, and, occasionally for several months, sole symptom of the disease[88]. Epidermal deposition of bile acids resulting from cholestasis with PBC patients is a cause of skin itch[89].

At the same time, all fractions of conjugated bile acids increase in the blood[90] while in the skin 50%-85% of bile acids are not conjugated with glycine or taurine[91] and less than 20% of bile acids are in the form of sulfoesters[92]. Sulfation and glucuronization of bile acids diminish their toxic properties and increase their urinary and fecal excretion. The processes of acid bile sulfation and glucuronization, which occur in PBC, seem to be a response to the toxic effect of excess bile acids and to aim at enhancing their elimination through the skin, kidney, and intestine and at lowering their detergent and irritant effect on cells, tissues, and organs. On entering the skin, bile acids induce skin itch. The latter's intensity depends on the ratio of sulfated (glucuronized) and nonsulfated (nonglucuronized) bile acids accumulating in the skin.

The sera of patients with PBC show a higher ratio of trihydroxy-/dihydroxycholanic acids and a lower glycine/taurine coefficient[90]. Garcia-Marin JJ *et al*[93] have indicated that taurine salts of bile acids stimulate the production of micelles with cholesterol and lecithin, promoting the inactivation of cholanic acids. Greim *et al*[94,95] have shown that cholic (trihydroxy-) bile acid has smaller detergent properties than dihydroxy- (deoxycholic, chenodeoxycholic) bile acids. The higher ratio of trihydroxy-/dihydroxy- bile acids in PBC as a compensatory detoxifying mechanism becomes apparent.

"Atypical", "nonphysiological" bile acids appear in the blood and urine of patients with PBC[96]. The appearance of "atypical" bile acids suggests that bile acid biosynthesis accomplished by the participation of 7-α and 12-α hydroxylases is impaired[80]. This may be due to the involvement into the process of biosynthesis of primary bile acids, apart from specific and nonspecific $P_{450}$-dependent monooxygenases[58].

"Atypical" bile acids have a more potent irritant effect on the nerve receptors located in the skin. The intensity of skin itch also depends on the amount of "nonphysiological" ("atypical") bile acids in the skin of patients with PBC.

PBC affects mainly middle-aged, able-bodied females[97]. What is associated with is still unknown so far. Alterations in the content of sex hormones referring to as steroid ones are one of the possible causes of PBC in females[98]. There may be a change in the number or sensitivity of sex hormone receptors. The low expression of estrogen receptors-alpha in PBC and their disappearance in the advanced histological stages suggests that an estrogenic deficiency could favor the evolution of this disease toward ductopenia[99].

A study of steroids hormones in the plasma of patients with PBC has demonstrated a statistically significant reduction in the levels of progesterone and cortisol[100]. The found changes seem to be associated with the delayed biosynthesis of steroid hormones in late-stage PBC. This may be due to the competitive inhibitions of monooxygenases (by enhancing the biosynthesis of bile acids from cholesterol) that participate in the biosynthesis of progesterone and cortisol. Changes in sex hormone profile are secondary to hepatic dysfunction in PBC[98]. Lower cortisol concentrations in the blood of patients with PBC may cause a change in the fluidity of membranes[101,102], including hepatocytic cytoplasmic membranes. The changed fluidity of hepatocytic membranes leads to impaired bile excretion through the canalicular portion of a hepatocytic cytoplasmic membrane, which aggravates and enhances intrahepatic cholestasis.

Deficient entry of bile acids into the intestinal lumen substantially diminishes the absorption of fats and fat-soluble vitamins (A, D, and K)[103]. Along with insufficient vitamin D absorption, there is a lower formation of 1,25-dihydroxyvitamin $D_3$ on cytochrome $P_{450}$ (competitive inhibition of monooxygenases due to the enhanced biosynthesis of bile acids). This all brings about the inadequate calcium ion absorption in the small bowel and impaired phosphorus-calcium metabolism. Impaired hepatocytic calcium metabolism may affect the contractility of intracellular microtubules and microfilaments, which may cause a decrease in the contractility of bile capillaries and enhance cholestasis[77].

Impaired phosphorus-calcium metabolism gradually results in osteodystrophy[104]. Signs of osteoporosis are most commonly detected in patients with end-stage PBC by bone X-ray studies[105,106] and morphological studies of bone biopsy specimens[87,107,108].

The pathogenesis of osteoporosis in cholestatic lesions of the liver appears to be multifactorial[109] and involves impairments of vitamin $D_3$ absorption and metabolism[85], decreased intestinal calcium ion absorption[108,110], genetic predisposition[111], and impact of corticosteroid therapy[112].

Recent data suggest that serum leptin is associated with bone mineral density (BMD). F Szalay *et al* found a lower

serum leptin level and a higher soluble leptin receptor in patients with PBC, which could not be explained by the difference in body mass index. As leptin was associated with BMD, it may be hypothesized that leptin is involved in the complex regulation of bone metabolism in PBC[113]. There was a clear increase in serum leptin levels according to the histological stage of PBC[114].

Glucocorticosteroids (GCSs) diminish intestinal calcium absorption, by reducing the production of 1, 25$(OH)_2$-$D_3$, increasing urinary calcium excretion, and depressing canalicular reabsorption. As a result, there is a compensatory increase in the production of parathyroid hormone (PTH) and bone resorption. In addition, GCSs directly increase PTH release and enhance its sensitivity. Furthermore, GCSs inhibit bone formation indirectly, by suppressing the synthesis of testosterone in gonads and by decreasing the production of growth hormone, insulin-like growth factor, and accordingly type 1 collagen, as well as directly by suppressing the function of osteoblasts[115,116]. Impaired fat absorption brings about steatorrhea - a daily fecal fat loss of more than 7 gram.

The altered composition of bile acids secreted into bile in patients with PBC leads to the impaired ratio of cholesterol, bile acids, and lecithin (in favor of cholesterol), which changes bile viscosity, and increases the bile lithogenicity index. This enhances evolving cholestasis in the bile capillaries and cholangioles. Blood bile reflux occurs. Another clinical sign of PBC, such as jaundice, develops. The development of jaundice is favored by attenuated interhepatocytic interactions and membrane fluidity changes leading to the impaired transport of bilirubin and copper through the canalicular portion of the hepatocytic cytoplasmic membrane. There is accumulation of copper in the body, activation of the copper-containing enzyme tyrosinase, an increase in the synthesis of melanins and in their skin deposition. Skin hyperpigmentation develops.

The serum concentration of bile acids increases and skin itch occurs in patients with PBC long before hyperbilirubinemia and jaundice develop in them. In end-stage PBC, an increase in the level of bilirubin occurs mainly due to its conjugated fraction. These data suggest that the development of hyperbilirubinemia in PBC is most likely to be associated with bile reflux from the bile capillary lumen or hepatocyte into blood and due to its impaired secretion through the apical portion of the hepatocytic cytoplasmic membrane. The altered fluidity of the liver cell membrane in PBC may promote impaired bilirubin transport and enhanced hyperbilirubinemia. Down-regulation of uptake transporters may contribute to the impaired hepatobiliary elimination in advanced PBC, and partially altered localization of MRP2 may reflect the onset of changes leading to icteric PBC[117]. It is clear to say that the glycosylating function of liver cells is retained at early stages of the disease.

At the late stage of the disease, hyperbilirubinemia may also develop due to erythrocytic hyperhemolysis caused by excess serum bile acids in patients with PBC. As potent detergents, bile acids (they are absent in systemic circulation in the normal state) can solubilize the cytoplasmic membranes of erythrocytes and other blood formed elements, by causing their hemolysis. This in turn leads to the development of erythrocytopenia, thrombocytopenia, hypochromic anemia, and other hematological symptoms occurring in advanced stages of the disease with the significant increase in the serum bile acid levels[118]. The degree of hyperbilirubinemia is characterized by not only the conjugated fraction of bilirubin, but also by its unconjugated fraction.

Thus, an autoimmune pathological process occurring for unknown reasons triggers the development of intrahepatic cholestasis in PBC. A gradual progression of the latter contributes to the occurrence of immunological, biochemical, morphological, and clinical signs of the disease. The mechanisms responsible for triggering and developing the major signs of PBC are given in the scheme (Figure 4).

## TREATMENT OF PBC PATIENTS

The presented diagram is far from perfection. The diagram contains many debatable representations, but it gives an insight into the mechanism of development of major clinical signs in PBC and into their sequence and into that it is necessary to break a "closed vicious circle" in order to prevent the progression of the disease. This can be done by externally administering the missing content of bile acids into the intestinal lumen. The given scheme shows that use of ursodeoxycholic acid (UDCA) is a pathogenetically justified treatment for PBC, which promotes the stabilization of the disease process.

UDCA is a 7-beta-epimer of chenodeoxycholic acid (CDCA) (Figure 5). The OH group at carbonic atom 7 is at the alpha-position in CDCA, one of the primary bile acids, and that is at the beta-position in UDCA. It is precisely these structural differences that are at first glance minor, inducing essential dissimilarities of their chemical properties: UDCA is more hydrophilic and less hepatotoxic. With administration of UDCA agents, there is systemic improvement, diminished weakness, and reduced skin itch intensity. Moreover, it should be noted that some patients report greater itching in the first month of an agent's administration and when temperature drastically drops in the transition fall-winter period. Intensified skin itching at the beginning of a drug's administration seems to be associated with the incorporation of UDCA into enterohepatic circulation of bile acids and with their blood concentrations. However, then there is a gradual substitution of endogenous bile acids for administered UDCA and alleviation of skin itch.

The use of UDCA drugs in cholestatic liver lesions results in stabilization of clinical and biochemical signs of the disease. The stabilizing effect of UDCA in patients with PBC is associated with the delivery of missing bile acids into the small bowel (replacement therapy). The drug also inhibits the hepatocytic biosynthesis of endogenous bile acids from cholesterol. The "closed vicious circle" is discontinued. UDCA is particularly effective in early-stage[119,120] and asymptomatic PBC[121]. The early use of the drug prevents these histologic features of PBC[122]. In 30% of patients with PBC, UDCA causes full biochemical normalization, while 70% are incomplete responders[123]. In

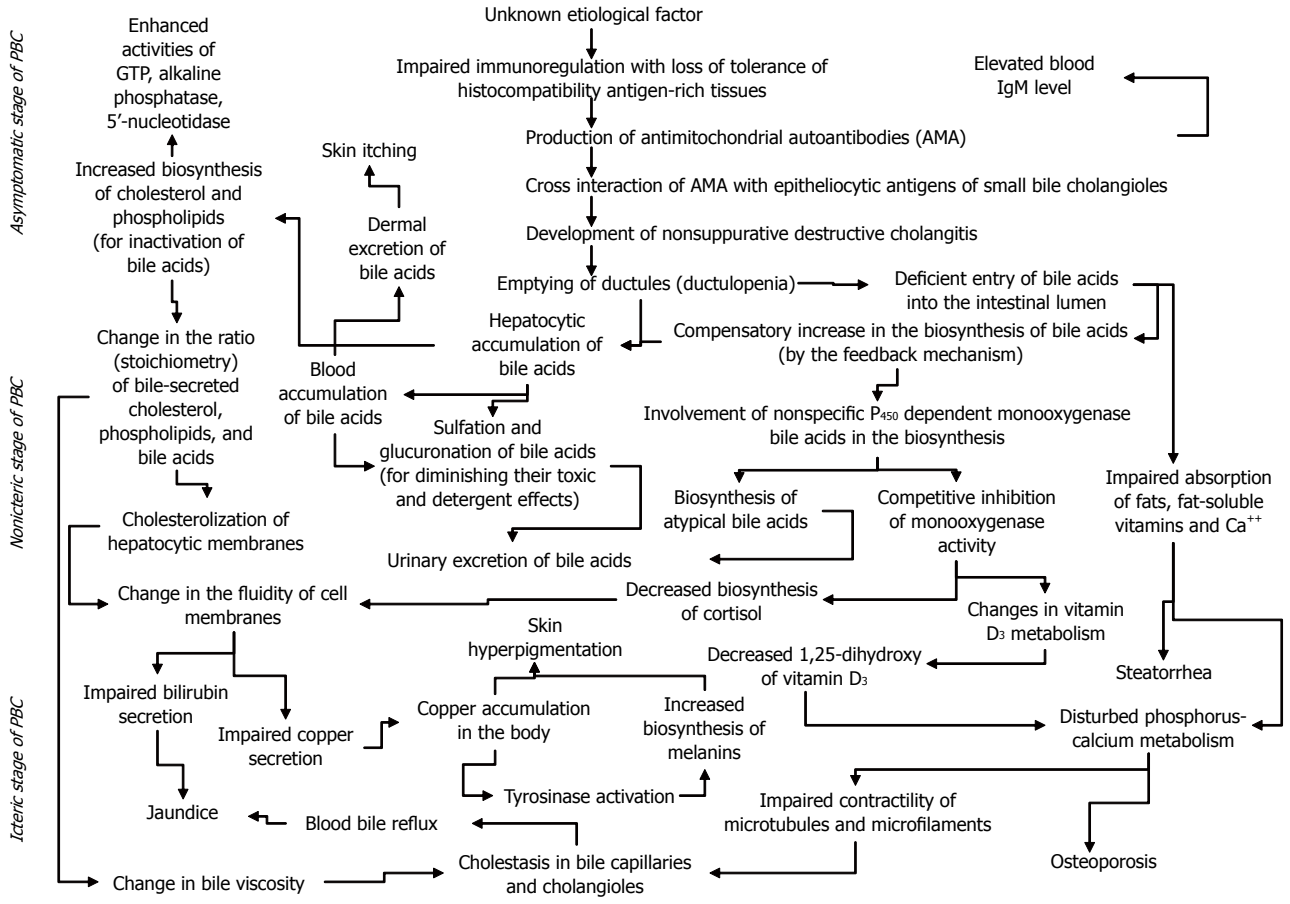


**Figure 4** The scheme of mechanisms responsible for triggering and developing the major signs of PBC.

early PBC, higher dose ursodeoxycholic acid appears to be more effective than the currently recommended doses[124].

UDCA has a positive effect on the prognosis of PBC and can slow down the progression to end- stage liver disease[125]. Treatment with UDCA prevents the deposition of perisinusoidal collagen and reduces the apoptotic activity in PBC patients after 2 years of therapy[126]. Increased endothelin-2 is an early defect in PBC that is significantly reduced by UDCA treatment[127]. Budesonide combined with UDCA improves liver histology, whereas the effect of UDCA alone is mainly on laboratory values[128]. UDCA is not a relevant inducer of CYP3A enzymes in humans[129].

More significant UDCA-induced changes are observed at early stages of the disease. The use of UDCA in patients with PBC is pathogenetically substantiated. However, this treatment modality does not eliminate the cause of the disease and patients with PBC have to take UDCA agents continuously at short intervals.

There are available reports wherein the authors state that UDCA agents show a low efficacy[130,131].

Excess of endogenous blood bile acids can be eliminated by plasmapheresis, followed by a mandatory transfusion of fresh-frozen plasma of the analogous blood group and/or albumin. Plasmapheresis enhances and accelerates the positive therapeutic effect of UDCA agents.

Ion-exchange resins [cholestyramine (vasosan, quantalan), bilignin, questran] are used to control skin itching in patients with PBC. These drugs are not absorbed by enterocytes. By binding to bile acids in the intestinal lumen, ion-exchange resins clear them from the body by transit, by excluding from enterohepatic circulation. This causes a decrease in bile acid levels in blood and, accordingly, in the skin, which lowers the intensity of skin itching.

Ion-exchange resins may not be co-administered with UDCA agents since the latter will combine and be cleared from the body by transit, without achieving a therapeutic effect.

The given diagram also allows an understanding of the pathogenetic mechanisms of the efficiency of use of S-adenosyl-L-methionine in patients with PBC. S-adenosyl-L-methionine is a product of metabolism of the essential sulfur-containing aminoacid methionine (Figure 6). The latter is an officinal drug recommended for the treatment of chronic liver disease. Moreover, methionine is either built into new-synthesized proteins or metabolized to form an active form of S-adenosyl-L-methionine.

The use of S-adenosyl-L-methionine results in relieved skin itching and slightly stabilized clinical and biochemical signs of the disease. The beneficial effect of the drug on the intensity of skin itch is more pronounced when it is parenterally administered.

The mechanism of action of S-adenosyl-L-methionine in chronic cholestatic liver diseases is not completely elucidated so far. This compound is known to play an important role in the body's cells as a source of methyl and sulfonate groups. Therefore it may be suggested that S-adenosyl-L-methionine participates in the formation of

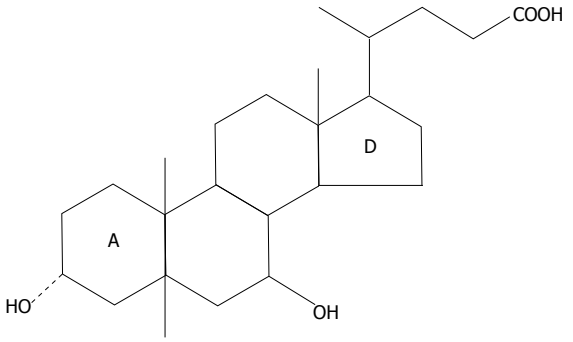


**Figure 5** Ursodeoxycholic acid.

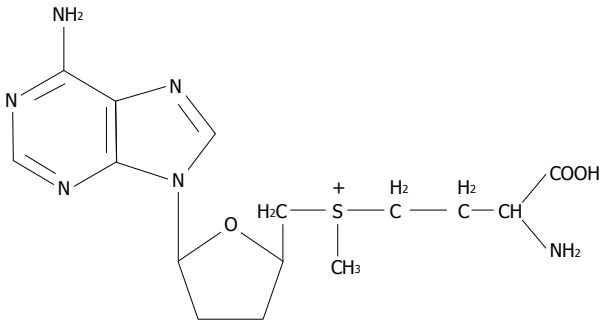


**Figure 6** S-adenosyl-L-methionine.

sulfated bile acids that, unlike nonsulfated ones, may be renally cleared from the body. Moreover, their content in the blood and tissues of the body may decrease, which should yield a positive therapeutic effect. S-adenosyl-L-methionine may also participate in the cellular biosynthesis of phospholipids. This may contribute to the formation of micellar-lamellar structures composed of phospholipids and bile acids. The formation of such structures enables the toxic effect of excess bile acids to be neutralized. Reasoning from these assumptions and the mechanism of development of intrahepatic cholestasis in PBC (see the scheme), the use of S-adenosyl-L-methionine in chronic liver diseases accompanied by intrahepatic cholestasis is pathogenetically justified. Its use is perhaps long and conceivably continuous, as the administration of UDCA agents.

It is advisable to co-administer UDCA and S-adenosyl-L-methionine[132]. This should lead to the potentiation of a therapeutic effect of each of the drugs individually.

Since the etiology and pathogenesis of cholestatic liver lesions remain unknown so far, their treatment is generally symptomatic and aimed at eliminating skin itching[133-137] and jaundice, diminishing steatorrhea, and simultaneously affecting osteoporosis[83,138-140]. The assumed autoimmune nature of PBC has served as the basis for using prednisolone[141] and other immunomodulators[142-144]. However, a rapid rise in bone changes, a great deal of side effects, and a low therapeutic efficacy of these agents have not permitted these drugs to be commonly used for the treatment of PBC[141].

Liver transplantation currently remains to be the most radical treatment for PBC[145,146]. However, selection of patients for surgery is a difficult task. The patient generally gives his/her consent to surgery when his/her condition causes a drastic reduction in the quality of life. Severe weakness, intolerable skin itch, and persistent severe jaundice make the patient be at home and complicate his/her relations with his/her peoples and relatives. This condition is aggravated by the occurrence of ascitis, bleedings from the varicose veins of the esophagus and stomach, or encephalopathy[147]. Liver transplantation may be expected to show the best prognosis in this condition[148,149]. As a rule, the postoperative rehabilitative period is fair[150].

After liver transplantation, there is a change in the titer of anti-mitochondrial autoantibodies: the AMA titers generally drop in the first postoperative months then tend to return to their original values[9].

Whether the disease recurs remains to be solved[151]. Histological changes typical of PBC are detectable in the needle liver biopsy specimens in 9 of 10 patients a year after surgery; of them, 4 patients are then observed to have skin itch and/or other clinical signs[152]. On the contrary, Demetris AJ *et al*[153] detected no histological data of a recurrence in patients with PBC after liver transplantation. Changes in the bile ducts may be induced by other causes: hepatitis C or G viruses; cytomegalovirus; a gradual rejection of the organ or problems associated with biliary anastomotic changes.

With this, 25% of patients needed liver retransplantation due to the development of "bile duct loss" syndrome.

In conclusion, a great body of new data on primary biliary cirrhosis has been recently accumulated. However, inadequate efficiency of its therapy has given impetus to further studies of the pathogenetic mechanisms of the disease[154] and on their basis to searches for new treatment options[155]. No specific therapy that effectively stops or reverses disease progression has been identified, thus it behooves investigators to aggressively pursue identification of the etiology of PBC[156].

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# Effect of ligand troglitazone on peroxisome proliferator-activated receptor γ expression and cellular growth in human colon cancer cells

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## Abstract

**AIM:** To investigate the effect of troglitazone on peroxisome proliferator-activated receptor γ (PPARγ) expression and cellular growth in human colon cancer HCT-116 and HCT-15 cells and to explore the related molecular mechanism.

**METHODS:** Human colon cancer HCT-116 and HCT-15 cells cultured *in vitro* were treated with troglitazone. Reverse transcription-polymerase chain reaction (RT-PCR) and Western blot were employed to detect the effect of troglitazone on PPARγ expression. The proliferative activity was determined by MTT assay, cell cycle and apoptosis were detected by flow cytometry. Apoptosis-related genes, cell cycle regulatory genes and p53 were examined by RT-PCR and Western blot respectively.

**RESULTS:** The expression of PPARγ in colon cancer HCT-116 and HCT-15 cells was up-regulated by troglitazone. Troglitazone inhibited proliferation, induced apoptosis and cell cycle G1 arrest in colon cancer cells. Troglitazone induced p53 expression in HCT-116 cells, but not in HCT-15 cells. The down-regulation of survivin and bcl-2 was found in both cell lines and up-regulation of bax was found only in HCT-116 cells, being consistent with growth inhibition in HCT-116 cells but not in HCT-15 cells. Troglitazone increased expression of $p21^{WAF1/CIP1}$ (p21), $p27^{KIP1}$ (p27) and reduced cyclin D1 in HCT-116 cells while only a minor decrease of cyclin D1 was found in HCT-15 cells.

**CONCLUSION:** Troglitazone is an inductor of PPARγ in colon cancer cells and inhibits PPARγ-dependently proliferation, which may attribute to cell cycle G1 arrest and apoptosis in colon cancer cells. Troglitazone may induce p53-independent apoptosis and p53-dependent expression of p21 and p27. Depending on cell background, different activation pathways may exist in colon cancer cells.



**Key words:** Peroxisome proliferator-activated receptor γ; Colon cancer; Troglitazone; Cellular growth; Cell cycle; Apoptosis

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## INTRODUCTION

Nowadays, there is increasing interest in the use of specific agonists of peroxisome proliferator-activated receptor γ (PPARγ) as a new antineoplastic approach. PPARγ is a member of the nuclear receptor superfamily of ligand-dependent transcriptional factors[1-3]. It has been shown that PPARγ plays an important role in the differentiation of adipocytes and monocytes/macrophages, as well as in cell proliferation, apoptosis and carcinoma cell arrest[4-6]. Many investigators have focused on the role of PPARγ in colon cancer because PPARγ is highly expressed in human colon and colorectal cancer[4,5].

PPARγ is an excellent target for cancer chemotherapy because it expresses highly in tumors and its activation results in decreased cell proliferation and progression from G0-G1 to S phase, increased differentiation and apoptosis. However, within each cancer type, individual cell lines have been found to respond differently to distinct PPARγ ligands according to ligand structure and cell context. For example, PPARγ-activated C-DIMs could induce p21 expression in Panc-28 but not in other pancreatic cell lines[7]. Rosglitazone, ciglitazone and PGJ2 are all potent agonists of PPARγ transactivation in lung adenocarcinoma cell lines but have no effect on squamous cell or large

cell carcinomas of the lung[8]. It is somewhat paradoxical about the induction of these responses by PPARγ agonists and the relative contributions of these pathways are often not well-defined. The PPARγ ligand, troglitazone, one of the thiazolidinediones which are used in the treatment of type II diabetes, is a potent and selective activator of PPARγ. The role of troglitazone in growth of cancer cells has been elucidated in some studies[3,9]. It has been shown that troglitazone has negative effects on the proliferation of malignant tumor cells, including colon cancer cells[3]. However, Lucarelli *et al*[9] found that troglitazone could stimulate growth of osteosarcoma cells. Up to now, whether troglitazone inhibits or stimulates the growth of cancer cells is still unclear. Whether troglitazone activates PPARγ-dependent and/or –independent pathways, which may be beneficial for cancer chemotherapy, has not been elucidated.

This study was to detect the effect of troglitazone on PPARγ expression and colon cancer cell growth and to explore its molecular mechanism. In order to elucidate the effect of troglitazone on colon cancer cells, we chose colon cancer HCT-116 and HCT-15 cells, because these two cell lines are wild-type (WT) and mutant-type p53 respectively[10].

## MATERIALS AND METHODS

### Materials

Two established human colon cancer cell lines, HCT-116 and HCT-15 (Central Chinese Type Culture Collection, Wuhan, China) were used in this study. All standard culture reagents were obtained from Gibco BRL Inc., USA. Troglitazone was obtained from Alexis Corporation (Switzerland) and dissolved in dimethyl sulfoxide (DMSO) at a final concentration of 0.1% DMSO in the culture medium. 3, [4, 4-dimethylthiazol-2-yl] 2, 5 diphenyltetrazolium bromide (MTT) was purchased from Serva (Germany). Annexin V-FITC and TRIzol were obtained from Pharmingen and Invitrogen (USA). Anti-PPARγ, anti-p53, anti-p21, anti-p27, anti-cyclin D1, anti-survivin, anti-bcl-2, anti-bax and anti-β-actin were purchased from Santa Cruz Biotechnology (USA). All primers were obtained from Shanghai Sangon Biotechnology (China).

### Cell culture

HCT-116 and HCT-15 cells were maintained in Dulbecco modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 0.238% N-2-hydroxy-ethlpiperazine-N-2-ethanesulfonic acids (HEPES), 100 U/mL penicillin G and 0.1 mg/mL streptomycin. Cells were incubated at 37℃ in the 5% $CO_2$ incubator. Before the experiment, the cells were incubated in a serum-free medium for 12 h and treated with experimental reagents.

### MTT assay for cell proliferation

Cell proliferation was evaluated by MTT assay. Briefly, cells were seeded at a density of 5000 cells/well in 96-well plates with or without troglitazone. DMSO was used as vehicle control. MTT solution in phosphate-buffered saline (PBS) was added to reach a final concentration of 0.5 mg/mL and incubated for 4 h at 37℃. Medium was removed and cells were resuspended in 100 μL DMSO, and then the absorbance was measured at 570 nm using a microplate reader. The ratios of the absorbance of treated cells relative to those of control wells were calculated and expressed as proliferation percentage.

**Table 1 Sequences of PCR primers used for amplifications**

| Genes | Primer sequences (5′-3′) | PCR products (bp) |
|---|---|---|
| PPARγ | Forward CTCTCCGTAATGGAAGACC | 474 |
| | Reverse GCATTATGAGACATCCCCAC | |
| P21 | Forward GCGATGGAACTTCGACTTTGA | 354 |
| | Reverse GGGCTTCCTCTTGGAGAAGAT | |
| P27 | Forward AATAAGGAAGCGACCTGCAA | 451 |
| | Reverse CCTCCCTTCCCCAAAGTTTA | |
| Cyclin D1 | Forward GGCAACGGAGGTCTGCG | 323 |
| | Reverse GTCGGTGGTAGATGCACAGCTT | |
| Survivin | Forward CACCGCATCTCTACATTCAA | 345 |
| | Reverse CACTTTCTTCGCAGTTTCCT | |
| Bcl-2 | Forward GGCAATGTGACTTTTTCCAA | 137 |
| | Reverse GGCTGATATTCTGCAACACTG | |
| Bax | Forward CCAGCTGCCTTGGACTGT | 135 |
| | Reverse ACCCCCTCAAGACCACTCTT | |
| GAPDH | Forward CCATGGAGAAGGCTGGGG | 200 |
| | Reverse CAAAGTTGTCATGGATGACC | |

### Flow cytometric detection for cell cycle and apoptosis

The cells were collected by centrifugation and washed with PBS. The pellets were resuspended in ice cold 70% ethanol and fixed at 4℃ for 24 h. The cells were centrifuged and washed repeatedly with PBS for removal of ethanol. The cell pellets were resuspended in 1 mL DNA staining reagent and kept at 4℃ for 30 min. DNA contents were analyzed using a FACS 440 flow cytometer (Becton Dickinson). Apoptosis was evaluated by Annexin V-FITC and flow cytometer. Cells were harvested and washed with PBS, and then stained with Annexin V-FITC and 2 μg/mL PI in binding buffer for 15 min at 37℃ in the dark. Finally, the samples were analyzed by using a FACS 440 flow cytometer.

### Semi-quantitative reverse-transcription polymerase chain reaction (RT-PCR)

Total RNA was isolated from human colon cancer cells with TRIzol reagent following the manufacturer's instructions. Concentration of the RNA was detected by the absorbance at 260 nm, and the integrity was verified by electrophoresis on formaldehyde gels. RT-PCR was carried out as described previously[11]. Total RNA was reverse-transcribed into complementary deoxyribonucleic acid (cDNA) which was subjected to PCR for measurement of messenger RNA (mRNA). The product of PCR was checked by 2% agarose gel electrophoresis for a single band of the expected size. The abundance of each mRNA was detected and normalized to that of GAPDH mRNA. The sequences of all primers used in this project are shown in Table 1.

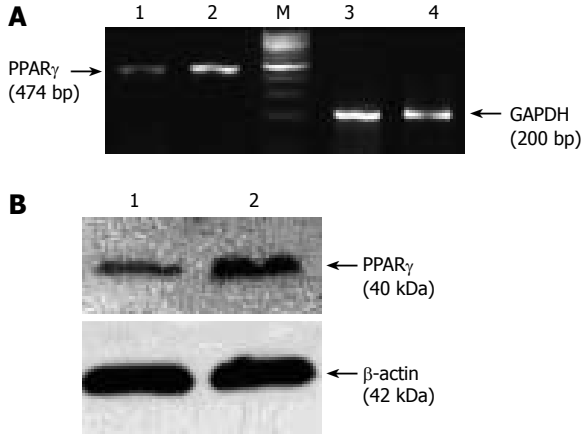



**Figure 1** PPARγ expression in HCT-116 and HCT-15 cells detected by RT-PCR (**A**) and Western blot (**B**). Lane 1: HCT-15 cells; lane 2: HCT-116 cells; M: marker; lanes 3 and 4: GAPDH.

### *Cell protein extraction and Western blot analysis*

Nuclear and cytoplasmic extractions were carried out as previously described[12]. Protein concentrations were measured with a Micro BCA protein assay reagent kit. After being boiled in electrophoresis SDS sample buffer, 40 μg proteins were separated on 10% polyacrylamide gel and transferred onto nitrocellulose membranes. Nonspecific binding sites were blocked with blocking buffer (5% fat-free skimmer milk with 0.1% Tween 20) for 2 h. Subsequently, the membranes were washed with TBST buffer and incubated with the particular primary antibodies diluted in the blocking buffer overnight at 4℃. Then the membranes were washed with TBST buffer and incubated with secondary antibodies. The proteins were detected by enhanced chemiluminescence reagent according to its manufacturer's instructions. The abundance of each protein was detected and normalized to that of β-actin.

### *Statistical analysis*

All experiments were performed in triplicate and the data were expressed as mean ± SD. Statistical analysis was performed by one-way analysis of variance (ANOVA) using SPSS11.0 for windows and $P < 0.05$ was considered statistically significant.

## RESULTS

### *Effect of troglitazone on PPARγ expression in colon cancer cells*

The expression of PPARγ in colon cancer cells was detected by RT-PCR and Western blot. Before exposure to troglitazone, PPARγ was expressed in both HCT-116 and HCT-15 cells, while the mRNA and protein levels of PPARγ in the former were higher than those in the latter (Figure 1A and 1B). After cells were treated with 10 μmol/L troglitazone, the mRNA level of PPARγ increased by 3.03-fold (3.12 ± 0.55 *vs* 1.03 ± 0.31, $P < 0.01$) and 1.52-fold (1.02 ± 0.35 *vs* 0.67 ± 0.25, $P < 0.05$) (Figure 2A) and the protein level increased by 1.86-fold (2.33 ± 0.53 *vs* 1.25 ± 0.42, $P < 0.05$) and 1.48-fold (1.95 ± 0.48 *vs* 1.32 ± 0.38, $P < 0.05$) respectively (Figure 2B).

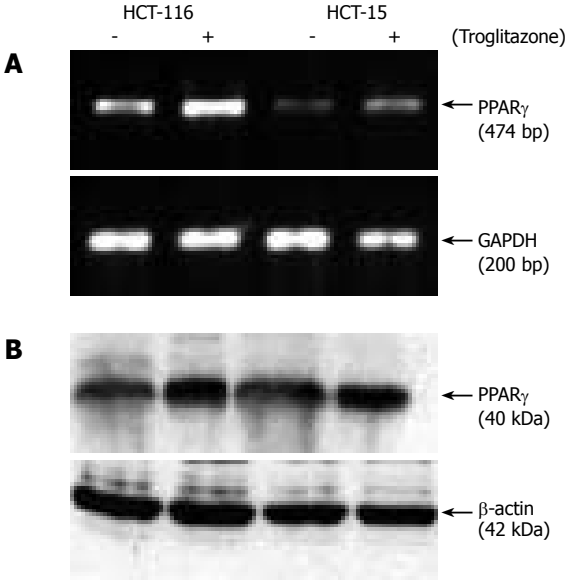



**Figure 2** Effect of troglitazone on PPARγ expression in HCT-116 and HCT-15 cells detected by RT-PCR (**A**) and Western blot (**B**).

### *Effect of troglitazone on proliferation of colon cancer cells*

MTT assay was used to determine the effect of troglitazone on growth of colon cancer. Two cell lines were treated with different concentrations (1, 10 and 30 μmol/L) of troglitazone for different periods of time (12, 24 or 48 h). The results illustrated that troglitazone inhibited significantly proliferation of both HCT-116 and HCT-15 cells in a concentration- and time-dependent manner, which were observed as early as 12 h in HCT-116 cells and 48 h in HCT-15 cells after treatment with 10 μmol/L troglitazone. Significant proliferation inhibition of HCT-15 cells could be only found at 24 h with 30 μmol/L troglitazone (Figure 3).

### *Effect of troglitazone on cell cycle and apoptosis in colon cancer cells*

To determine whether the decrease in cell proliferation was associated with cell cycle arrest or apoptosis, cells were treated with 10 μmol/L troglitazone for 12, 24 or 48 h, then cell cycle and apoptosis were analyzed by using a flow cytometer. Ten μmol/L troglitazone led to increase in percentage of cells in G1 phase and decrease in S phase at 12 h in HCT-116 cells (Figure 4A) and at 48 h in HCT-15 cells (Figure 4B), and there was no significant change in the proportion of cells in $G_2$/M phase. Moreover, the apoptosis rate for HCT-116 cells increased at 12 h (2.52-fold) while that for HCT-15 cells increased at 48 h (2.09-fold), albeit the apoptosis rate gradually increased time-dependently in both cell lines (Figure 4). These results were consistent with the growth inhibition of HCT-116 and HCT-15 cells (Figure 3), suggesting that the anti-proliferation effect of troglitazone should be associated with cell cycle G1 arrest and apoptosis in colon cancer cells.

### *Effect of troglitazone on p53 and apoptosis-related genes*

It is well-known that p53 plays an important role in the induction of apoptosis and growth inhibition, so we

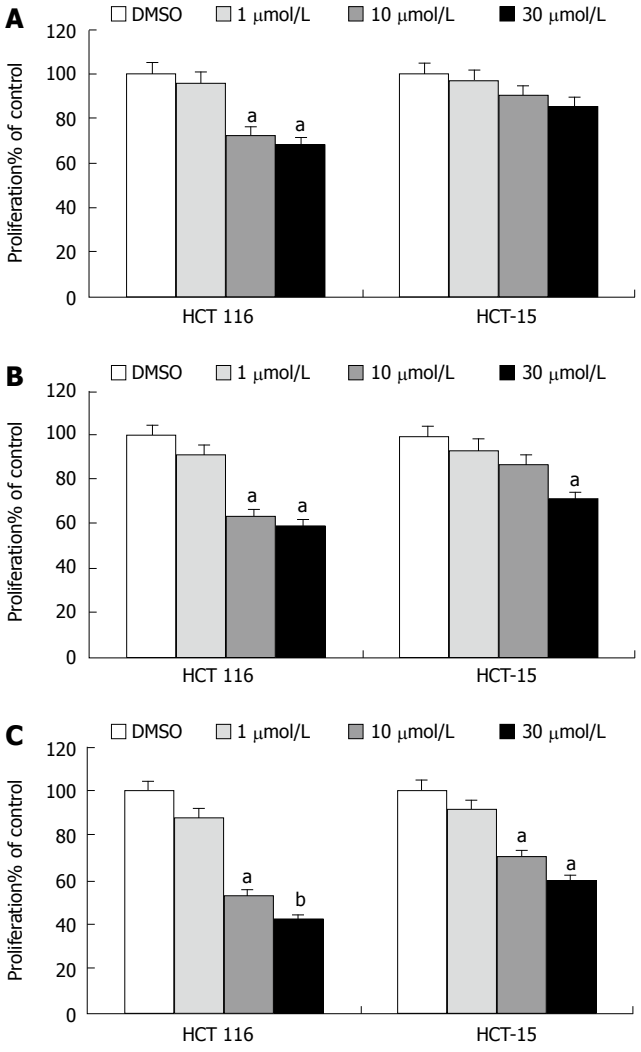
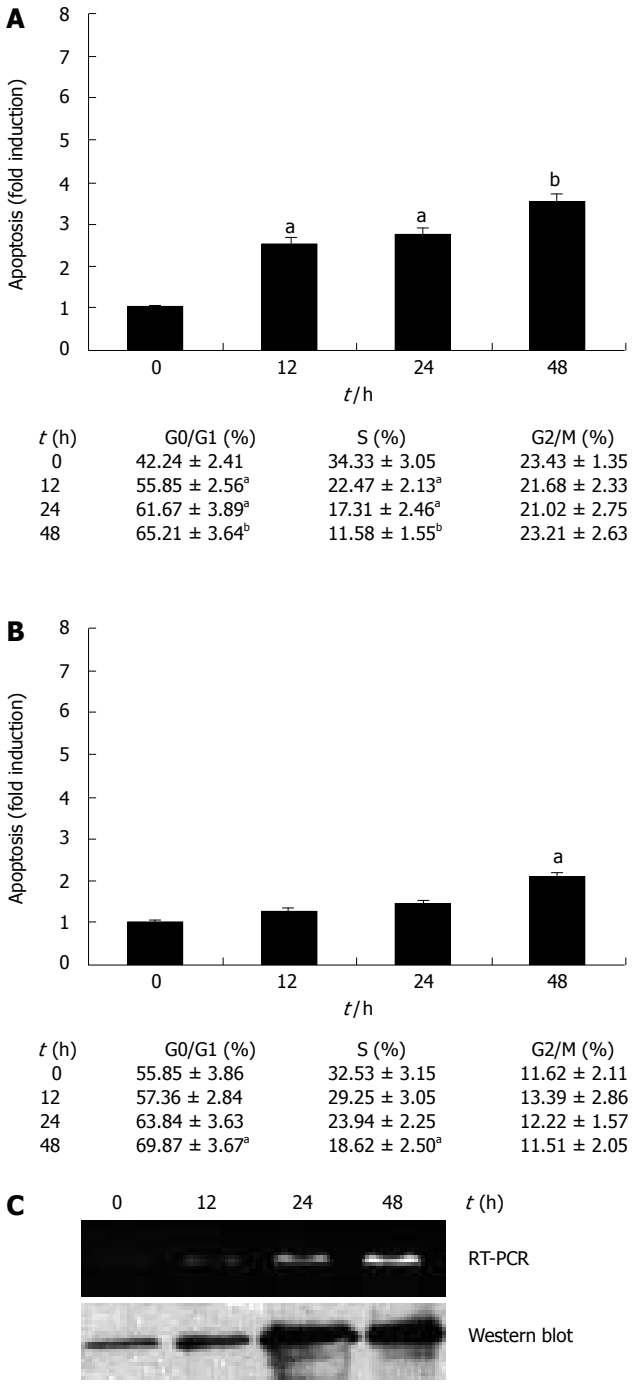



**Figure 3** Effect of troglitazone on proliferation of colon cancer cells at 12 h (**A**), 24 h (**B**), 48 h (**C**). The percentages of cell growth were compared with DMSO (solvent control was set at 100%). $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* corresponding control group respectively.




| *t* (h) | G0/G1 (%) | S (%) | G2/M (%) |
|---|---|---|---|
| 0 | 42.24 ± 2.41 | 34.33 ± 3.05 | 23.43 ± 1.35 |
| 12 | 55.85 ± 2.56[a] | 22.47 ± 2.13[a] | 21.68 ± 2.33 |
| 24 | 61.67 ± 3.89[a] | 17.31 ± 2.46[a] | 21.02 ± 2.75 |
| 48 | 65.21 ± 3.64[b] | 11.58 ± 1.55[b] | 23.21 ± 2.63 |

| *t* (h) | G0/G1 (%) | S (%) | G2/M (%) |
|---|---|---|---|
| 0 | 55.85 ± 3.86 | 32.53 ± 3.15 | 11.62 ± 2.11 |
| 12 | 57.36 ± 2.84 | 29.25 ± 3.05 | 13.39 ± 2.86 |
| 24 | 63.84 ± 3.63 | 23.94 ± 2.25 | 12.22 ± 1.57 |
| 48 | 69.87 ± 3.67[a] | 18.62 ± 2.50[a] | 11.51 ± 2.05 |

**Figure 4** Effect of troglitazone on cell cycle and apoptosis in HCT-116 (**A**) and HCT-15 (**B**) cells and p53 expression in HCT-116 cells (**C**). $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* corresponding control group respectively.

tried to investigate the potential mechanism by which troglitazone causes apoptosis and to clarify whether p53 is related with troglitazone-induced apoptosis in colon cancer cells. After cells were treated with 10 μmol/L troglitazone for 12, 24 or 48 h, mRNA and protein levels of p53 and apoptosis-related genes, such as survivin, bcl-2 and bax, were analyzed by RT-PCR and Western blot. P53 expression was found to be affected by troglitazone in a time-dependent manner in HCT-116 cells (Figure 4C) but not in HCT-15 cells. As shown in Figure 5A, the mRNA levels of survivin and bcl-2 in HCT-116 and HCT-15 cells were down-regulated at 12 h and 24h, respectively. Moreover, the up-regulation of bax was found only in HCT-116 cells, but not in HCT-15 cells. The protein levels detected by Western blot were consistent with the mRNA levels in the three genes (Figure 5B). After exposure to troglitazone for 24 h, the decreased survivin and bcl-2 expression and the unchanged bax expression in HCT-15 cells without corresponding apoptosis suggested that neither survivin or bcl-2 nor bax might be the key regulators for troglitazone-induced apoptosis in HCT-15 cells.

### *Effect of troglitazone on cell cycle regulatory genes*

After cells were treated with 10 μmol/L troglitazone, various markers of cell cycle progression, such as $p21^{WAF1/CIP1}$ (p21), $p27^{KIP1}$ (p27) and cyclin D1 were measured at different time points for verifying the effect of troglitazone on cell cycle regulatory genes. It was observed that the effect of troglitazone on the induction of p21 at mRNA and protein levels in HCT-116 cells was time-dependent. The transcriptional up-regulation of p27, another type of cyclin-dependent kinase inhibitor, was also observed. In addition, cyclin D1 expression was also down-regulated in a time-dependent manner (Figure 6). These results were

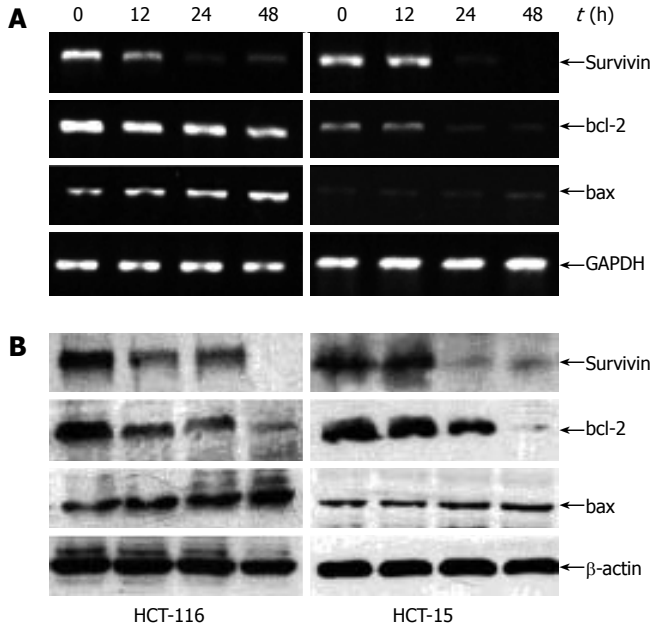



**Figure 5** Effect of troglitazone on survivin, bcl-2 and bax expression by RT-PCR (**A**) and Western blot (**B**) in HCT-116 and HCT-15 cells.

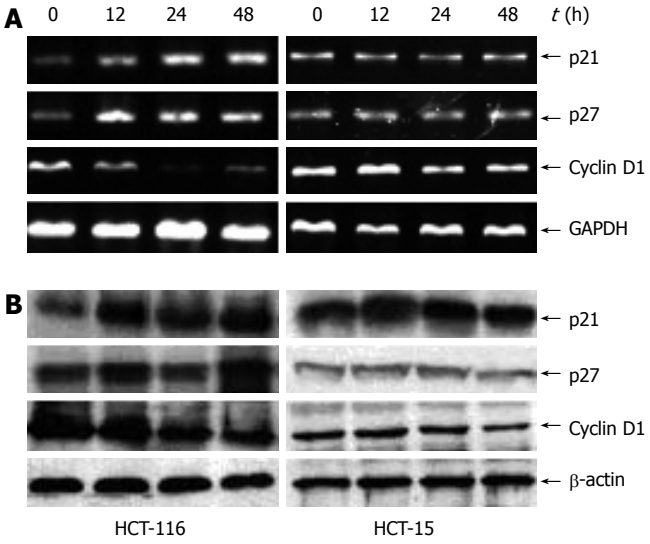



**Figure 6** Effect of troglitazone on p21, p27, and cyclin D1 expression by RT-PCR (**A**) and Western blot (**B**) in HCT-116 and HCT-15 cells.

consistent with cell cycle G1 arrest in HCT-116 cells (Figure 4A). Neither p21 nor p27 was found to be significantly altered in HCT-15 cells, but a minor down-regulation of cyclin D1 was observed (Figure 6). The reasons why p21 and p27 in the two cell lines reacted to troglitazone differently were unclear. Troglitazone could not induce p21 or p27 in HCT-15 cells but could induce p21 and/or p27 expression in a p53-dependent manner.

## DISCUSSION

PPARγ ligands have been shown to regulate gene networks involved in control of cell growth, differentiation, apoptosis and cell cycle[1-3]. Structurally diverse PPARγ agonists inhibit the growth of multiple cancer cell lines[7,13-15], including colon cancer cells. However, the mechanism of growth inhibition is dependent on ligand structure and cell context.

This study was therefore undertaken in an effort to provide additional support for the hypothesis that PPARγ is a negative growth regulator of colon cancer cells. The results showed that PPARγ was expressed in colon cancer HCT-116 and HCT-15 cells, which is consistent with previous report that PPARγ was expressed highly in colon cancer cells[15]. The fact that troglitazone treatment resulted in increased PPARγ mRNA and protein levels in both cell lines suggests that PPARγ signaling is functional. Furthermore, the enhancement of PPARγ in HCT-116 cells was higher, indicating that HCT-116 cells may be more sensitive to troglitazone treatment than HCT-15 cells.

The effect of troglitazone on growth of colon cancer cells was determined by MTT assay. Although troglitazone inhibited growth of both cell lines in a concentration- and time-dependent manner, there were significant differences in the responsiveness of colon cancer cells to the treatment with troglitazone. The growth of HCT-116 cells was inhibited significantly at 12 h (Figure 3) by 10 μmol/L troglitazone, which is consistent with that by 5 μmol/L troglitazone reported by Baek *et al*[16]. In contrast, the growth of HCT-15 cells could be only inhibited by high concentrations of troglitazone (30 μmol/L) at 24 h (Figure 3). A recent study in HCT-15 cells found that only 50 μmol/L troglitazone could induce the same responses, indicating that activation of some (K422Q) PPARγ-dependent mutant responses in HCT-15 cells may be observed with higher concentrations of thiazolidinediones[17]. In our study, troglitazone decreased cell growth in both cell lines, but only high concentration could inhibit growth of HCT-15 cells, suggesting that HCT-15 cells are less responsive to the growth inhibition effect of troglitazone than HCT-116 cells.

There is growing evidence that cell cycle arrest and apoptosis play a key role in the development of human cancer. Troglitazone and other PPARγ-modulating drugs are known to induce cell cycle G1 arrest and apoptosis in some cell types[18]. Our results showed that troglitazone resulted in a remarkable increase of cells in G1 phase and induced apoptosis at 12 h in HCT-116 cells (Figure 4A), but significant increase in G1 phase and induction of the fraction apoptosis were found at 48 h in HCT-15 cells (Figure 4B). This phenomenon was coincident with the growth inhibition in both cell lines. Therefore, the inhibitory effect of troglitazone on cell growth of both colon cancer cell lines may be partly due to cell cycle G1 arrest and apoptosis. Similar effects of troglitazone on other colon cancer cells, liver cancer cells and bladder cancer cells have been reported[3,19,20].

Since troglitazone could inhibit growth and induce G1 arrest and apoptosis in HCT-116 and HCT-15 cells, more attention has been paid to its potential mechanism. Wild type p53 is a potential mediator of apoptosis and more sensitive to DNA-damaging treatment than mutant p53[21]. It is also demonstrated that wild type p53 in human colon epithelial cells is functionally dominant over mutant p53 and restoration of wild type p53 expression is insufficient

to trigger apoptosis of transformed colonic cells[22]. The fact that troglitazone induced p53 in HCT-116 cells (wild type p53) but not in HCT-15 cells (mutant p53), along with induction of apoptosis in both cell lines, suggests that troglitazone-induced apoptosis in both cell lines *in vitro* may be p53-independent. Different results in human gastric cancer MKN-74 cells suggest that p53 might be involved in troglitazone-induced apoptosis[23], which may lie in the different cell context.

Survivin, a member of inhibitors of apoptosis family, plays an important role in cell proliferation and cell death. Down-regulation of survivin may cause a cell-cycle defect that leads to programmed cell death[24]. It has been reported that troglitazone can cause a marked decrease of survivin in glioblastoma cell lines[25]. Other critical regulators of apoptosis, such as bcl-2, bcl-xL (which promotes survival) and bax (which promotes apoptosis), also play a different role in programmed cell death[26-28]. Some researches on PPARγ ligand-induced apoptosis in human myeloid leukemia K562 and HL-60 cell lines showed that up-regulation of bax as well as down-regulation of survivin and bcl-2 may be the important mechanism underlying the induction of apoptosis[26]. However, other reports have shown different mechanisms, depending on the target cell type, PPARγ agonists, duration of treatment, dosage, and the presence of other mitogenic factors[27,28]. In our study, troglitazone decreased survivin and bcl-2 expression and increased bax expression at 12 h in HCT-116 cells, which were consistent with corresponding apoptosis, further suggesting that these apoptosis-associated genes may participate in troglitazone-induced apoptosis in HCT-116 cells. Similar results have also found in breast cancer cells[29] and hepatic cancer HepG2 cells[30]. Troglitazone decreased survivin and bcl-2 expression in HCT-15 cells at 24 h and had no impact on bax expression, which did not correlate with any alteration in troglitazone-induced apoptosis. These data indicate that survivin, bcl-2 and bax do not play a major role in the regulation of troglitazone-induced apoptosis of HCT-15 cells. Chintharlapalli *et al*[27] have reported that the cell context-dependent differential induction of caveolins 1 and 2 may be connected with this process, which is under further investigation.

Inhibition of cell cycle has been considered a target for the management of cancer. We detected the effect of troglitazone on modulation of p53 and its natural target genes p21, p27 and studied the role of two types of p53 in colon cancer cells by examining their effect on cell cycle regulatory genes. P21 and p27 are the downstream effectors of p53. Generally, p21 regulates CDK2-cyclin complexes at the G1 phase of cell cycle following DNA damage or nucleotide pool perturbation, while p27 is a key regulator of the G1/S phase entry and involves the response of cells to environmental mitogen stimulation[31-33]. Once activated by cellular signals, p53 brings about transcription of several cell-cycle regulatory genes, including p21 and p27[34]. P21 expression can be up-regulated by p53-independent or p53-dependent mechanisms, and mutant type p53 induces lower expression of p21 than non-mutant p53[35,36]. Increased p53 expression and transcription-induced p21 and p27 in response to troglitazone treatment at 12 h in HCT-116 cells were found in our study. However, troglitazone had no significant influence on HCT-15 cells, indicating that p21 and p27 play an important role in cell cycle arrest by suppressing HCT-116 cell proliferation. It might be reasonable to consider that troglitazone-induced p21 and p27 may function in a p53-dependent manner. In addition, cyclin D1 is another major positive regulator of G1-S transition by binding to and activating cyclin-dependent kinase 4 or 6, which then phosphorylates and thereby inactivates the tumor suppressor protein pRB[37]. In our study, troglitazone decreased cyclin D1 expression at mRNA and protein levels in HCT-116 cells but only slightly decreased cyclin D1 expression in HCT-15 cells (Figure 6), demonstrating that p21, p27 and cyclin D1 may play a role in the regulation of troglitazone-induced G1 arrest in HCT-116 cells, which are different from some studies on the other type of PPARγ ligands, such as ciglitazone[38]. Meantime, the difference in activation of specific cell cycle regulatory genes by troglitazone was observed between HCT-116 and HCT-15 cells, indicating that different activation pathways may exist in different cell responses to troglitazone-induced G1 arrest, further investigations are underway to clarify the mechanism.

In conclusion, troglitazone is an inductor of PPARγ, a member of nuclear receptor superfamily of ligand-dependent transcriptional factors that has pro-apoptotic and antitumorigenic properties in colon cancer cells. PPARγ-dependent proliferation inhibited by troglitazone is predominant and the growth inhibition attributes partly to cell cycle G1 arrest and apoptosis. Troglitazone may induce p53-independently apoptosis but p53-dependently expression of p21 and p27. Different activation pathways may exist in colon cancer cells.

## ACKNOWLEDGMENTS

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## COMMENTS

### Backgrounds

Peroxisome proliferator-activated receptor γ (PPARγ) is a member of the nuclear receptor superfamily of ligand-dependent transcriptional factors. There is increasing interest in the use of specific agonists of PPARγ as a new antineoplastic approach.

### Research frontiers

PPARγ is an excellent target for cancer chemotherapy because it expresses highly in tumors and its activation results in decreased cell proliferation, decreased G0-G1 to S phases progression, increased differentiation and apoptosis. However, within each cancer type, individual cell lines were found to respond differently to distinct PPARγ ligands according to ligand structure and cell context.

### Innovations and breakthroughs

Troglitazone is an inductor of PPARγ in colon cancer cells and it inhibits PPARγ-dependently proliferation, which may attribute to cell cycle G1 arrest and apoptosis in colon cancer cells. Troglitazone may induce p53-independently apoptosis and p53-dependently expression of p21 and p27. Depending on cell background, different activation pathways may exist in colon cancer cells.

### Applications

To conclude, this results in this article qualify PPARγ ligands as promising antineoplastic agents and form the basis for future PPARγ ligands-mediated therapeutic approaches.

### Terminology

PPARγ: peroxisome proliferator-activated receptor gamma. PPARγ is a member of the nuclear receptor superfamily of ligand-dependent transcriptional factors, It has been showed that PPARγ plays an important role in the differentiation of adipocytes and monocytes/macrophages, as well as in cell proliferation, apoptosis and carcinoma cell arrest.

### Peer review

The main objectives of the study were to determine the exression of PPAR gamma in colon cancer HCT-116 and HCT-15 cells and to focus on the proliferation and apoptosis under the influence of troglitazone. Expression of PPAR gamma was demonstrated by RT-PCR and immunoblotting. Proliferation was evaluated by MTT assay. Cell cycle arrest and apoptosis were measured by immunoblotting and FACS analysis. Involvement of p53 was determined by RT-PCR and genes associated with p53 activation were also measured by immunoblotting (p21, p27 and cyclin D1). Overall this is a complete story providing strong evidences that PPARgamma agonist may be useful in colon cancer therapy. The authors point out that not all cancer cell lines react to these agonists in the same fashion and thus each cell line should be taken under examination.

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# Differences in viral kinetics between genotypes 1 and 3 of hepatitis C virus and between cirrhotic and non-cirrhotic patients during antiviral therapy

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## Abstract

**AIM:** To evaluate the impact of hepatitis C virus (HCV) infection with genotype 1 or 3 and the presence or absence of liver cirrhosis (LC) in the early viral kinetics response to treatment.

**METHODS:** Naive patients ($n$ = 46) treated with interferon-α (IFN-α) and ribavirin and followed up with frequent early HCV-RNA determinations were analysed. Patients were infected with genotype 1 ($n$ = 28, 7 with LC) or 3 ($n$ = 18, 5 with LC).

**RESULTS:** The first phase decline was larger in genotype 3 patients than in genotype 1 patients (1.72 *vs* 0.95 log IU/mL, $P < 0.001$). The second phase slope decline was also larger in genotype 3 patients than in genotype 1 patients (0.87 *vs* 0.15 log/wk, $P < 0.001$). Differences were found in both cirrhotic and non-cirrhotic patients. Genotype 1 cirrhotic patients had a slower 2nd phase slope than non-cirrhotic patients (0.06 *vs* 0.18 log/wk, $P < 0.02$). None of genotype 1 cirrhotic patients had a 1st phase decline larger than 1 log (non-cirrhotic patients: 55%, $P < 0.02$). A similar trend toward a slower 2nd phase slope was observed in genotype 3 cirrhotic patients but the 1st phase slope decline was not different. Sustained viral response was higher in genotype 3 patients than in genotype 1 patients (72% *vs* 14%, $P < 0.001$) and in genotype 1 non-cirrhotic patients than in genotype 1 cirrhotic patients (19% *vs* 0%). A second phase decline slower than 0.3 log/wk was predictive of non-response in all groups.

**CONCLUSION:** Genotype 3 has faster early viral decline than genotype 1. Cirrhosis correlates with a slower 2nd phase decline and possibly with a lower 1st phase slope decline in genotype 1 patients.



**Key words:** Hepatitis C virus; Treatment; Early kinetics; Cirrhosis; Genotypes

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## INTRODUCTION

Chronic hepatitis C virus (HCV) infection is a major health problem with about 170 million infected subjects worldwide[1]. It is the most common cause of liver cirrhosis (LC), hepatocellular carcinoma, end-stage liver failure, and liver transplantation around the world. Achieving a sustained viral response (SVR), which is defined as undetectable HCV-RNA in serum 24 wk after the end of treatment, is the most effective way to prevent disease progression. Interferon-alpha (IFN-α) plus ribavirin (RBV) therapy (combined therapy) achieves an overall response rate ranging from 42% to 82% in naïve patients[2]. Treatment outcome is highly influenced by dosage and IFN-α presentation, use of RBV, and HCV genotype. For HCV genotype 1-infected patients, optimal SVR rates are achieved with pegylated IFN-α plus RBV for 48 wk (a SVR rate of 40%-55%)[3]. For patients infected with HCV genotypes 2 and 3, a high SVR rate (65%-80%) is observed with standard IFN-α and RBV treatment for 24 wk[4]. Various baseline factors, including histological status and the presence of cirrhosis also affect the outcome of treatment[5].

Hepatitis C viral kinetics has provided an explanation

for the differential response among the different genotypes and allows to evaluate the response rates early during therapy with conventional or pegylated interferons[6]. The effects of treatment on the replication of HCV genotype 1 and the clearance of free virions and infected cells have been investigated in previous studies of viral kinetics[7-9]. Blocking virion production from infected cells with daily IFN-α treatment results in a biphasic viral decline pattern[8] which can predict the treatment outcome[10,11]. Studies of viral kinetics in patients infected with HCV genotype 2 showed that IFN-α treatment is more effective in blocking viral production, and results in faster clearance of virions and infected cells[12,13]. Genotype 4 viral dynamics parameters appear similar to HCV-1 and are slower than those of HCV-2 and HCV-3 infected patients, who should be grouped with those with HCV-1 infection when therapeutic schemes are considered in relation to genotype[14]. Other baseline parameters, such as viral load before treatment, race, gender, and age, have been studied for their effect on HCV viral kinetics[11,15].

However, viral kinetics and the effect of cirrhotic status on viral kinetics in patients infected with HCV genotype 3 have not been extensively studied yet. In a previous report analysing the viral kinetics response to standard or pegylated interferon α2a, HCV-3 patients were grouped as "HCV non-1" with HCV-2, -4 and -5 patients and compared to HCV-1 infected patients[16]. Genotype 3 deserves special attention as it is particularly common in some regions of the world, such as South and South East Asia[17], Australia[18] and Brazil[19], as well as in intravenous drug users in Europe[20].

In this study, considering the frequency of HCV-3 infection in our country, we compared the viral kinetics of early response to treatment by taking frequent samples from naïve patients infected with HCV genotypes 1 and 3. Furthermore, we assessed the effects of LC in the early viral kinetics.

## MATERIALS AND METHODS

### Patients

Forty-six naïve chronic hepatitis C patients from the Hepatology Branch, Department of Gastroenterology, University of São Paulo School of Medicine, São Paulo, Brazil were enrolled. Inclusion criteria were patients infected with HCV genotype 1 or 3 who did not undergo previous treatment, with an age of 18 to 70 years, those with their viraemia detectable by reverse transcriptase-polymerase chain reaction (RT-PCR) and their alanine aminotransferase (ALT) serum levels being 1.5 times the upper normal limit, and those having no other significant pathological findings. Histological activity grade and fibrosis stage were evaluated according to the METAVIR scoring system[21]. In particular, cirrhosis was considered when stage F4 was detected. Normal hepatic function and a liver biopsy within six months before treatment demonstrating necroinflammatory stage A2 or A3 were required. Standard inclusion and exclusion criteria for chronic hepatitis C treatment with IFN and ribavirin were applied. Written informed consent was obtained from all patients. The study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in a *priori* approval by the Ethical Committee of the University of São Paulo School of Medicine and the Instituto Adolfo Lutz.

### Therapy groups

Patients were randomised into different IFN-α treatment schedule groups (Table 1). All patients received conventional IFN-α (Roferon®, F. Hoffmann-La Roche Ltd., Basel, Switzerland) to allow a better comparison among the kinetic parameters, but the treatment was continued for 48 wk to allow a better response in all the studied groups. All patients received a first dose of 9 Mega units (MU) of IFN-α. Group "3 TIW" (11 patients) received additional 3 MU doses of IFN-α three times a week until wk 48, while group "3 Q2D" (10 patients) received the same dose every other day. Group "3 QD" (13 patients) received 3 MU daily after the first dose until d 4, followed by 3 MU every other day until wk 48. Finally, group "9 QD" (12 patients) received 9 MU daily after the first dose until d 14, followed by 3 MU every other day until the end of treatment (48 wk). All groups received two doses of 500 mg ribavirin daily for 48 wk.

### Sampling

Serum samples were collected just before IFN-α injections (with a tolerance of 15 min) in the first 2 wk and then every other day until d 28. Samples were processed within 2 h after collection and stored at -80℃.

### Viral load quantitation

HCV viral loads were determined using a commercially available quantitative assay (COBAS AMPLICOR MONITOR™ 2.0, lower limit of detection 600 IU/mL, upper limit of linear quantitation 850 000 IU/mL, Roche Molecular Systems, CA, USA). Samples below the limit of detection were subjected to a qualitative PCR assay (AMPLICOR™, limit of detection 50 IU/mL, Roche Molecular Systems, CA, USA) while samples above the upper limit of detection were retested at 10 or 100 fold dilution in normal serum. Viral load determinations were performed at the same time using the same lot of the kit for all patients. Viral load results were transformed to log10.

### HCV genotyping

Viral RNA was extracted from 100 μL of serum using guanidine isothiocyanate-phenol-chloroform as previously described[22]. The 5' untranslated region (5' UTR) of HCV genome was amplified, PCR products were subjected to cycle sequencing reactions using dideoxynucleotides (ddNTPs) with fluorescent markers (Kit ABI Prism® BigDye™ terminator cycle sequencing ready reaction, Applied Biosystems, Foster City, CA, USA). Genotyping was carried by aligning both sequenced strands (sense and antisense), obtaining a consensus sequence and comparing this consensus sequence to a database[19].

### Mathematical analysis

Kinetic data were analysed using a previously published mathematical model[8], which assumes that there is a pre-

**Table 1 Interferon administration schedule (interferon dosage in MU)**

| Group | d 0 | d 1 | d 2 | d 3 | d 4 | d 5 | d 6 | d 7 | d 8 | d 9 | d 10 | d 11 | d 12 | d 13 | d 14 | d 15 | d 16 | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 3 TIW | 9 | - | 3 | - | 3 | - | - | 3 | - | 3 | - | 3 | - | - | 3 | - | 3 | 3 TIW until wk 48 |
| 3 Q2D | 9 | - | 3 | - | 3 | - | 3 | - | 3 | - | 3 | - | 3 | - | 3 | - | 3 | 3 Q2D until wk 48 |
| 3 QD | 9 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | - | 3 | |
| 9 QD | 9 | 9 | 9 | 9 | 9 | 9 | 9 | 9 | 9 | 9 | 9 | 9 | 9 | 9 | 9 | - | 3 | |

All patients received a first dose of 9 Mega units (MU) of IFN-α. Group "3 TIW" received 3 MU doses of IFN-α three times a week until wk 48; group "3 Q2D" received 3 MU doses of IFN-α every other day; group "3 QD" received 3 MU daily after the first dose until d 14, followed by 3 MU every other day until wk 48; group "9 QD" received 9 MU daily after the first dose until d 14, followed by 3 MU every other day until wk 48. All groups received two doses of 500 mg ribavirin daily for 48 wk. -:Interferon was not administered that day.

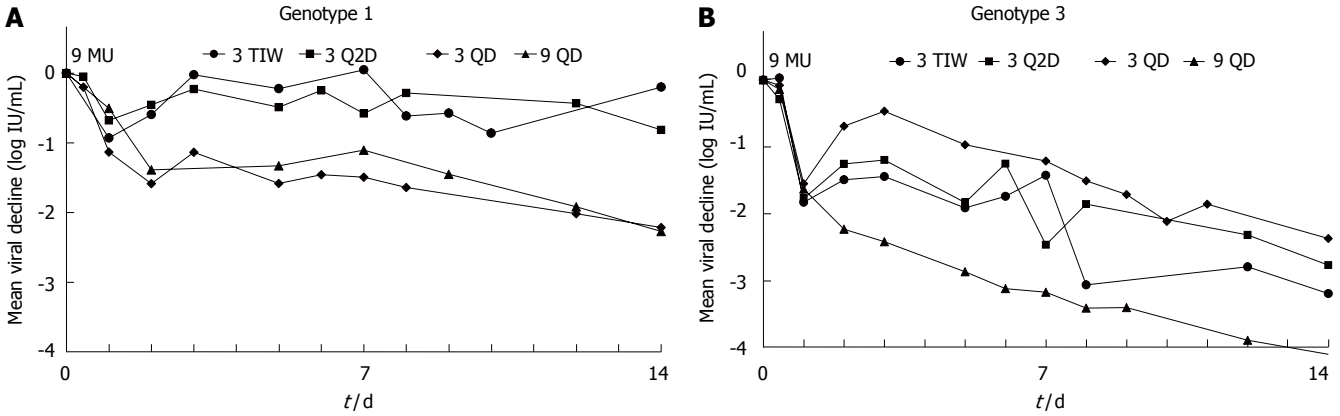


**Figure 1** Mean viral decline over time in each IFN dosing schedule group for HCV genotype 1 (**A**) and genotype 3 (**B**) non-cirrhotic patients. Note that on the first day (vertical line) all groups received 9 MU IFN and had a similar 1st phase decline. A transient rebound was then observed until d 3 in patients who switched to a lower 3 MU IFN dose. Furthermore, patients on a dosing schedule of 3 TIW or 3 Q2D showed viral rebounds on the days they did not receive IFN (d 5-7 for the 3 TIW group and d 5-6 and 7-8 for the 3 Q2D group) in both genotype 1 and 3 patients. However, patients with genotype 3 continued a rapid 2nd phase decline albeit the dosing related transient viral rebounds.

treatment steady state and that the primary effect of IFN-α is to block viral production from infected cells. The first phase decline was defined as the log decline in viral load in the first 24 h, which was equal for all dosing schedule groups. The second phase slope decline was calculated from d 4 (96 h after the first injection) to d 14 to avoid effects of the transient rebound observed after switching from the 9 MU dose to the 3 MU dose on d 2 and differences between the study arms.

### *Statistical analysis*

The statistical significance of differences between groups was assessed with the Mann-Withney non-parametric U-test for the distribution of quantitative variables and the Fisher's exact test for the categorical variables. Multivariable analysis was not performed since the small number of patients per sub-group did not allow any statistical power. $P < 0.05$ was considered statistically significant.

## RESULTS

Forty-six HCV infected patients were included in this study. Twenty-eight of the patients were infected with genotype 1 virus, 7 of them (24%) were cirrhotic. Eighteen patients were infected with genotype 3 virus, 5 of them (27.7%) were cirrhotic. Four different IFN dosing schedules were tested (Table 1). There was no difference in baseline viral load (mean 5.9 log IU/mL) between the genotypes.

### *Effect of genotype and interferon treatment schedule on viral kinetic parameters in patients without cirrhosis*

For non-cirrhotic patients, the kinetics of viral decline for each HCV genotype and treatment schedule is shown in Figure 1. As seen in this figure, oscillations in the viral load were found in patients of groups 3 TIW and 3 Q2D. Each treatment group had particular viral kinetic patterns in accordance with the IFN-α schedule. However, the decline was faster in genotype 3 patients than in genotype 1 patients (Table 2, Figures 2A and 2B). In patients without cirrhosis, the first phase decline after the first 9 MU dose which was given for all groups together, was significantly ($P < 0.001$) larger in genotype 3 patients (1.65 log) than in genotype 1 patients (1.03 log). Only 2 (15%) out of the 13 patients with genotype 3 had a 1$^{st}$ phase decline less than 1 log, compared with 9 (43%) out of the 21 patients with genotype 1. Also the decline of the second phase slope was significantly ($P < 0.001$) faster in genotype 3 patients (0.99 log per week) than in genotype 1 patients (0.18 log per week). None of the genotype 3 patients had a slope slower than 0.3 log per week compared with 16 out of the 21 (76%) genotype 1 patients.

The effect of different treatment schedules on viral kinetics was also compared separately between the two

**Table 2 Mean viral kinetic parameters of each HCV genotype and treatment schedule group in non-cirrhotic patients**

| | IFN dose and schedule | *n* | 1st phase decline[1] (log IU/mL) | 2nd phase slope[2] (log/wk) |
|---|---|---|---|---|
| HCV genotype 1 | 3 TIW | 5 | | 0.09 |
| | 3 Q2D | 4 | | 0.12 |
| | 3 QD | 7 | | 0.15 |
| | 9 QD | 5 | | 0.27 |
| Total non-cirrhotic | | 21 | 1.03 | 0.18 |
| HCV genotype 3 | 3 TIW | 3 | | 0.9 |
| | 3 Q2D | 3 | | 0.78 |
| | 3 QD | 2 | | 0.96 |
| | 9 QD | 5 | | 1.11[b] |
| Total non-cirrhotic | | 13 | 1.65[b] | 0.99[b] |

[1]Treatment during the 1st phase decline was equal in all dosing groups and thus was not given separately. [2]There was no significant difference in the 2nd phase decline slope between dosing groups per genotype. [b]$P < 0.001$ *vs* genotype 1.

**Table 3 Mean viral kinetic parameters for each HCV genotype and histological status**

| HCV genotype | Histology status | *n* | Baseline viral load (log IU/mL) | 1st phase decline[1] (log IU/mL) | 2nd phase slope[1] (log/wk) |
|---|---|---|---|---|---|
| 1 | cirrhotics | 7 | 5.9 | 0.69 | 0.06 |
| | non-cirrhotics | 21 | 6 | 1.03[a] | 0.18[a] |
| Total | | 28 | 5.9 | 0.95 | 0.15 |
| 3 | cirrhotics | 5 | 6.4[c] | 1.89[b] | 0.57[b] |
| | non-cirrhotics | 13 | 5.7 | 1.65[b] | 0.99[b] |
| Total | | 18 | 5.9 | 1.72[d] | 0.87[d] |

[1]Treatment during the 1st phase decline was equal in all dosing groups and no significant difference was found in the 2nd phase decline slope between the dosing groups (Table 2), thus the effect of histological status was studied in all dosing groups together. [a]$P < 0.02$ *vs* cirrhotic genotype 1 patients; [c]$P < 0.03$ *vs* all other patients; [b]$P < 0.01$, [d]$P < 0.001$ *vs* genotype 1 patients.

genotypes (Table 2, Figures 2A and 2B). As expected there was no difference in the 1st phase decline between the treatment groups since the treatment on the 1st day was the same for all patients. Also for the 2nd phase slope, no significant difference was found in the treatment groups, although there was a trend toward faster decline with the daily dose schedules (3 QD and 9 QD) in genotype 1 patients. Indeed, among non-cirrhotic genotype 1 patients receiving daily IFN-α (3 QD and 9 QD), 4 (36%) out of 11 patients had a slope faster than 0.3 log/wk as compared with 1 out of 10 (10%) in the 3 TIW or 3 Q2D group. In each treatment group, genotype 3 patients had a faster second phase slope than genotype 1 patients, with a statistical significance for the 9 QD group even with a small number of patients (Table 2).

Lastly, a lower viral load at the end of the first phase was strongly correlated with a faster second phase slope in all patients ($P < 0.001$; $r = 0.6$).

### *Effect of liver histology on viral kinetic parameters*

The impact of cirrhosis status on cirrhotic and non-cirrhotic patients was addressed by comparing parameters of viral kinetics for each HCV genotype. Patients of all treatment groups were analysed together since there

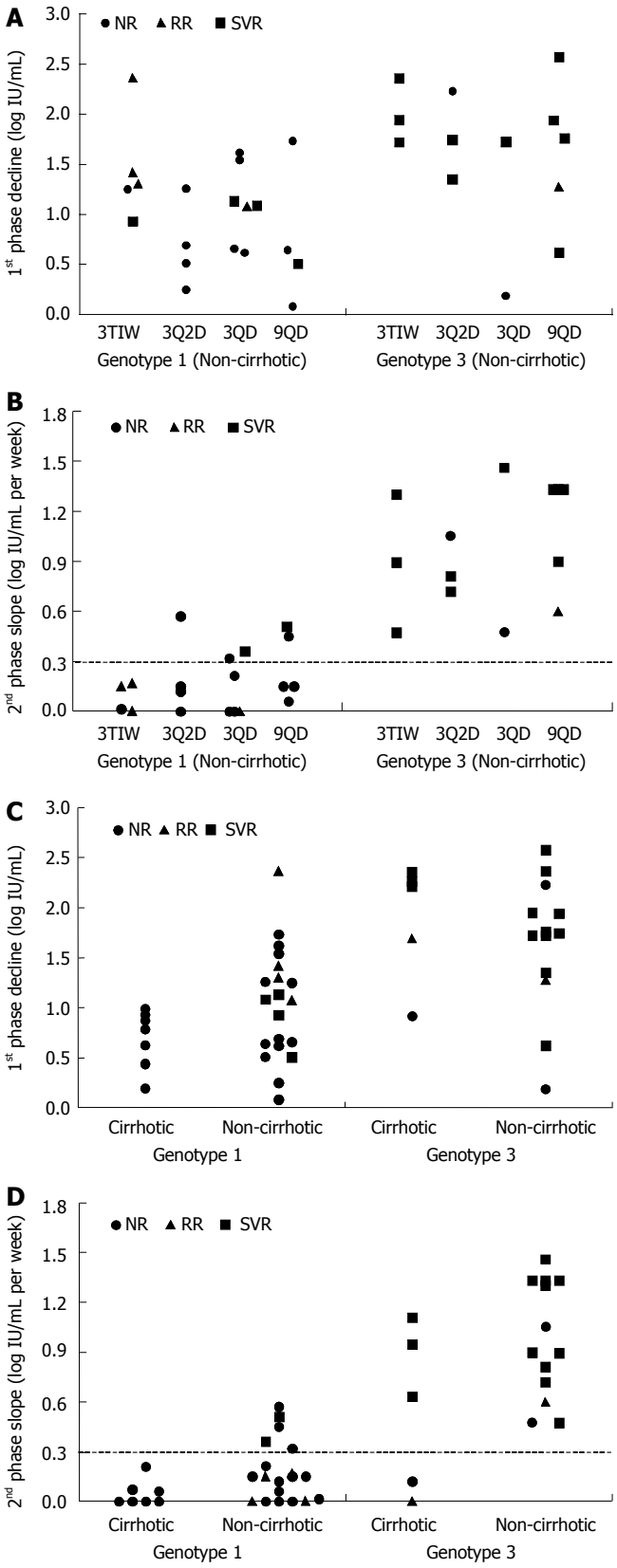


**Figure 2** Distributions of the 1st phase decline (**A** and **C**) and the 2nd phase slope (**B** and **D**) and their correlation with virological end-points given per HCV genotype and dosing schedule for non-cirrhotic patients (**A** and **B**) and each HCV genotype and histological status (**C** and **D**). As in previous studies a 2nd phase decline slope slower than 0.3 log IU/mL per week is predictive of non-SVR (horizontal dashed line in **B** and **D**).

**Table 4 Virological end-points per HCV genotype and histological status *n* (%)**

| HCV genotype | Histology status | *n* | No response | Relapse | Sustained virological response |
|---|---|---|---|---|---|
| 1 | cirrhotics | 7 | 7 (100) | 0 (0) | 0 (0) |
| | non-cirrhotics | 21 | 13 (62) | 4 (19) | 4 (19) |
| Total | | 28 | 20 (72) | 4 (14) | 4 (14) |
| 3 | cirrhotics | 5 | 1 (20) | 1 (20) | 3 (60)[a] |
| | non-cirrhotics | 13 | 2 (15) | 1 (8) | 10 (77)[b] |
| Total | | 18 | 3 (17) | 2 (11) | 13 (72)[b] |

[a]$P < 0.05$, [b]$P < 0.001$ *vs* genotype 1.

was no significant difference in viral kinetic parameters between the interferon treatment schedules. There was no difference in baseline viral load between cirrhotic (5.9 log) and non-cirrhotic (6.0 log) genotype 1 patients (Table 3). Interestingly, genotype 3 cirrhotic patients had a significantly ($P < 0.03$) higher baseline viral load (6.4 log) than non-cirrhotic (5.7 log) genotype 3 patients and genotype 1 patients (Table 3).

A lower first phase decline was observed in genotype 1 cirrhotic patients (0.69 log) than in non-cirrhotic patients (1.03 log), but the difference was not statistically significant (Table 3). Nevertheless, as shown in Figure 2C, none (0%) of genotype 1 cirrhotic patients had a first-phase decline larger than 1 log IU/mL, compared with 55% of the non-cirrhotic genotype 1 patients ($P < 0.02$). Furthermore, a significantly ($P < 0.02$) slower second-phase decline was observed in genotype 1 cirrhotic patients (0.06 log IU/mL per week) when compared with non-cirrhotic patients (0.18 log per week) (Table 3). None of the cirrhotic genotype 1 patients had a slope faster than 0.3 log per week compared with 5 (24%) of 21 non-cirrhotic genotype 1 patients (Figure 2D).

As shown in Table 3, no difference in first phase decline was observed in genotype 3 patients as a function of cirrhosis status (1.89 *vs* 1.65 log IU/mL, $P < 0.05$). However, cirrhotic patients with the genotype 3 virus did have a trend towards a slower second-phase decline (0.57 log IU/mL per week) compared with non-cirrhotic patients (0.99 log per week), and notably the only 2 genotype 3 patients with a flat second phase were cirrhotic patients (Figure 2D).

Cirrhotic patients (Table 3) infected with genotype 1 virus had a less intense first phase ($P < 0.01$) and a slower second phase slope ($P < 0.01$) when compared with genotype 3 infected patients. When cirrhotic and non-cirrhotic patients were pooled together the differences in the genotypes became even more significant (Table 3).

### *Effect of liver histology and HCV genotype on virological end-points*

Only 4 (14%) of 28 genotype 1 patients achieved a SVR. In contrast, 13 (72%) of 18 genotype 3 patients ($P < 0.001$) achieved a SVR (Table 4 and Figure 2). The difference in the genotypes was also seen between cirrhotic and non-cirrhotic patients.

The 4 (19%) of 21 genotype 1-infected patients who achieved a SVR were non-cirrhotic. In contrast, none (0%) of cirrhotic patients achieved a SVR. Interestingly, all the patients who relapsed were also non-cirrhotic (none of the cirrhotic patients achieved a SVR). On the other hand, 3 (60%) out of 5 cirrhotic genotype 3 patients achieved a SVR, which was comparable with 77% of non-cirrhotic genotype 3 patients. Only 1 genotype 3 patient was a true non-responder. A cirrhotic patient and 2 non-cirrhotic non-responders discontinued their treatment early (Table 4 and Figure 2).

Lastly, none of the patients with a second phase slope slower than 0.3 log IU/mL per week, irrespective of genotype, histology status or treatment group, achieved a sustained virological response (negative predictive value = 100%, Figure 2).

## DISCUSSION

Hepatitis C viral kinetics has become an important tool both for investigating the effects of drugs on HCV viral replication[7-10,23,24] and for making clinical decisions about HCV treatment with interferon plus ribavirin therapy[2]. Previous studies have addressed the kinetics of HCV genotypes[7-10,12-16,23-25]. To our knowledge, only one study has addressed the viral kinetics of the early response in patients infected with genotype 3[25] and in that study, HCV-3 patients were grouped together with HCV-2, -4 and –5 patients and viral kinetic data were shown for the "HCV non-1" patients. Our results clearly showed that the 1st and 2nd phase declines were faster in genotype 3 patients than in genotype 1 patients, which were independent of the treatment schedule used or the cirrhosis status when compared to HCV-1. The data relavant to geographic areas were genotypes 1 and 3 which are the most frequent in Brazil, Australia, South and South East Asia, *etc*. To our knowledge, this is the first study to evaluate the effect of LC on viral kinetic parameters.

As conventional IFN-α is still used in many regions of the world particularly in developing countries due to its lower cost, we consider that these data are always valuable for understanding the viral response to conventional IFN-α as the response to treatment of genotype 3 patients is similar to the response to pegylated IFN-α. To ensure a better comparison to genotype 1-infected patients, they were also treated with standard IFN-α, but a 48 wk course was chosen to ensure a better response among all the studied patients in our study.

These results agree with clinical trials of IFN-α (standard or pegylated) and ribavirin treated patients, demonstrating that SVR rates are significantly higher in genotype 3 HCV-infected patients than in genotype 1-infected patients[2,4,5,12]. These results are also consistent with the differences found in viral kinetics between genotype 1 and 2 patients[12,13], as it is known that genotypes 2 and 3 have a similar response rate. We found that the effectiveness in blocking viral production was higher in genotype 3 and the rate of loss of infected cells was faster. Unfortunately, the frequency of sampling in the first 2 d in our study was not enough to study the clearance of free viruses. A detailed comparison between viral kinetics of genotypes 2 and 3 is still of interest.

We observed in this study an intriguing higher baseline viral load in genotype 3 cirrhotic patients than in genotype 3 non-cirrhotic patients or in genotype 1 patients in general. This result was not demonstrated in genotype 1 cirrhotic patients and non-cirrhotic patients, or in genotype 1 or 3 non-cirrhotic patients. The difference observed in genotype 3 cirrhotic patients may be related to the higher steatosis found in these patients[26].

One of the most important results of this study is the observed effect of liver histology on viral kinetics. We have clearly demonstrated that LC adversely impacts early viral kinetics, especially the slope of the second phase viral decline. This is the first viral kinetics study demonstrating a significant difference in the second phase, which is related to the rate of loss of infected cells and is predictive of SVR as a function of the histological status of the liver. The slower decline in cirrhotic patients, which is statistically significant in genotype 1 and genotype 3, can explain the sub-optimal results obtained with interferon and ribavirin in this difficult-to-treat subgroup, even with pegylated interferons[5]. The mechanism behind this effect may be due to a lower immune infiltration to all parts of the liver and/or a lower potency of the immune response to clear infected cells in the cirrhotic environment. Further experimental studies are needed to clarify the mechanism.

On the other hand, our observation that the first phase decline was limited in genotype 1 patients with cirrhosis, but not in genotype 3 patients, was unexpected. The mechanism behind this observation is not understood. Since resistance to IFN-$\alpha$ can be viral or cellular, a possible hypothesis is that hepatocytes in a cirrhotic liver of HCV genotype 1 patients are more resistant to IFN-$\alpha$, which is different from cirrhosis due to genotype 3 that is strongly related to steatosis[25-28]. Steatosis among genotype 3-infected patients does not appear to be related to the presence of HCV antigens within single hepatocytes but to indirect mechanisms, possibly mediated by cytokine[29]. Two HCV proteins, core and NS5A seem to be sufficient to induce lipid accumulation in hepatocytes[30,31], especially in genotype 3[32]. Steatosis is associated with the development of fibrosis[29], which is related to genotype 3[26] and may accelerate fibrosis progression. In genotype 3 patients, steatosis can regress when HCV replication is inhibited supporting a cytophatic effect of genotype 3[33], but the mechanisms underlying the influence of steatosis in response remain to be determined.

In summary, infection with genotype 3 or genotype 1 virus is a more important factor in determining viral kinetics than cirrhosis status or IFN-$\alpha$ treatment schedule. However, within genotypes 1 and 3, patients with cirrhosis have a slower 2$^{nd}$ phase decline, and among genotype 1 patients the 1$^{st}$ phase has a trend toward a lower decline. Thus, advanced histological status although multi-factorial, has a significant adverse effect on viral kinetics, especially in genotype 1 cirrhotic patients. Our results suggest that both genotype 1 and 3 patients with cirrhosis may need more aggressive treatment than the current standard treatment for each genotype. In our study, early prediction of sustained viral response using a second phase slope slower than 0.3 log per week had a negative predictive value of 100%, which is in accordance with previous studies [6]. Thus early viral kinetic prediction can work for cirrhotic patients, since the net effects of all factors are summarized into the first and second phases of viral kinetics.

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*H pylori*

# Relationship between the severity of hepatitis C virus-related liver disease and the presence of *Helicobacter* species in the liver: A prospective study

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## Abstract

**AIM:** To determine the presence of *Helicobacter* species DNA in the liver of chronic hepatitis C (CHC) patients with and without cirrhosis as compared to controls, and to identify the bacterial species involved.

**METHODS:** Seventy-nine consecutive patients (HBV and HIV negative) with a liver sample obtained after liver biopsy or hepatic resection were studied: 41 with CHC without cirrhosis, 12 with CHC and cirrhosis, and 26 controls (HCV negative). Polymerase chain reactions (PCRs) targeting *Helicobacter* 16S rDNA and species-specific were performed on DNA extracted from the liver. A gastric infection with *H pylori* was determined by serology and confirmed by $^{13}$C-urea breath test.

**RESULTS:** Overall, *Helicobacter* 16S rDNA was found in 16 patients (20.2%). Although positive cases tended to be higher in CHC patients with cirrhosis (41.6%) than in those without cirrhosis (17.0%) or in controls (15.4%), the difference was not statistically significant ($P$ = 0.08). *H pylori*-like DNA was identified in 12 cases and *H. pullorum* DNA in 2, while 2 cases remained unidentified. Gastric infection with *H pylori* was found in only 2 of these patients.

**CONCLUSION:** Our results do not confirm the association of *Helicobacter* species DNA in the liver of CHC patients with advanced liver disease. The lack of correlation between positive *H pylori* serology and the presence of *H pylori*-like DNA in the liver may indicate the presence of a variant of this species.



**Key words**: Hepatitis C virus; Hepatitis; Cirrhosis; *Helicobacter*

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## INTRODUCTION

Chronic hepatitis C virus (HCV) infection is a major public health problem with over 170 million people infected worldwide. It is the leading cause of chronic liver disease and the main indication for liver transplantation in the Western world[1]. Approximately 80% of patients with acute infection develop chronic hepatitis. Chronic hepatitis C is associated with a wide spectrum of liver histological lesions ranging from mild chronic hepatitis to cirrhosis and hepatocellular carcinoma (HCC)[2]. The course of HCV-related hepatic disease varies markedly from one patient to another. Several factors including age at exposure, duration of infection, alcohol intake, male gender and more recently steatosis have been shown to be associated with fibrosis progression[3-6]. However, even in the absence of these factors, disease progression may be observed in some

patients, suggesting the role of other factors which remain to be identified. Host genetic factors or environmental factors, such as a bacterial coinfection, could be involved.

Several *Helicobacter* species colonize the liver of animals and induce hepatitis[7]. In the past few years, the emergence of new *Helicobacter* species associated with the pathogenesis of human enterohepatic diseases has been observed[8-10]. *H pylori* and *Helicobacter pullorum* (*H. pullorum*) DNA have been detected in the liver tissue of patients with chronic hepatitis C and HCC, suggesting that these bacteria could be implicated in the progression of chronic hepatitis C to cirrhosis and HCC[11]. In addition, we have shown recently in a cross-sectional retrospective study, a significant association between the presence of *Helicobacter* species DNA in the liver and HCV-related cirrhosis with and without HCC[12]. Given the limitation of retrospective studies, the aim of this new study was to prospectively determine the prevalence of *Helicobacter* infection of the liver in HCV-infected patients with and without cirrhosis as compared to controls.

## MATERIALS AND METHODS

### Study population

All patients with chronic hepatitis C undergoing a percutaneous liver biopsy at the Hepatology Clinics of Bordeaux University between March 2002 and December 2003 were eligible. Chronic hepatitis C was defined by detection of HCV antibodies using a third generation test (Ortho HCV 3.0 ELISA Monolisa anti-HCV, BioRad, Marne la Coquette, France), detectable HCV RNA (Cobas amplicor HCV 2.0, Roche Diagnostics, Neuilly sur Seine, France) in serum, and elevated alanine aminotransferase (ALT) levels for more than 6 mo. Exclusion criteria were age under 18 years, co-infection with hepatitis B virus (HBV) or human immunodeficiency virus (HIV).

The following data were collected at the time of enrolment of patients with chronic HCV infection: age, gender, route of HCV transmission, duration of HCV infection, alcohol intake, aspartate aminotransferase (AST), ALT, gamma-glutamyl transpeptidase (GGT), HCV genotype and viral load.

All patients seronegative for HCV and undergoing hepatic resection for benign tumors or metastatic tumors during the study period, were eligible and served as controls. All patients gave their informed consent and the study protocol conformed to the 1975 declaration of Helsinki and was approved by the Ethics Committee of our institution.

### Processing of liver tissues

Fresh liver tissues obtained either from needle biopsies or from surgical specimens during usual diagnostic or therapeutic procedures, were immediately cut into three parts with three different conditionning protocols: formalin fixation for routine histology (at least 1 cm for needle biospies), freezing in liquid nitrogen-cooled isopentane followed by storage at -80℃ before molecular biology, and immersion in a specific culture medium for culture characterization of the bacterium.

### Pathological study

For all patients, conventional liver histology was performed on formalin-fixed liver tissues. Sections were stained with hematein-eosin-safran, Masson's trichromic stain and reticulin stain. In patients with chronic hepatitis C, liver fibrosis and necroinflammatory activity were evaluated semi-quantitatively according to the METAVIR scoring system[13]. Fibrosis was staged on a 0-4 scale as follows: F0 = no fibrosis; F1 = portal fibrosis without septa; F2 = portal fibrosis and few septa; F3 = numerous septa without cirrhosis; F4 = cirrhosis. Activity was graded as follows: A0 = none; A1 = mild; A2 = moderate; A3 = severe. The diagnosis of hepatocellular carcinoma was based on usual criteria[14].

### H pylori infection diagnosis

Serum samples from all patients were tested for anti *H pylori* IgG antibodies, using the commercially available kit Pyloriset EIA-G Ⅲ (Orion Diagnostica, Espoo, Finland) and the Western blot assay HELICO BLOT 2.1 (Genelabs Diagnostics®, Singapore). When antibodies were present, gastric infection with *H pylori* was searched by $^{13}$C-urea breath test (UBT) or by culture on gastric biopsy specimens obtained during upper gastrointestinal endoscopy when indicated for diagnosis of esophageal varices in patients with cirrhosis. UBT was performed after overnight fasting, 75 mg of urea was ingested after citric acid. Air samples were obtained before and 30 min after urea ingestion and analyzed by isotope ratio mass spectrometry. Serology and UBT have 95% sensitivity and specificity[15].

### Helicobacter culture from liver tissue

Standard *Helicobacter* culture methods were used including Wilkins Chalgren agar with antibiotics[16] and chocolate agar without antibiotics. The plates with fresh liver tissues were incubated at 37℃ for 4 d, one set in a microaerobic atmosphere and another in an anaerobic atmosphere.

To maximize the culture of *Helicobacter* from the biopsies, a flask of confluent murine hepatic (CCl 9.1) cells was systematically inoculated with liver samples and observed during a week for a color change or opacification of the medium, or for a cytopathic effect. The medium was also observed microscopically before discarded.

### DNA extraction from liver tissues

DNA from frozen liver material (20 to 25 mg/specimen) was extracted by using the QIAamp kit (Qiagen Inc., Chatsworth, CA) as previously described[12].

### PCR conditions

Standard PCR amplifications were carried out as previously reported and PCR products were analyzed on a 1%-4% agarose gel, depending on the amplicon size, and stained with ethidium bromide[12].

Two real time PCRs were performed using the TaqMan® or SYBR® Green chemistries. The TaqMan real time PCR amplification and hybridization reactions were carried out in a final volume of 10 μL containing 5 μL of TaqMan® Universal PCR Master Mix, 0.3 μmol/L of each primer, 0.2 μmol/L of the labeled probe Helico-spp-16S and 1 μL

of purified DNA in a ABI PRISM® 7000 thermocycler (Applied Biosystems, Foster City, CA). DNA was amplified using the following cycling parameters: heating at 95℃ for 10 min followed by 40 cycles of a two-stage temperature profile of 95℃ for 15 s and 60℃ for 1 min. The SYBR® Green real time PCR amplification and melting curve analysis were carried out in a final volume of 10 μL containing 5 μL of SYBR® Green PCR Master Mix, 0.3 μmol/L of each primer and 1 μL of purified DNA in a ABI PRISM® 7000 thermocycler (Applied Biosystems). DNA was amplified using the following cycling parameters: heating at 50℃ for 2 min, then at 95℃ for 10 min followed by 40 cycles of a two-stage temperature profile of 95℃ for 15 s and 60℃ for 1 min. Then a dissociation of the amplicon from 60℃ to 95℃ was followed during 20 min.

### *Escherichia coli* PCR amplification

A PCR targeting a 130-bp sequence of the malate deshydrogenase (mdh) gene of *E. coli* was applied to the liver tissue as previously described[12].

### *Helicobacter* genus- and species-specific PCR amplification

*Helicobacter* genus-specific primer pairs C97/C98[17] and HS1/HS2[8,18] were used to generate 16S rDNA amplicons of approximately 400 bp. Moreover, a new PCR targeting the *Helicobacter* genus 16S rRNA gene was developed using the TaqMan chemistry. Sequences of different *Helicobacter* species such as *H pylori*, *Helicobacter heilmannii*, *Helicobacter fennelliae*, *Flexispira rappini*, *Helicobacter bilis*, *Helicobacter cinaedi*, *Helicobacter winghamensis*, *Helicobacter pullorum*, and *Helicobacter bizzozeronii* were aligned. Closely related 16S rRNA genes from various bacteria, such as *Campylobacter coli*, *Campylobacter jejuni*, C*ampylobacter fetus*, *Wolinella succinogenes,* were also included and a search was carried out to identify conserved regions specific to the *Helicobacter* genus. As a result, a set of primers (AS2-Helico-TQM: 5'-CCGTGTCTCAGTTCCAGTGTGT-3' and S1-Helico-TQM: 5'-GATCAGCCTATGTCCTATCAGCTTGT-3') were designed to amplify a 106-bp sequence in *Helicobacter* species. After the specificity of the PCR was ensured, a probe was also designed (Helico-spp-16S FAM-TCACCCTCTCAGGCCGGATACCC-TAMRA). TaqMan probes were synthesized with the FAM reporter dye covalently linked to the 5'P ends and the TAMRA quencher dye at the 3'P ends which were phosphorylated to prevent probe extension. Primers and probes used for the TaqMan assays synthesized by Proligo (Paris, France) were designed using a Primer Express software package PE-ABI (Applied Biosystems).

Samples generating a positive result with the *Helicobacter* genus-specific PCR were subsequently analyzed with seven different sets of primers for the detection of four species previously found in human liver, i.e. *H. sbilis*, *H. spullorum*, *H pylori* and *F. rappini*[12]. For *H. sbilis* and *H. spullorum* primers amplifying a 151-bp product and a 140-bp product on the *cdt*B gene were used, respectively[12]. For *F. rappini*, primers amplifying a 101-bp product on the *ure*B gene were used[12]. For *H pylori*, four real time PCRs were performed for detecting a 267-bp product on the 23S rRNA gene[19], a 146-bp product on the *glm*M (*ure*C) gene[20], a 231-bp product on the *ure*A gene[21], and a 303-bp product on the 26 kDa specific antigen gene[22].

### Purification and cloning of PCR products for 16S rDNA sequencing

Given the possibility of the presence of different strains in the same sample, PCR products were cloned prior to sequencing. *Helicobacter* species 16S rDNA samples were amplified, cloned and sequenced as previously reported[12]. *Helicobacter* genus-specific and species-specific amplified primer-less sequences were compared to the GenBank database with the Blast program at the National Center for Biotechnology Information Computer server[23].

### Statistical analysis

Descriptive statistics are provided as means ± SD. One way analysis of variance (ANOVA) or the Mann-Whitney test was used when necessary for statistical comparison of quantitative data, and the chi-square test or Fisher's exact test for qualitative data. $P < 0.05$ was considered statistically significant.

## RESULTS

### Characteristics of the study population

A total of 79 patients with a liver sample obtained after percutaneous liver biopsy or hepatic resection were included. The median length of the needle biopsy specimens was 1.4 cm (mean 1.35 cm). The characteristics of the patients are shown in Table 1.

Fifty-three patients who had chronic hepatitis C were divided into two groups: group 1 consisting of patients without cirrhosis ($n = 41$), group 2 consisting of patients with cirrhosis ($n = 12$). One patient in group 2 also had HCC. As expected, patients with cirrhosis (group 2) were significantly older than those without cirrhosis (group 1) (60 ± 11 years *vs* 50 ± 13 years, respectively; $P < 0.03$) and had significantly higher AST levels (3.7 ± 2.4 upper limit of normal value *vs* 1.5 ± 1.1 upper limit of normal value, respectively; $P = 0.001$) and ALT levels (3.7 ± 2.8 ULN *vs* 1.7 ± 1.3 ULN, respectively; $P < 0.003$). Conversely, they did not differ in gender distribution, route of HCV transmission, duration of infection, alcohol intake, GGT levels, HCV genotype and viral load.

Group 3 consisted of HCV negative control patients ($n = 26$) in whom liver tissues were taken from the non-tumoral part of hepatectomy specimens after resection for hepatic benign tumors ($n = 11$; i.e. 4 focal nodular hyperplasias, 2 hydatic cysts, 1 cavernous hemangioma, 1 liver cell adenoma, 1 biliary cyst, 1 cystadenoma, 1 abcess) or metastatic tumors ($n = 15$; i.e. all 15 of colorectal origin).

### *Helicobacter* culture

Despite our efforts, it was impossible to grow any *Helicobacter* strain from the liver, either on the plate media or in the tissue culture flasks.

### *Helicobacter* genus detection by PCR

Overall, *Helicobacter* genus DNA was detected in the liver of 16 patients (20.2%). These positive cases were found in

**Table 1 Characteristics of the 79 patients at the time of inclusion**

| Characteristics | Group 1 (Hepatitis) | Group 2 (Cirrhosis) | Group 3 (Controls) |
|---|---|---|---|
| Patients (*n*) | 41 | 12 | 26 |
| Age (yr) | 50 ± 13 | 60 ± 11[a] | 49 ± 12 |
| Gender (M/F) | 14/27 | 5/7 | 13/13 |
| Route of transmission, *n* (%) | | | |
| Transfusion | 10 (24%) | 5 (42%) | - |
| IVDU | 11 (27%) | 3 (25%) | - |
| Other or unknow | 20 (49%) | 4 (33%) | - |
| Duration of infection (yr) | 19 ± 9 | 23 ± 5 | - |
| Alcohol intake (g/d) | 12 ± 23 | 12 ± 14 | - |
| AST (xULN) | 1.5 ± 1.1 | 3.7 ± 2.4[b] | - |
| ALT (xULN) | 1.7 ± 1.3 | 3.7 ± 2.8[b] | - |
| GGT (xULN) | 1.4 ± 0.7 | 2.0 ± 1.9 | - |
| HCV genotype, *n* (%) | | | - |
| 1 | 24 (58%) | 8 (67%) | - |
| 2/3 | 11 (27%) | 4 (33%) | - |
| Other | 6 (15%) | 0 (0%) | - |
| HCV viral load (IU/mL) | 827 889 ± 123 741 | 923 026 ± 181 866 | - |
| Histological lesions (Metavir) | | | |
| Activity, *n* (%) | | | |
| A0-1 | 16 (39%) | 0 (0%) | - |
| A2-3 | 25 (61%) | 12 (100%)[a] | - |
| Fibrosis, *n* (%) | | | |
| F0-1 | 16 (39%) | - | - |
| F2 | 23 (56%) | - | - |
| F3 | 2 (5%) | - | - |
| F4 | - | 12 (100%) | - |

IVDU: Intravenous drug use; ULN: Upper limit of normal value; [a]$P < 0.05$, [b]$P < 0.01$ *vs* hepatitis group.

**Table 2 Factors associated with *Helicobacter* DNA presence in the liver**

| Factor | *Helicobacter* DNA | | *P* |
|---|---|---|---|
| | Present | Absent | |
| Patients (*n*) | 12 | 41 | |
| Age (yr) | 46 ± 13 | 54 ± 13 | 0.05 |
| Gender (M/F) | 5/7 | 12/29 | NS |
| Route of transmission | | | |
| Transfusion | 3 (25%) | 12 (29%) | |
| IVDU | 3 (25%) | 11 (27%) | NS |
| Other or unknown | 6 (50%) | 18 (44%) | |
| Duration of infection (yr) (*n* = 36) | 18 ± 7 | 21 ± 8 | NS |
| Alcohol intake (g/d) | 5 ± 8 | 14 ± 24 | NS |
| AST (xULN) | 2.2 ± 2.1 | 1.9 ± 1.5 | NS |
| ALT (xULN) | 2.7 ± 2.9 | 2.0 ± 1.5 | NS |
| GGT (xULN) | 1.3 ± 0.5 | 1.6 ± 1.2 | NS |
| HCV genotype | | | |
| 1 | 9 (75%) | 23 (56%) | |
| 2/3 | 2 (17%) | 13 (24%) | NS |
| Other | 1 (8%) | 5 (20%) | |
| HCV viral load (IU/mL) | 663 824 ± 569 933 | 972 157 ± 1 388 853 | NS |
| Histological lesions (Metavir score) | | | |
| Activity | | | |
| A0-1 | 4 (33%) | 12 (29%) | |
| A2-3 | 8 (67%) | 29 (71%) | NS |
| Fibrosis | | | |
| F0-2 | 7 (58%) | 32 (78%) | |
| F3-4 | 5 (42%) | 9 (22%) | NS |

IVDU: Intravenous drug use; ULN: Upper limit of normal value; NS: Not significant.

7/41 (17.0%) patients of group 1, 5/12 (41.6%) patients of group 2, and 4/26 (15.4%) patients of group 3 (Figure 1). Although positive cases tended to be more frequent in patients with cirrhosis than in those without cirrhosis or in controls, the difference did not reach statistical significance ($P = 0.08$). Among the 79 liver specimens studied, none was positive by PCR for *E. coli*, a frequent colonizer of the gut.

A number of factors were tested for their association with the presence of *Helicobacter* DNA in the liver of 53 patients with chronic hepatitis C (with or without cirrhosis). None of these factors was associated with the presence of *Helicobacter* (Table 2). Positive patients were significantly younger than negative patients (46 ± 13 years *vs* 54 ± 13 years, respectively; $P = 0.05$).

### *Helicobacter pylori* serology and urea breath test

Overall, anti *H pylori* IgG antibodies were detected in 20/76 (26.3%) cases. These positive cases were found in 12/41 (29.2%) patients of group 1, 3/11 (27.0%) patients of group 2 and 5/24 (20.8%) patients of group 3. As shown in Table 3, among the 16 patients positive for *Helicobacter* DNA in the liver, anti *H pylori* IgG antibodies were detected in 2/7 (28.5%) patients of group 1, 1/4 (25.0%) patients of group 2 and 1/4 (25.0%) patients of group 3. Serology was not performed in one positive patient. All the results obtained by ELISA were also confirmed using an immunoblot specific for *H pylori*. Gastric infection was confirmed by UBT in the 2 positive patients of group 1 but not in the positive patient of group 2. UBT was not performed in one positive control patient.

### Identification of Helicobacter species

Material from 16 *Helicobacter* genus positive patients was tested with species-specific primers (Table 3). Neither *H. bilis* nor *H. rappini* was found (data not shown). *H pylori*-like organisms were identified in 12 (75%) cases and H. spullorum-like organisms were identified in 2 cases (12.5%), 2 samples remained unidentified. *H pylori*-like organisms were present in 4/7 (57%) patients of group 1, 4/5 (80.0%) patients of group 2, and 4/4 (100%) patients of group 3. These results were confirmed by sequencing the 16S rRNA gene in the 7 cases where it was carried out. Among the positive samples by sequencing or by *H pylori* specific PCR, none of them reacted with the highly conserved primer designed to amplify the specific *vac*A gene of *H pylori* and variable results were obtained with other *H pylori* specific targets, such as *ure*A, *glm*M. Among the 7 samples for which the near complete 16S rRNA gene was sequenced, the 2 nucleotide polymorphisms in position 92 and 130 which were found to be associated with *Helicobacter* 16S rDNA sequences from the liver described by Verhoef *et al*[29] were detected in 5 instances: 3 sequences harboured the 2 polymorphisms simultaneously, 2 sequences

**Table 3 Results of *H pylori* serology, urea breath test (UBT) and species identification in the liver, for the 16 patients positive for *Helicobacter* genus-specific PCR in the liver**

| Groups | Patient No. | *H pylori* infection | | *H pylori* PCR | | | | *H. pullorum* PCR | Conclusion |
|---|---|---|---|---|---|---|---|---|---|
| | | Serology | UBT | 23S rDNA | *ure*A | 26 kDa | *glm*M | *cdt*B | |
| Group 1 | 1 | - | - | - | - | - | - | - | Not identified |
| (Hepatitis) | 2 | - | - | - | - | + | - | + | "*H. pullorum*"-like[1] |
| | 3 | - | - | + | + | + | - | - | "*H pylori*"-like[1] |
| | 4 | + | + | + | + | - | - | - | "*H pylori*"-like |
| | 5 | + | + | + | + | + | - | - | "*H pylori*"-like[1] |
| | 6 | - | - | - | - | - | - | - | Not identified |
| | 7 | - | - | + | - | - | - | - | "*H pylori*"-like[1] |
| Group 2 | 8 | - | - | + | + | + | - | - | "*H pylori*"-like[1] |
| (Cirrhosis) | 9 | - | - | + | + | + | + | - | "*H pylori*"-like[1] |
| | 10 | ND | - | + | + | + | + | - | "*H pylori*"-like |
| | 11 | - | - | - | - | + | - | - | "*H pylori*"-like |
| | 12 | + | - | - | - | + | - | + | "*H. pullorum*"-like |
| Group 3 | 13 | - | - | - | - | + | - | - | "*H pylori*"-like |
| (Controls) | 14 | - | - | - | - | + | - | - | "*H pylori*"-like[1] |
| | 15 | + | ND | - | + | + | - | - | "*H pylori*"-like |
| | 16 | - | - | + | + | + | - | - | "*H pylori*"-like |

[1]Also confirmed by 16S rDNA sequence; ND: Not determined. The 16S rRNA amplified primer-less sequences of approximate 1370 bp for biopsies 2, 3, 5, 7, 8, 9 and 14 were submitted to GenBank and assigned accession numbers DQ062210 to DQ062216, respectively.

harboured one of these 2 polymorphisms and 2 sequences did not present these polymorphisms.

## DISCUSSION

In a previous retrospective cross-sectional study[12], we showed that DNA from *H pylori*- and *H. spullorum*-like organisms was present in the liver of patients with hepatitis C cirrhosis with or without HCC, suggesting that *Helicobacter* species could be a co-risk factor for progression of HCV chronic liver diseases. However, a limitation of this preliminary study was its retrospective nature which did not allow us to determine whether *H pylori* was present in the stomach of these patients and to gather all of the clinical and biological information on HCV genotype and duration of infection, *etc*, which is needed for a more accurate analysis.

In this prospective study, we were able to better characterize the presence of *Helicobacter* species in the liver of a group of consecutive patients with HCV infection with or without cirrhosis, using both the tools which were developed in the retrospective study as well as new tools.

The results of the present study only partially confirm those obtained previously. Although the prevalence of *Helicobacter* DNA tended to be higher in the liver samples from patients with hepatitis C cirrhosis than in those from HCV-infected patients without cirrhosis or from controls, the difference did not reach statistical significance ($P = 0.09$ and $P = 0.08$, respectively). However, we cannot exclude the fact that given the limited number of patients with HCV- induced cirrhosis, this result may be due to a lack of statistical power. The negative results obtained with regard to the detection of *E. coli* DNA in liver material confirm that *H pylori* DNA detected is not the result of a non specific transport or failure in elimination by a non functional liver. Indeed, since *E. coli* is constantly present in the human intestine, if a non specific bacterial translocation occurred, we could find *E. coli* DNA in the liver.

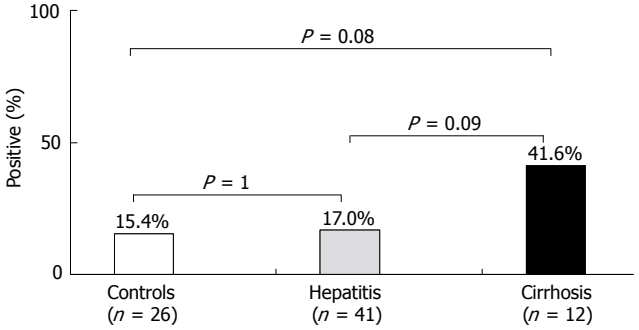


**Figure 1** Percentage of positive biopsies for *Helicobacter* genus by DNA detection. Group 1 corresponds to hepatitis ($n = 41$), group 2 corresponds to cirrhosis ($n = 12$), and group 3 corresponds to controls ($n = 26$). *Helicobacter* genus positivity and negativity was compared using Fisher's exact test.

This study did not enable us to solve the issue of identifying specific *Helicobacter* species involved since no positive culture occurred on the media usually used for *Helicobacter* culture. Indeed, in another study bacteria closely related to *H pylori* morphologically were visualized in the liver of 6 out of 20 patients with HCC[24]. Furthermore, Oliveira *et al* only found *H pylori* in two studies on ulcerative colitis[25] and Crohn's disease patients[26]. For the latter, *H pylori* was more frequent than in the intestinal mucosa of the control group. These two studies were however carried out in a country of high *H pylori* prevalence, Brazil.

In the current study, we also had the possibility to perform *H pylori* serology on most patients. Interestingly, among the 15 patients (out of 16) positive for *Helicobacter* DNA in the liver, only 4 (26.6%) had *H pylori* antibodies.

Given this surprising result, immunoblot was performed and the results were confirmed.

Unfortunately, the protocol did not include a UBT when *Helicobacter* DNA was present in the liver, but only when *H pylori* serology was positive. Indeed, 2 out of 3 positive serology cases were confirmed by UBT. As the sensitivity of our serological kit is 95%[15,27], it is surprising that such a high number of patients (11/15) did not produce *H pylori* antibodies, which casts a doubt on the identification of the species present. Two potential explanations can be given: (1) *H pylori* is truly present in the liver, but not in the stomach, and at an insufficient load to stimulate an immune response; and (2) another *Helicobacter* species which is closely related to *H pylori* but does not cross-react with *H pylori* antigens, is present in the liver. These patients did not receive *H pylori* eradication therapy, which could explain the eradication of the bacteria. The possibility of immunodeficiency in HCV positive patients, especially with cirrhosis can also be considered as an explanation of the lack of serological response but we have not found arguments for this hypothesis.

The 1370 bp sequence of the 16S rRNA gene of six patients with *H pylori* DNA shows 99% homology with *H pylori*, but it is known that 16S rDNA sequences do not have a good discriminatory power in the *Helicobacter* genus. A recent taxonomic analysis showed for example that *H. felis, H. salomonis* and *H. bizzozeronii* could not be differentiated on the basis of the 16S rRNA gene sequence[28].

There are a number of genes which are theoretically specific for *H pylori*, eg *ure*A, *glm*M, and *vac*A. Although they were looked for in this study, they were not uniformly present. The absence of the *H pylori* specific *vac*A detected by a PCR targeting a short sequence is particularly surprising. Furthermore, a striking feature in the 16S rDNA sequences obtained from liver material is the presence of 2 nucleotide polymorphisms at the positions 92 and 130 as described by Verhoef *et al*[29]. Indeed, this polymorphism was already present in the sequences first described by Avenaud *et al*[8] and Ponzetto *et al*[11], and was present in most of our cases (5/7). It is important to note that in the study of Verhoef *et al*[29], *H pylori* was grown from the stomach of 3 out of 9 (33%) patients with this *Helicobacter* 16S rDNA polymorphism in the liver, and the corresponding gastric strains also had this polymorphism and were confirmed to be *H pylori*. It is therefore most likely that this species corresponds to a variant of *H pylori* with different properties including bile resistance and the ability to colonize the liver. This polymorphism is also present in the only strain grown from liver material[10]. The study of strains obtained from the same patients several years apart has shown a greater diversity than in other bacterial species studied due to high rates of mutations and recombinations[30]. This observation has led to the concept of quasi species which could correspond to the situation found in the liver.

Despite the doubt concerning the reality of the presence of *H pylori* in the liver (absence of culture and negative-*H pylori* specific PCR), the presence of *H. spullorum*-like organisms in the liver is much more likely as has been confirmed by *H. spullorum* specific *cdt*B PCR and by sequencing of the 16S rRNA gene. The identification of *H. pullorum* is easy, given that this bacterium has been designated as a separate species on the basis of 16S rRNA gene sequencing[31]. *H. pullorum* isolates were initially recovered from the cecal content of broiler chickens and from the liver and intestinal content of laying hens with vibrionic hepatitis, suggesting that this bacterium can infect the liver and that poultry may serve as the source of human infection[31]. *H. pullorum* has also been cultured from immunocompetent patients with gastroenteritis and one HIV-infected patient[31]. One individual, in addition to having diarrhea, developed elevated liver enzyme levels and hepatomegaly which, although not proven, may have been induced by *H. pullorum* invasion of the liver in a manner similar to the organism's ability to cause hepatitis in chickens[32]. There is clearly a potential for zoonotic foodborne transmission of *H. pullorum* to humans, as is known to occur with *Campylobacter* species.

In conclusion, *Helicobacter* DNA can be present in the liver of patients with liver disease, with a tendency to have a higher prevalence in those with cirrhosis. Although *H. pullorum* appears to be regularly found at a low rate, the exact nature of the main *Helicobacter* species present is still uncertain. It is most likely a variant of *H pylori* which has acquired specific properties. Further studies exploring both the liver and stomach of patients with liver diseases need to be carried out to unravel the mystery of this intriguing association.

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*BASIC RESEARCH*

# Expression pattern and action analysis of genes associated with the responses to chemical stimuli during rat liver regeneration

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## Abstract

**AIM:** To study the genes associated with the responses to chemokines, nutrients, inorganic substances, organic substances and xenobiotics after rat partial hepatectomy (PH) at transcriptional level.

**METHODS:** The associated genes involved in the five kinds of responses were obtained from database and literature, and the gene expression changes during liver regeneration in rats were checked by the Rat Genome 230 2.0 array.

**RESULTS:** It was found that 60, 10, 9, 6, 26 genes respectively participating in the above five kinds of responses were associated with liver regeneration. The numbers of initially and totally expressed genes occurring in the initial phase of liver regeneration (0.5-4 h after PH), $G_0/G_1$ transition (4-6 h after PH), cell proliferation (6-66 h after PH), cell differentiation and structure-functional reconstruction (66-168 h after PH) were 51, 19, 52, 6 and 51, 43, 98, 68 respectively, illustrating that the associated genes were mainly triggered in the initiation and transition stages, and functioned at different phases. According to their expression similarity, these genes were classified into 5 groups: only up-regulated (47), predominantly up-regulated (18), only down-regulated (24), predominantly down-regulated (10), and up- and down-regulated (8). The total times of their up-regulated and down-regulated expression were 441 and 221, demonstrating that the number of up-regulated genes is more than that of the down-regulated genes. Their time relevance and gene expression patterns were classified into 14 and 26 groups, showing that the cell physiological and biochemical activities were staggered, diversified and complicated during liver regeneration in rats.

**CONCLUSION:** The chemotaxis was enhanced mainly in the forepart and metaphase of LR. The response of regenerating liver to nutrients and chemical substances was increased, whereas that to xenobiotics was not strong. One hundred and seven genes associated with LR play important roles in the responses to chemical substances.



**Key words**: Partial hepatectomy; Rat Genome 230 2.0 array; Responses to chemical substances; Genes associated with liver regeneration

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## INTRODUCTION

The liver has tremendous capacity to regenerate itself[1,2]. Liver cells proliferate rapidly to compensate the lost liver tissues after liver injury or chemical stimulus, which is called liver regeneration (LR)[1,3]. Generally, based on the cell physiological activities, the regeneration process is classified into 4 phases: the initiation (0.5-4 h after hepatectomy, PH), the transition from $G_0$ to $G_1$ (4-6 h after PH), the cell proliferation (6-66 h after PH), the cell differentiation and structure-functional reorganization (66-168 h after PH)[2]. According to the time course, it is divided into 4 phases: forepart (0.5-4 h after PH), prophase (6-12 h after PH), metaphase (16-66 h after PH), and anaphase (72-168 h after PH)[4], involving hepatic cell activity, dedifferentiation, proliferation and its regulation, redifferentiation, structure-functional reorganization[5], which are regulated by many factors including chemical substances[6].

Liver is a vital organ where chemical substances undergo transformation[7-9], it also can respond rapidly to chemical stimulus[10]. This process involves more than 400

genes, so high-throughput biological analysis is needed[11,12]. The pathogenesis of drug-induced liver disease, toxic liver injury and the characteristics of responses of regenerating liver to drug, toxin, oxidation and unfolded protein have been studied at transcriptional level[13]. In this paper, the expression changes of the genes in rat regenerating liver after PH were detected by Rat Genome 230 2.0 array[14]. Two hundred genes were related to the response to chemical substances, in which, 107 genes were observed to be associated with liver regeneration[11]. Expression changes, patterns and action of the genes were primarily analyzed.

## MATERIALS AND METHODS

### Regenerating liver preparation

Healthy SD rats weighing 200-250 g were obtained from the Animal Center of Henan Normal University. The rats were separated into groups randomly, each group consisting of 6 rats (male: female = 1:1). PH was performed according to Higgins and Anderson[15], the left and middle lobes of liver were removed. Rats were killed by cervical vertebra dislocation at 0.5, 1, 2, 4, 6, 8, 12, 16, 18, 24, 30, 36, 42, 48, 54, 60, 66, 72, 96, 120, 144 and 168 h after PH and the regenerating livers were observed at corresponding time point. The livers were rinsed three times in PBS at 4℃, then 100-200 mg livers from middle parts of right lobe (total 0.1-0.2 × 6 g liver tissues, six samples for each group) were collected and stored at -80℃. The sham-operation (SO) groups were the same with PH groups except that the liver lobes were not removed. The laws of animal protection of China were enforced strictly.

### RNA isolation and purification

Total RNA was isolated from frozen livers according to the manual of Trizol kit (Invittrogen)[16] and then purified based on the instructions of RNeasy mini-kit (Qiagen)[17]. Total RNA samples were found to exhibit a 2:1 ratio of 28S to 18S rRNA intensities by agarose electrophoresis (180 V, 0.5 h). Total RNA concentration and purity were estimated by optical density measurement at 260/280 nm[18].

### cDNA, cRNA synthesis and purification

As template, 1-8 μg total RNA was used for cDNA synthesis. cDNA and cRNA synthesis was carried out based on method of Affymetrix[19]. cRNA labeled with biotin was synthesized using 12 μL of the above synthesized cDNA as the template, and cDNA and cRNA were purified according to the sample purification procedure of GeneChip Analysis[19]. Measurement of the concentration, purity and quality of cDNA and cRNA were the same as above[18].

### cRNA fragmentation and microarray detection

Fifteen μL (1 μg/μL) cRNA incubated with 5 × fragmentation buffer at 94℃ for 35 min was digested into 35-200 bp fragments. The hybridization buffer was added to the prehybridized Rat Genome 230 2.0 microarray produced by Affymetrix, then hybridization was carried out for 16 h at 45℃ on a rotary mixer at 60 rpm. The microarray was washed and stained by GeneChip fluidics station 450 (Affymetrix Inc., USA). The chips were scanned by GeneChip Scan 3000 (Affymetrix Inc., USA), and the signal values of gene expression were observed[14].

### Microarray data analysis

The normalized signal values, signal detections (P, A, M) and experiment/control (Ri) were obtained by quantifying and normalizing the signal values using GCOS1.2[14].

### Normalization of microarray data

To minimize error in the microarray analysis, each analysis was performed three times by Rat Genome 230 2.0 microarray. The results in which the total ratio($R^m$) was maximal and the average of the three housekeeping genes β-actin, hexokinase and glyseraldehyde-3-phosphate dehydrogenase approached 1.0 ($R^h$), were taken as a reference. The modified data were generated by applying a correction factor ($R^m/R^h$) multiplying the ratio of every gene in $R^h$ at each time point. To remove spurious gene expression changes resulting from errors in the microarray analysis, the gene expression profiles at 0-4 h, 6-12 h and 12-24 h after PH were reorganized by NAP software (normalization analysis program) according to the cell cycle progression of the regenerating hepatocytes. Data statistics and cluster analysis were done using GeneMath, GeneSpring, Microsoft Excel software[14,20,21].

### Identification of genes associated with liver regeneration

The nomenclature of the responses to chemical substances was adopted from the GENEONTOLOGY database (www.geneontology.org), and input into the database at NCBI (www. ncbi.nlm.nih.gov) and RGD (rgd.mcw.edu) to identify the rat, mouse and human genes associated with the responses to chemical substances. According to KEGG (www.genome.jp/kegg/pathway.html#amino) and BIOCARTA (www.biocarta.com/genes/index.asp), the genes associated with the biological process were collated. The results of this analysis were codified, and compared with the results obtained for mouse and human searches in order to identify human and mouse genes which are different from those of rat. Comparing these genes with the analysis output of the Rat Genome 230 2.0 array, those genes which showed a greater than twofold changes in expression level, and observed as meaningful expression changes[22], were referred to as rat homologous or rat specific genes associated with the responses to chemical substances under evaluation. Genes, displaying reproducible results with three independent analyses with the chip and showing a more than twofold change in expression level in at least one time point during liver regeneration with significant difference ($0.01 \leqslant P < 0.05$) or extremely significant difference ($P \leqslant 0.01$) between PH and SO, were considered as associated with liver regeneration.

## RESULTS

### Expression changes of genes associated with responses to chemical substances during liver regeneration

According to the data from databases at NCBI, GENEMAP, KEGG, BIOCARTA and RGD, 403 genes

**Table 1 Expression abundance of 107 genes associated with responses to chemical substances during rat liver regeneration**

| Gene Abbr. | Associated to others | Fold difference | Gene Abbr. | Associated to others | Fold difference | Gene Abbr. | Associated to others | Fold difference | Gene Abbr. | Associated to others | Fold difference |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 Chemotaxis | | | Fosl1 | | 2.3 | Utrn | | 2.4 | Ppp2cb | | 4.5 |
| C3ar1 | | 0.3, 2.3 | G1p2 | | 22.6 | Vegfa | | 0.1, 4.5 | Rela | | 0.5 |
| C5r1 | | 0.4, 2.6 | Gpr2 | | 0.4, 4 | Xcl1 | | 0.1, 3.7 | 5 Response to xenobiotic | | |
| Ccl17 | | 0.1 | Htr6 | | 9.2 | 2 Response to nutrient | | | Abhd6 | | 0.1 |
| Ccl19 | | 3.9 | Ifng | | 6.5 | Cckar | | 0.3, 3.2 | Afp | | 0.1, 3.7 |
| Ccl2 | | 128 | Il16 | | 0.4, 3.1 | Enpp1 | | 2.2 | Ahr | | 2.2 |
| Ccl20 | | 8 | Il1b | | 0.4 | Gipr | | 0.3, 2.3 | Ahrr | | 0.1 |
| Ccl21B | | 0.1, 2 | Il4 | | 0.1, 2.6 | Gnai2 | | 2.3 | Akr1c12 | | 0.2, 2.2 |
| Ccl24 | | 4 | [1]Itga1 | | 2.9 | [1]Kpna2 | | 4.3 | Apoa2 | | 2.9 |
| Ccl27 | | 0.3 | Itgam | | 3.4 | Ogt | | 2.7 | Arnt2 | | 0.4, 6.8 |
| Ccl4 | | 0.2, 3 | Itgb2 | | 0.5 | Sst | | 0.2, 4.3 | Cda | | 0.1 |
| Ccl7 | | 22.6 | Kit | | 0.4 | Sstr2 | | 0.4, 4.9 | Chga | | 2.3 |
| Ccr1 | | 0.4, 27.9 | Lsp1 | | 0.3 | Stc1 | | 2.3 | Cyp1a1 | | 59.7 |
| Ccr6 | | 0.3, 4.3 | Map2k1 | | 9.1 | Stc2 | | 4 | Cyp2c | | 52.6 |
| Ccrl1 | | 3.7 | Mapk1 | | 2.7 | 3 Response to inorganic | | | Cyp3a13 | | 0.2 |
| Cklf1 | | 8.3 | Plau | | 0.4, 3 | substance | | | Defb3 | | 0 |
| Cklfsf1 | | 0.3, 2.1 | Plaur | | 13.9 | Als2 | | 0.5 | Ephx1 | | 0.4, 2.8 |
| Cklfsf3 | | 4.2 | Pld1 | | 2.3 | [1]Aqp9 | 4 | 0.4, 3.7 | Gstm2 | | 4.5 |
| Cmklr1 | | 2.4 | Plp2 | | 0.4, 4.3 | Arsb | | 2 | Gstm4 | | 7.5 |
| Cmkor1 | | 7.1 | Ppbp | | 0.1, 2.1 | Arse | | 0.2, 2.5 | Laptm4a | | 2.5 |
| Creb3 | | 8.4 | [1]Prkca | | 4.6 | As3mt | 5 | 0.5 | Lpo | | 3.4 |
| Cx3cr1 | | 0.4, 6.8 | Ptafr | | 7.1 | Asna1 | | 0.5 | Mbp | | 0.4 |
| Cxcl10 | | 0.3, 9.2 | Rac2 | | 0.5 | Atp7a | | 0.4 | Pap | | 68.6 |
| Cxcl12 | | 0.2 | Rala | | 2.3 | Drg1 | 5 | 0.4 | Prg2 | | 0.2 |
| Cxcl16 | | 0.3 | Scg3 | | 0.5, 2.2 | Zd10b | | 5.6 | Ugt1a5 | | 2.1 |
| Defb1 | 5 | 0.4, 2.5 | Serpind1 | | 0.1 | 4 Response to organic substance | | | Wbscr21 | | 0.5 |
| Ela2 | | 0.5, 52 | Spn | | 0.2, 4 | Accn1 | | 0.3, 10.1 | | | |
| Enpp2 | | 6.2 | Spp1 | | 0.5, 2.7 | Accn3 | | 0.5, 4 | | | |
| Fgf2 | | 0.5, 2.1 | Tgfb2 | | 0.5, 2.9 | Ppp2ca | | 3 | | | |

[1]Reported genes associated with liver regeneration; Associated to others: involved in the other kind of responses to chemical substances.

were involved in the responses to chemical substances, 200 of them were contained in the Rat Genome 230 2.0 array. Among the 200 genes, 107 displayed meaningful changes at least at one time point after PH, there was significant difference or extremely significant difference in expression between PH and SO groups, and results were reproducible from the three checks with Rat Genome 230 2.0 array, suggesting that the genes were associated with liver regeneration. The up-regulation was 2-128 folds, and the down-regulation was 2-10 folds that of controls (Table 1). The analysis indicated that 47 genes were up-regulated, 24 down-regulated, and 36 up/down-regulated during liver regeneration. The total up- and down-regulated times were 441 and 221, respectively (Figure 1A). At the initial phase, 34 genes were up-regulated and 16 down-regulated; at the transition phase from $G_0$ to $G_1$, 24 genes were up-regulated and 14 down-; at the cell proliferation phase, 47 genes were up-, 26 down- and 24 up/down-regulated; at cell differentiation and structure-function reorganization phase (66-168 h after PH), 44 genes were up-, 17 down- and 6 up/down-regulated (Figure 1B).

### *Initial expression time of genes associated with responses to chemical substances during liver regeneration*

At each time point of liver regeneration, the numbers of initially up-, down-regulated and totally up-, down-regulated genes were in the following sequence: both 10 and 3 at 0.5 h; 11, 6 and 21, 8 at 1 h; 6, 1 and 18, 4 at 2 h; 8, 6 and 25, 8 at 4 h; 3, 2 and 19, 11 at 6 h; 1, 1 and 14, 9 at 8 h; 2, 3 and 20, 11 at 12 h; 10, 4 and 28, 7 at 16 h; 2, 10 and 26, 22 at 18 h; 2, 0 and 24, 12 at 24 h; 5, 1 and 19, 7 at 30 h; 0, 0 and 20, 15 at 36 h; 1, 0 and 17, 4 at 42 h; 0, 0 and 26, 18 at 48 h; 1, 1 and 22, 18 at 54 h; 1, 0 and 24, 11 at 60 h; 2, 0 and 22, 9 at 66 h; 1, 0 and 19, 10 at 72 h; 2, 0 and 25, 10 at 96 h; 0, 1 and 14, 10 at 120 h; 0, 0 and 15, 7 at 144 h; 0, 0 and 13, 7 at 168 h (Figure 2). Generally, gene expression changes occurred during the whole liver regeneration, and the up- and down-regulated times were 441 and 221, respectively. The initially up-regulated genes were predominantly expressed in the forepart, prophase and pre-metaphase, and only few genes were initially expressed in the metaphase, pre-anaphase and mid-anaphase, whereas no initial expression in the late anaphase.

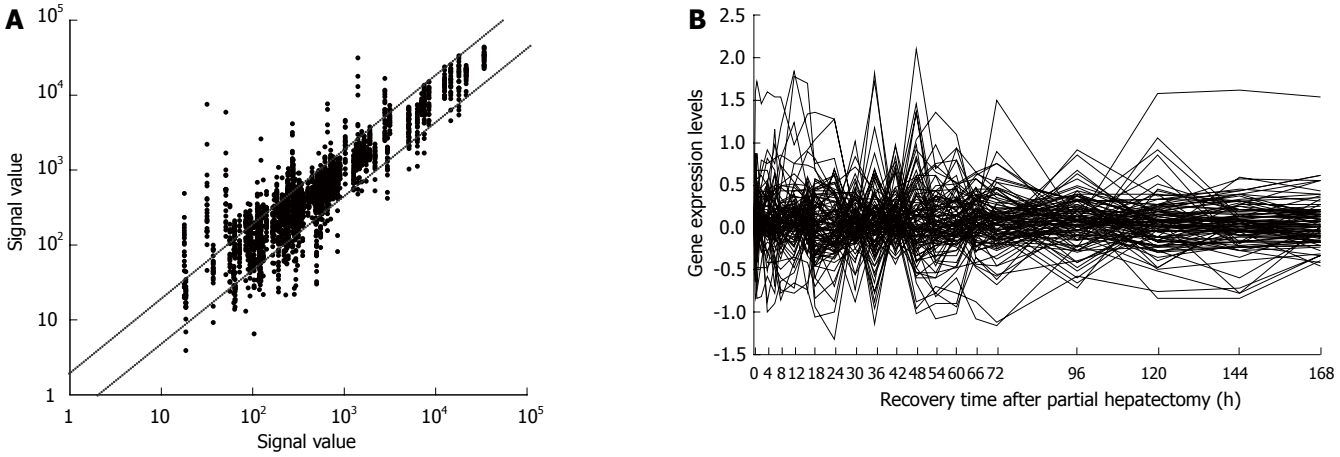


**Figure 1** Expression frequency, abundance and changes of 107 genes associated with responses to chemical substances during rat liver regeneration. Detection data of Rat Genome 230 2.0 array were analyzed and graphed by Microsoft Excel. **A**: Gene expression frequency. The dots above bias represent the genes up-regulated more than 2-fold, those under bias represent the ones down- more than 2-fold, and total times of up- and down-regulation were 441 and 221; the ones between two diagonal lines represent the genes without alteration in expression; **B**: Gene expression abundance and changes. Eighty-three genes were 2-128 folds up-regulated, and sixty genes 2-10 folds down-regulated.

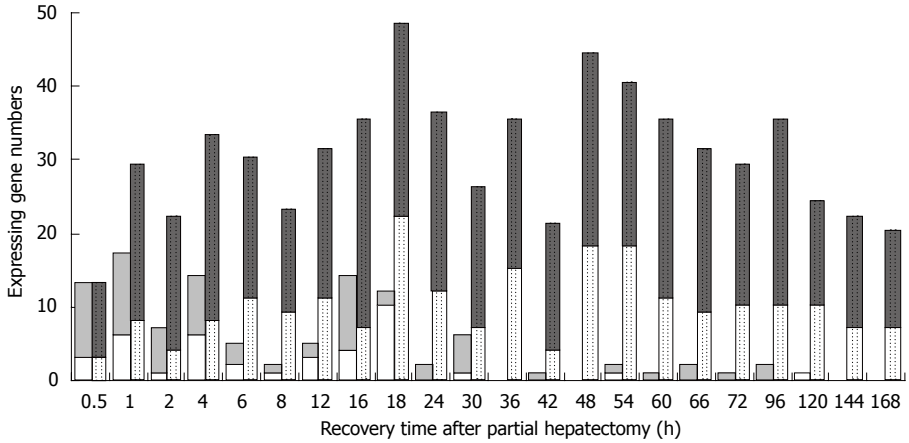


**Figure 2** The initial and total expression profiles of 107 genes associated with responses to chemical substances at each time point of liver regeneration. Grey bars: up-regulated genes; White bars: down-regulated genes. Blank bars represent initially expressing genes, in which up-regulation genes are predominantly in the forepart, prophase and early metaphase, and few genes initially expressed in the metaphase, early anaphase and middle anaphase, whereas no initial expression in the late anaphase. Dotted bars represent the total expressing genes, in which some genes are up-expressed, and the others down-expressed during LR.

### *Expression similarity and time relevance of the genes associated with the responses to chemical substances during liver regeneration*

Among the 107 genes characterized, 47 were up-regulated, 18 predominantly up-, 24 only down-, 10 predominantly down-, 8 up/down-, based on their similarity in expression (Figure 3). According to the time relevance, they were classified into 14 groups: 0.5 and 1 h, 2 and 4 h, 6 h, 8 and 12 h, 16 h, 18 and 24 h, 30 and 42 h, 36 h, 48 h, 54 and 60 h, 66 h, 72 and 120 h, 96 h, 144 and 168 h. The up- and down-regulated times were 31 and 11, 43 and 12, 19 and 11, 34 and 20, 28 and 7, 50 and 34, 36 and 11, 20 and 15, 26 and 18, 46 and 29, 22 and 9, 36 and 20, 25 and 10, 28 and 14 (Figure 3). The up-expressed genes were mainly associated with chemotaxis, xenobiotics metabolism, protein transport and apoptosis development, and the down-expressed genes were mostly the ones associated with transportation and mucosal defense.

### *Expression patterns of genes associated with responses to chemical substances during liver regeneration*

The expressions of 107 genes were categorized into 26 patterns according to the expression trends. Figure 4A-4G shows the up-regulated trends of gene expression profiles after PH, involving 14 patterns (47 genes); Figure 4H-4K shows the opposite expression trends, involving 9 patterns (24 genes); Figure 4L,4M,4N respectively displays that 18 genes were predominantly up-regulated, 10 genes predominantly down-regulated and 8 genes similarly up/down-regulated, involving 3 patterns.

## DISCUSSION

Liver is an important site of substance transformation, and it can also rapidly respond to chemical stimuli. During the process, the proteins associated with chemotaxis are as following: nine receptors bind with corresponding chemokine ligand to activate chemotaxis[23]; eighteen chemokine factors and chemokine-like factors, such as chemokine CC ligand 2 (CCL2), bind to corresponding chemokine receptors to activate chemotaxis[24]; eleven proteins including protein kinase C alpha (PRKCA) participate in chemotaxis[25,26]; five proteins such as plasminogen activator urokinase receptor (PLAUR), accelerate cell migration[27]; three proteins including integrin alpha 1 (ITGA1), contribute to cell adhesion[28].

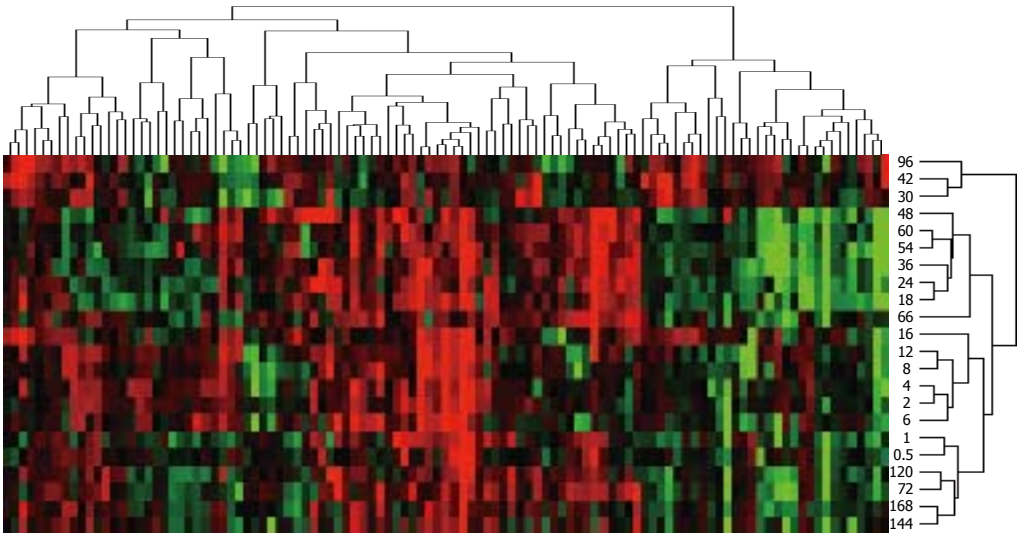


**Figure 3** Expression similarity and time relevance clusters of 107 genes associated with responses to chemical substances during LR. Detection data of Rat Genome 230 2.0 array were analyzed by H-clustering. Red represents up-regulated genes mainly associated with chemotaxis, xenobiotics metabolism, protein transport and apoptosis development; Green represents down- ones mainly associated with transportation and mucosal defense; Black: the genes having insignificant expression changes. The upper and right trees respectively show expression similarity and time series clusters, by which the above genes were classified into 5 and 14 groups.

The genes encoding the above proteins showed similar expression profiles at some points while different at other points, indicating that they may co-regulate chemotaxis. Among them, *prkca* was up-regulated at 16, 30, 42 and 96 h after PH, and reached a peak at 96 h that is 4.6 times higher than the control; *itga1* was up-regulated at 6-24 and 54 h, reaching a peak at 8 h, 2.9 times higher than the control. The two genes are generally consistent with the result reported by Dransfeld *et al*[11]. *ccr1* was up-regulated at 8-36, 48 and 120 h, and peaked at 48 h, 27.9 times that of control. *ccl2* was up-regulated at 0.5-1, 12-24, 36, 48-72 and 96 h, and reached a peak at 48 h, 128 times that control. *plaur* was up-regulated at 1, 6, 18-24, 48, 72 and 120 h, and peaked at 6 h, 13.9 times higher than the control, presuming that they play a key role in inflammation during liver regeneration.

Karyopherin alpha 2 (KPNA2) involved in glucose response, transports karyophilic proteins into the nucleus[29,30]. Cholecystokinin A receptor (CCKAR) promotes pancreatic enzyme secretion and smooth muscle contraction of stomach and gallbladder[31]. Three proteins including somatostatin (SST) inhibit gastrointestinal hormone release[32]. The meaningful expression profiles of the genes encoding the above-mentioned proteins show the identity or similarity at some points, whereas difference at others, presuming that they may co-regulate absorption and consumption of nutrients. Especially, *kpna2* displayed up-regulation at 16-24, 36 and 48-96 h, and had the highest abundance at 60 h, 4.3 times that of control. This result was basically in line with Dransfelds' result[11]. *cckar* appeared at 0.5-24, 36 and 48-54 h, and had peak expression at 8 h, 46.2 times that of control. *sst* was up-regulated at 4-24, 36, 48-60 and 120 h, and peaked at 12 h, 4.3 times that of control, presuming that they play a crucial role in nutrients assimilation and utilization in regenerating liver.

Aquaporin 9 (AQP9) influenced by inorganic substances, which could be inactivated by $Hg^{2+}$, stimulates transmembrane transportation of water and a variety of noncharged solutes, including urea[33]. It was up-regulated at 0.5-1, 24 and 54-72 h, and reached a peak at 66 h, being 3.7 times higher than the control, but the result was not consistent with Dransfelds' at 6 h[11], and need to be further analyzed with Northern blotting. Zinc responsive protein beta (ZD10B) stimulated by $Zn^{+}$ plays a role in apoptosis[34], and was up-regulated at 16-24, 36, 48-72 and 144-168 h, and reached a peak at 72 h, 5.6 times higher than control, indicating that the two play crucial roles in inorganic substances transport and removal in regenerating liver.

Amiloride-sensitive cation channel 1 (ACCN1) affected by organic substances, plays a part in neurotransmission and $Na^{+}$ transport[35], and is up-regulated at 30 and 42 h with the highest abundance of 10 folds at 30 h. Protein phosphatase 2a catalytic subunit beta (PPP2CB) stimulated by ceramide is implicated in the negative control of cell growth and division by dephosphorylation[36,37]. It was up-regulated at 16, 30, 42 and 120 h, and had abundance peak at 42 h, 4.5 folds of control, presuming that the two genes play a crucial part in response to organic substances in regenerating liver.

In response to xenobiotics, fourteen enzymes including cytochrome P450 family 1 subfamily a 1 (CYP1A1), metabolize xenobiotics[38]. Aryl hydrocarbon receptor nuclear translocator 2 (ARNT2) enhances expression of oxygen-responsive genes[39]. Alpha-fetoprotein (AFP) is involved in transportation of copper and nickel[40]. The meaningful expression profiles of the genes encoding the above proteins are the same or similar at some points while different at others, indicating that they may co-regulate the response to xenobiotics. Notably, *cyp1a1* was up-regulated at 0.5-24, 36, 48-72 and 120 h, and reached a peak at 12 h, 59.7 times higher than the control. *arnt2* was up-regulated at 30-42, 60, 72 and 120 h, and reached a peak at 30 h, 6.8 times higher than the control. *afp* was up-regulated at 1 and 54-72 h, and reached a peak at 66 h, 3.7 times higher than the control, showing that they play a critical role in xenobiotics biotransformation in regenerating liver.

In conclusion, the expression changes of the genes associated with chemotaxis, the responses to nutrients, inorganic substances, organic substances and xenobiotics during liver regeneration in rats were investigated by high-throughput gene expression analysis. It was primarily proved that chemotaxis and the responses to the chemical substances are enhanced during LR, that Rat Genome 230

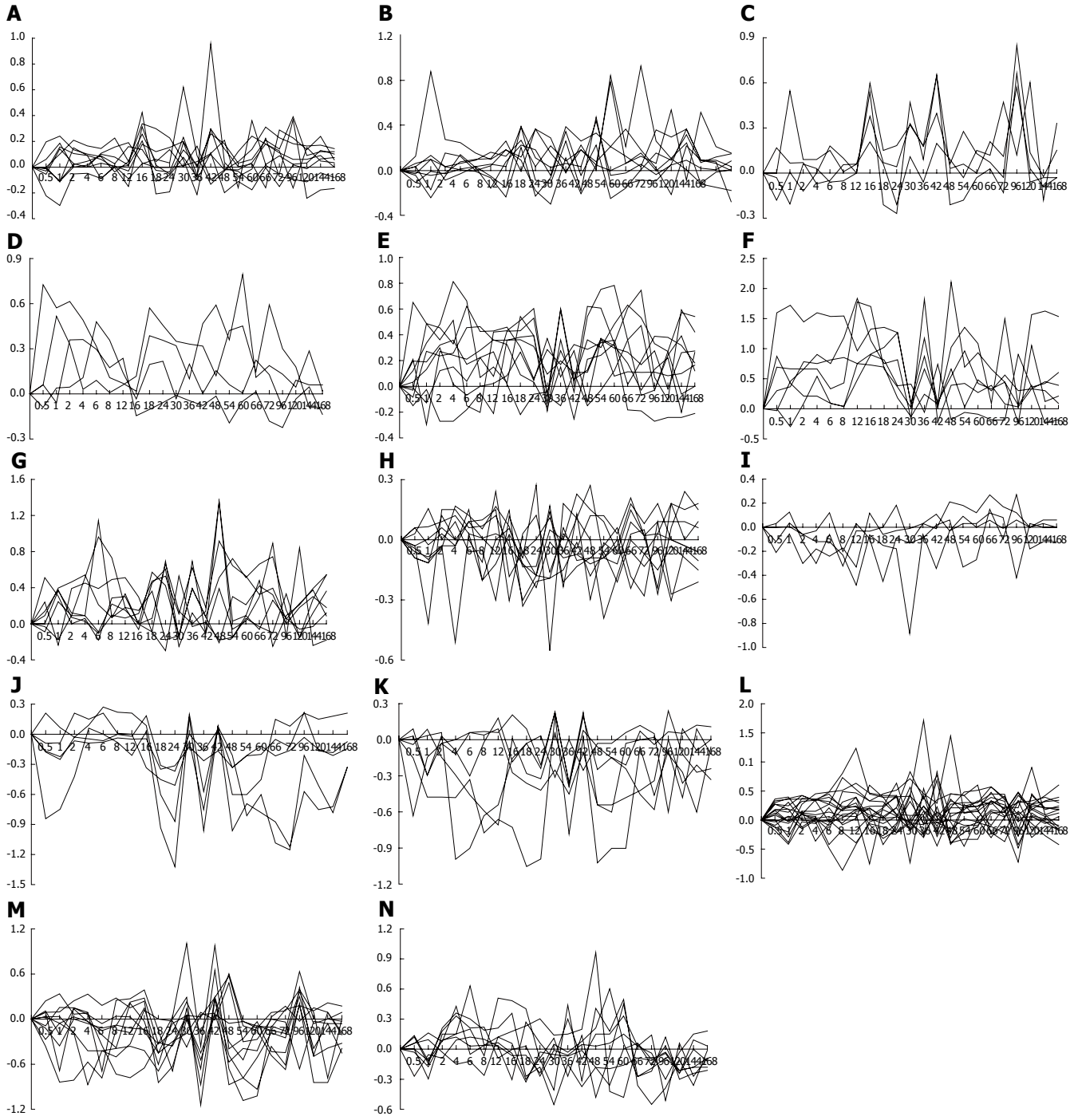


**Figure 4** Gene expression patterns of 107 genes associated with responses to chemical substances during LR. Twenty-six expression patterns were obtained by analysis of data detection of Rat Genome 230 2.0 array with Microsoft Excel. **A**-**G**: 47 up-regulated genes; **H**-**K**: 24 down-regulated genes; **L**-**N**: 36 up/down-regulated genes. X-axis represents recovery time after PH (h); Y-axis shows logarithm ratio of signal values of genes at each time point *vs* control.

2.0 array is a useful tool analyzing the above responses at the transcriptional level. However, the process DNA → mRNA→ protein was influenced by many factors including protein interaction. Therefore, the above results need to be further analyzed by the techniques, such as Northern blotting, protein chip, RNA interference, protein-interaction, *etc*.

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*BASIC RESEARCH*

# Transplantation of fetal liver epithelial progenitor cells ameliorates experimental liver fibrosis in mice

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## Abstract

**AIM:** To investigate the effect of transplanted fetal liver epithelial progenitor (FLEP) cells on liver fibrosis in mice.

**METHODS:** FLEP cells were isolated from embryonal day (ED) 14 BALB/c mice and transplanted into female syngenic BALB/c mice ($n$ = 60). After partial hepatectomy (PH), diethylnitrosamine (DEN) was administered to induce liver fibrosis. Controls received FLEP cells and non-supplemented drinking water, the model group received DEN-spiked water, and the experimental group received FLEP cells and DEN. Mice were killed after 1, 2, and 3 mo, and alanine aminotransferase (ALT), aspartate aminotransferase (AST), hyaluronic acid (HA), and laminin (LN) in serum, and hydroxyproline (Hyp) content in liver were assessed. Alpha-smooth muscle actin (α-SMA) of liver was tested by immunohistochemistry. Transplanted male mice FLEP cells were identified by immunocytochemistry for sry (sex determination region for Y chromosome) protein.

**RESULTS:** Serum ALT, AST, HA, and LN were markedly reduced by transplanted FLEP cells. Liver Hyp content and α-SMA staining in mice receiving FLEP cells were lower than that of the model group, which was consistent with altered liver pathology. Transplanted cells proliferated and differentiated into hepatocytes and bile duct epithelial cells with 30%-50% repopulation in the liver fibrosis induced by DEN after 3 mo.

**CONCLUSION:** Transplanted FLEP cells proliferate and differentiate into hepatocytes and bile duct epithelial cells with high repopulation capacity in the fiberized liver induced by DEN, which restores liver function and reduces liver fibrosis.



**Key words:** Fetal liver; Epithelial progenitor cells; Cell therapy; Proliferation; Liver fibrosis

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## INTRODUCTION

The incidence of hepatic injury and end-stage liver fibrosis is high in China. Cirrhosis represents a serious health care problem worldwide. The prognosis of patients with the disease is poor, although liver transplantation is a successful treatment for end-stage cirrhosis[1]. Counteracting the success of liver transplantation are problems such as lack of donors, operative damage, rejection, and high costs[2]. As a result, novel effective therapies are needed urgently.

Hepatocyte transplantation has been proposed as an alternative to whole organ transplantation[3]. It not only provides temporary liver function in patients waiting for liver transplantation but has also shown to be curative in certain metabolic conditions[4,5]. However, hepatocyte transplantation has very rarely produced therapeutic effects in human clinical trials, mainly because the number of transplanted hepatocytes is too small to achieve a biological effect[6]. Moreover, hepatocytes are quiescent cells that are difficult to maintain in culture and to cryopreserve.

Progenitor or facultative stem cells reside within or adjacent to the canals of Hering and comprise a quiescent compartment of dormant cells in adult livers. They can be activated to proliferate and differentiate into hepatocytes or bile duct epithelial cells when hepatocytes are impaired persistently[7]. Attempts have been made to identify their counterpart in fetal liver, and it has been suggested that the dormant stem-like cells originate most probably from bipotential fetal liver epithelial progenitor (FLEP) cells[8,9]. It was reported that embryonal day (ED) 14 rat liver

contains a subpopulation of bipotent epithelial cells that can differentiate into hepatocytes or bile duct epithelial cells[10-12]. These cells have characteristics of hepatic stem cell, self-renewal potential, and multiple differentiation capability to selectively repopulate the liver. FLEP cell transplantation could offer prospects of novel therapeutic strategies for patients with chronic liver disease or cirrhosis. However, there have been very few reports on the ability of these cells to reduce liver fibrosis after their transplantation.

This study aims to isolate and transplant FLEP cells into the liver of syngenic mice to explore the ability of FLEP cells to proliferate and differentiate into hepatocytes and bile duct epithelial cells and restore impaired liver function and liver fibrosis.

## MATERIALS AND METHODS

### Animals

Six to eight week old BALB/c mice were purchased from the Animal Breeding Center of Sun Yat-Sen University (Guangzhou, China). Mice were bred and maintained in an air-conditioned animal house with specific pathogen-free conditions, using an alternate 12 h cycle of daylight and darkness, and unlimited access to chow and water. All animal handling and experimental procedures were all in accordance with the Guidelines of the Animal Care and Use Committee at Sun Yat-Sen University.

### Isolation and transplantation of FLEP cells

FLEP cells were isolated on ED 14 from normal pregnant mice as described previously[8]. The cells were suspended at a concentration of $10^7$ cells/ml and cell viability (> 95%) was measured by trypan blue dye exclusion. The isolated FLEP cells were considered to be composed of 50% male cells and 50% female cells.

Anesthesia was performed with ether and partial hepatectomy (PH) was undertaken using the standard method for two-third resection[11]. Briefly, after ligation of the pedicule and resection of the two largest lobes (median and left), the remaining liver was composed of the caudate and epiploic lobes. Freshly isolated ED 14 FLEP cells were injected into the female liver via the superior mesenteric vein using insulin syringes after hepatectomy[13]. A total of $1 \times 10^6$ cells were injected per mouse.

### Flow cytometry

Approximately $2 \times 10^5$ FLEP cells were harvested and resuspended in 1 ml phosphate buffered saline (PBS) containing 1% bovine serum albumin (BSA). The cell suspension was incubated with goat anti-CK-19 monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit anti-AFP polyclonal antibody (Santa Cruz Biotechnology), or rabbit anti-albumin polyclonal antibody (Dako; Glostrup, Denmark), for 40 min at 4℃, then washed three times, and fixed in 2% paraformaldehyde. FITC-conjugated secondary antibody or PE-conjugated secondary antibody was used. FACScan was used for flow cytometry analysis (Becton-Dickinson, San Jose, CA) as previously reported[14].

### Diethylnitrosamine-induced liver injury

After partial hepatectomy and FLEP cells injection, mice were allowed to recover for one week. Thereafter, DEN (Sigma-Aldrich, St. Louis, MO) was continuously administered in drinking water at a final concentration of 100 μg/L for 12 wk[15]. The weight of the animals was carefully monitored during DEN administration.

Sixty female BALB/c mice were randomly divided into three groups (20 in each group). Mice in the normal control group were given FLEP cells and non-supplemented drinking water. The mice in the model group were continuously administered DEN in drinking water for liver injury induction. The experimental group received FLEP cells and DEN. Mice were killed under anesthesia and the livers were removed 1, 2, and 3 mo after cell transplantation. In most experiments, five animals were used at each time point. Blood was collected for evaluation of liver function and hepatic fibrosis at mo 3. Liver tissue was harvested for examination of hydroxyproline (Hyp) content and for histological evaluation.

### Serum parameters for liver function and fibrosis and hepatic Hyp content

Alanine aminotransferase (ALT), aspartate aminotransferase (AST), and total bilirubin in serum were assessed using routine laboratory methods. Hyaluronic acid (HA) and laminin (LN) in serum were also measured with a radioimmunoassay kit (Navy Medical Institute, Shanghai, China).

Hyp determination followed a method designed by Thirunavukkarasu *et al*[16]. Dried liver tissue after hydrolysis was oxidized by $H_2O_2$ and colored by p-dimethylaminobenzaldehyde and absorbance was determined at 540 nm. The amount of Hyp was expressed in mg/g liver.

### Liver histopathology and immunohistochemistry

To identify hepatocytes in fetal mouse liver sections, we immunostained AFP, albumin, and CK-19 with diaminobenzidine using standard techniques[17]. Liver sections were blocked with 1% BSA and 0.1% normal goat or rabbit sera at room temperature for 1 h, followed by incubation with rabbit anti-AFP polyclonal antibody (dilution 1:100; Santa Cruz Biotechnology), rabbit anti-albumin polyclonal antibody (1:200 dilution, Dako), or goat anti-CK-19 monoclonal antibody (dilution 1:100; Santa Cruz Biotechnology) at 4℃ overnight.

HE staining and immunohistochemistry for alpha-smooth muscle actin (α-SMA) were performed to determine the extent of liver inflammation and liver fibrosis development. Liver sections were tested with the antibody against α-SMA (dilution 1:200; NeoMarkers, Fremont, CA, USA) and immunoreactive materials were visualized with diaminobenzidine using a streptavidin-biotin staining kit (UltraSensitive SP kit; Maixin-Bio, Fujian, China).

To identify the origin of cells in the liver, immunohistochemistry was performed for sry (sex-determining region for Y chromosome) protein as described previously[18]. It was expected that transplanted cells (originating from fetal liver) would be approximately 50% male and 50% female.

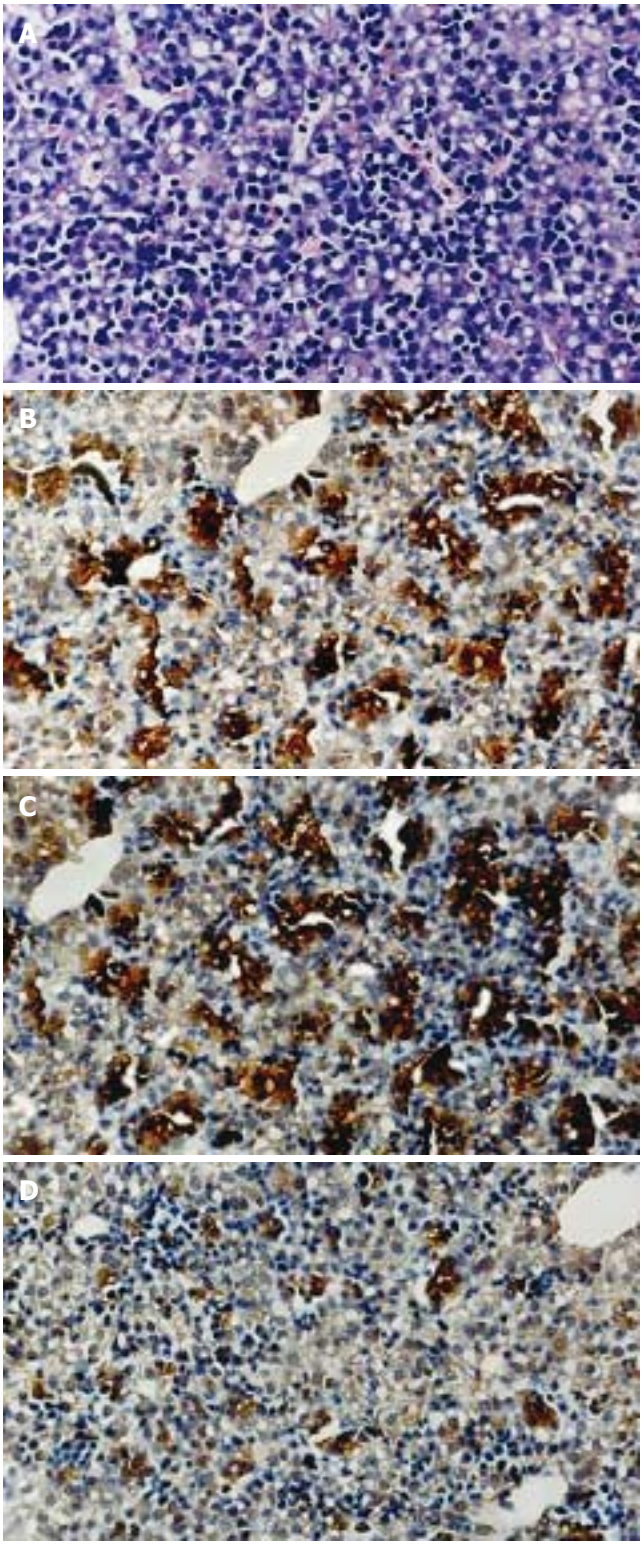


**Figure 1** Characteristics of ED 14 mouse FLEP cells. **A**: Fetal liver tissue, HE staining; **B**: AFP, immunohistochemical staining for FLEP cells; **C**: Albumin, immunohistochemical staining for FLEP cells; **D**: CK-19, immunohistochemical staining for FLEP cells. (Original magnification, x 200).

For histologic examination, several fields in each slide were randomly selected.

### *Statistical analysis*

Data were expressed as mean ± SD. Significant differences were determined using ANOVA in SPSS10.0. Results were considered significant when $P < 0.05$.

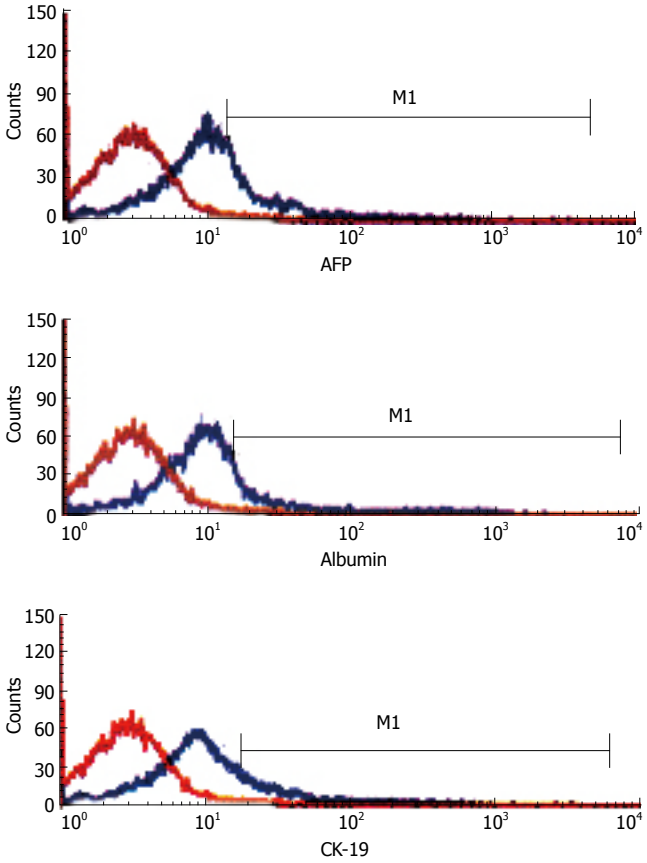


**Figure 2** Flow cytometric analysis performed on freshly isolated fetal mouse hepatocytes. Analysis of total cells after additional labeling for AFP, albumin, and CK-19. Fetal liver contained cell populations staining positive for AFP (15.5%), Albumin (13.9%), or CK-19 (6.4%).

## RESULTS

### *Characteristics of isolated FLEP cells*

Mouse liver at ED 14 contained immature epithelial cells and hematopoietic cells at different stages of differentiation (Figure 1A). Cells from ED 14 fetal liver were analyzed for expression of AFP, albumin, and CK-19. The percentage of FLEP cells was approximately 20% in the ED 14 fetal liver, determined as the number of cells expressing AFP (Figure 1B), albumin (Figure 1C), or CK19 (Figure 1D).

### *Flow cytometric analysis*

To further characterize the phenotype of immature liver epithelial cells, we tested for expression of AFP, albumin, and CK-19 in isolated ED 14 FLEP cells by flow cytometry. Results demonstrated the existence of at least three distinct subpopulations of epithelial cells in 14-d fetal mouse liver. The percentage of FLEP cells expressing AFP, albumin, and CK-19 was 15.5%, 13.9% and 6.4%, respectively (Figure 2).

### *Effects of FLEP cell transplantation on liver function and biochemical markers of liver fibrosis*

The survival rates were not different at the end of the 3-mo experiment in the two groups of DEN-treated mice ($P > 0.05$), with 90% of DEN-treated mice in the cell transplantation group and 80% of DEN-treated mice in

**Table 1 Liver function and biochemical markers of liver fibrosis (mean ± SD)**

| Groups | *n* | ALT (U/L) | AST (U/L) | TBIL (mg/L) | HA (μg/L) | LN (μg/L) | Hyp (mg/g) |
|---|---|---|---|---|---|---|---|
| Control | 8 | 42 ± 12 | 105 ± 35 | 8.8 ± 4.2 | 54 ± 19 | 58 ± 16 | 313 ± 118 |
| DEN | 8 | 341 ± 66[b] | 506 ± 81[b] | 12.9 ± 6.1 | 362 ± 83[b] | 232 ± 63[b] | 1255 ± 205[b] |
| DEN/FLEP cell | 8 | 176 ± 21[b,d] | 332 ± 83[b,d] | 14.0 ± 5.3 | 276 ± 65[b,c] | 176 ± 31[b,c] | 650 ± 173[b,d] |

[a]$P < 0.05$, [b]$P < 0.01$ *vs* control group; [c]$P < 0.05$, [d]$P < 0.01$ *vs* model group.

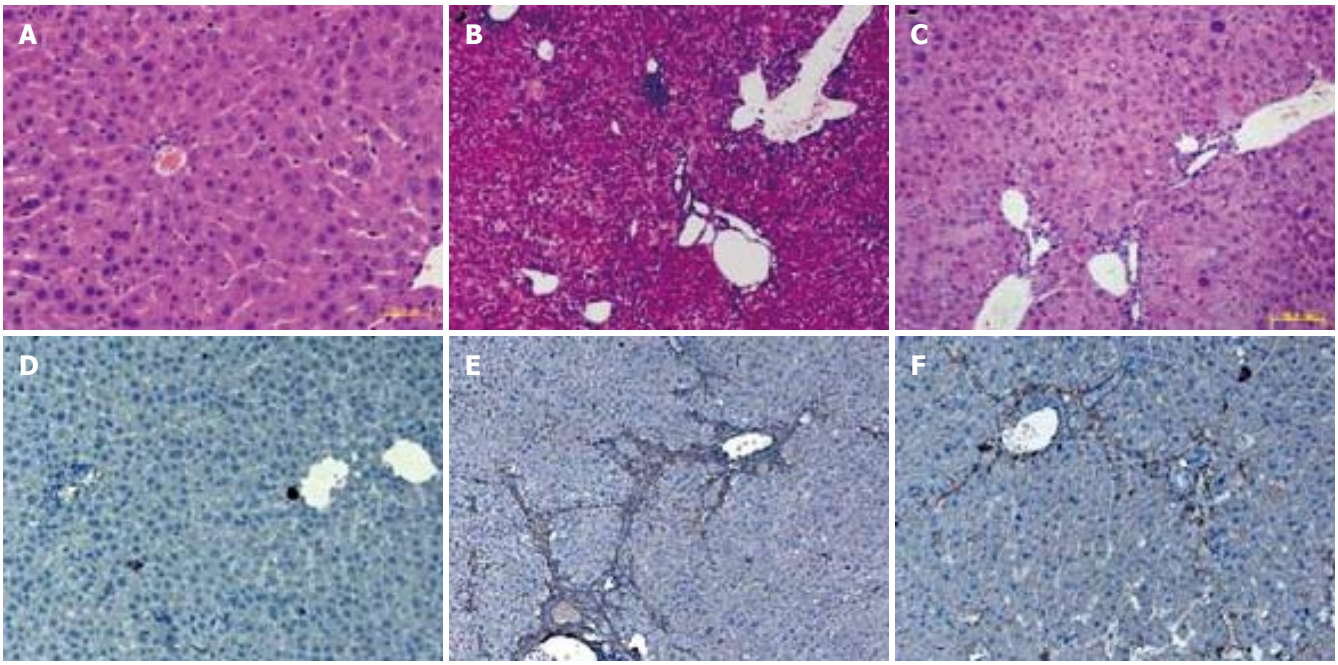


**Figure 3** Effect of FLEP cell transplantation on liver fibrosis by HE staining and immunohistochemistry of α-SMA. **A**: Normal liver tissue in control group (HE staining); **B**: Liver fibrosis in model group, fibrous tissue was formed with inflammatory cell infiltration in liver (HE staining); **C**: Liver fibrosis in FLEP cell transplantation group. The pathological change of liver was rather lighter compared with the model group (HE staining). **D**: Normal liver tissue, only α-SMA expression of vascular smooth muscle cell (immunohistochemical staining); **E**: Liver tissue in model group, displaying an increased amount of α-SMA expression with a dense network (immunohistochemical staining); **F**: Liver tissue in FLEP cell transplantation group, α-SMA expression was reduced compared with the model group (immunohistochemical staining). (original magnification, × 200).

the group without cell transplantation. Table 1 shows the values for the activities of the serum indicator enzymes and the markers of hepatic fibrosis.

DEN administration produced a marked increase in the activities of serum ALT and AST in mice ($P < 0.01$), compared with those of control group. The DEN plus FLEP cell transplantation group showed a significant decrease in the enzyme levels ($P < 0.01$), but the levels were still higher than those of control group ($P < 0.01$). Serum total bilirubin levels were similar among mice in all groups ($P > 0.05$) (Table 1).

We also examined levels of HA and LN in serum and Hyp contents in liver, which are biochemical markers of liver fibrosis. The serum levels of HA and LN in FLEP cell transplantation group was significantly decreased compared with the model group ($P < 0.05$), but the levels were still higher than those of control group ($P < 0.01$). Hyp contents of liver were significantly lower in FLEP cell transplantation group than in the model group ($P < 0.01$). However, the contents of liver in FLEP cell transplantation group were still higher than those of control group ($P < 0.01$) (Table 1).

### *Effects of FLEP cell transplantation on liver pathology*

We analyzed liver histology in mice with FLEP cell treatment using HE staining and immunohistochemistry. HE staining for sections of normal control group showed structural integrity without inflammation or fibrosis development (Figure 3A). However, DEN-induced liver injury in the model group showed fibrosis and inflammatory cell infiltration with the loss of structural integrity (Figure 3B). Nevertheless, results from DEN plus FLEP cell transplantation in the experimental group showed that FLEP cells could significantly alleviate DEN-induced fibrosis and inflammation compared with the model group (Figure 3C).

To further verify fibrosis, we analyzed α-SMA, an activating marker of mouse hepatic stellate cells by immunohistochemical staining of liver sections. Only α-SMA expression of vascular smooth muscle cell was observed in normal control group (Figure 3D). An increased amount of α-SMA expression with a dense network by activated HSC was observed in the model group (Figure 3E). However, the expression was reduced significantly in the FLEP cell transplantation group (Figure 3F).

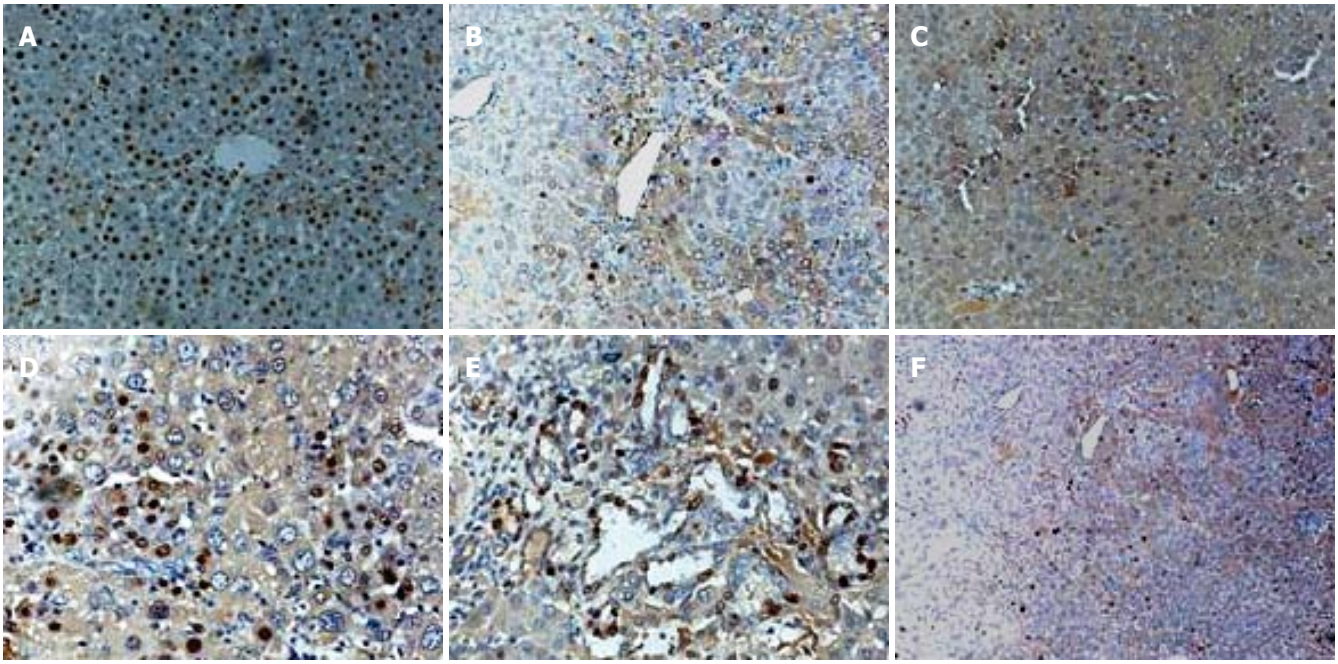


**Figure 4** Proliferation and differentiation of FLEP cells in DEN-treated mice and normal mice. **A**: Immunohistochemical positive control staining for sry protein; normal male mouse liver with nuclear positivity in the majority of hepatocytes (brown nuclei); **B**: One month after FLEP cell transplantation and DEN treatment, some cells stained for sry. **C**: Two months after FLEP cell transplantation and DEN treatment, sry$^+$ cells were present in liver parenchyma. **D** and **E**: Three months after FLEP cell transplantation and DEN treatment, sry$^+$ cells formed numerous mature hepatocytes showed canalicular structures (**D**), or bile duct epithelial cells in bile duct region, as evidenced by diffusely stained sry+ small epithelial cells in bile duct-like structures (**E**); **F**: Three months after FLEP cell transplantation without DEN treatment, scattered sry$^+$ cells were observed. Original magnifications, x 200 (**A-E**) and x100 (**D**).

### *Repopulation of liver by FLEP cells in DEN-treated mice and normal mice*

To follow the proliferation and differentiation of FLEP cells, we transplanted FLEP cells into the liver of normal and DEN-treated female mice subject to two-thirds PH. Detection of sry protein served as a marker for proliferation of transplanted FLEP cells. The sry$^+$ cells represented 50% of transplanted cells (originating from fetal liver).

In DEN-treated mice, sry$^+$ cells were distributed in the periportal regions of the liver after cell transplantation. One mo after FLEP cell transplantation into DEN-treated mice, some sry$^+$ cells were observed (Figure 4B). Two months after transplantation, sry$^+$ hepatocytes increased and were already present in the liver parenchyma (Figure 4C). Three months after transplantation, numerous sry$^+$ mature hepatocytes were observed, representing a substantial portion of the liver parenchyma (Figure 4D). We also found sry$^+$ cholangiocytes that formed mature sry positive bile ducts; sry$^+$ bile duct structures became evident as early as 1 mo after ED 14 FLEP cell transplantation, but became more numerous and fully developed in the portal regions at 3 mo (Figure 4E). The number of transplanted cells increased over time and repopulation ranged from 5%-10% at 1 mo, 15%-20% at 2 mo, and 30%-50% at 3 mo.

In the absence of DEN, sry$^+$ cells were still observed 3 mo after FLEP cell transplantation, but were less numerous and contained fewer cells (5%-10% of the total number of cells) than in DEN-treated mice (Figure 4F). In normal mice, PH was required for proliferation of transplanted FLEP cells.

## DISCUSSION

To determine whether ED 14 FLEP cells represent hepatic precursor cells, the characteristics of markers for various liver cell types have been explored, including AFP and albumin for fetal hepatocytes and CK19 for cholangiocytes. Three subpopulations of ED 14 FLEP epithelial progenitor cells have been detected. The first group of cells expressed AFP or albumin but not CK-19, comprising roughly 88% of FLEP cells. The second group (9% of total) expressed CK-19, but not AFP and albumin. The third and smallest group of cells (3% of total) expressed both AFP and albumin and CK-19[19]. Our immunohistochemical and flow cytometric analyses confirmed the existence of three distinct subpopulations of epithelial cells in 14-d fetal mouse liver. Furthermore, the present results demonstrate that immunostaining and flow cytometric analyses for the hepatic epithelial-specific markers AFP, ALB and CK-19 provided a definitive approach to characterize epithelial cell populations in the fetal liver.

We evaluated the anti-fibrosis effects of FLEP cells using a DEN-induced liver fibrosis model. DEN is a hepatotoxin, carcinogen, and mutagen, which can cause liver fibrosis. It provides a suitable experimental fibrosis model to examine human liver fibrosis[20,21]. Our results show that the transplantation of FLEP cells significantly restores the liver function and improves liver fibrosis index in DEN-injured mice. Transplanted FLEP cells can reduce necrosis collagen accumulation of liver and clearly inhibit the fibrosis formation. Moreover, levels of α-SMA positive staining of liver sections decrease significantly after FLEP cells administration in this model. This is exciting because

the expression of α-SMA in liver is an indicator of hepatic stellate cell activation, which is recognized as being critical in liver fibrogenesis[22,23]. FLEP cell transplantation thus appears to be effective for the suppression of liver inflammation and fibrosis formation.

We also investigated the underlying mechanisms regarding the repair mechanisms of transplanted FLEP cells to liver recovery after liver injury. We evaluated the stem cell properties and repopulation capacity of ED 14 FLEP cells after their transplantation into normal and chronically damaged livers. The effect of FLEP cell transplantation on reduction of liver fibrosis was studied using immunohistochemistry for sry (sex-determining region for Y chromosome) protein after sex-mismatched transplantation. Immunohistochemical staining for the sry protein offers the advantages of convenience and rapidity to enable the reliable detection and quantification of mixed chimerism in paraffin tissues[18]. We also provided evidence of sry detection in male cells, because the vast majority of cells in all male untreated control livers were positive for sry.

Stem cells are generally considered to exhibit self replication or renewal, differentiation into two or more specific cell phenotypes, and long-term repopulation of the host under appropriate circumstances. Hepatic stem cells include oval cells in canals of Hering of the adult liver and fetal liver hepatoblasts[24,25].

Our results show that FLEP cells selectively proliferate in the normal liver in response to a regenerative stimulus, such as partial hepatectomy. They differentiate into mature hepatocytes and incorporate into the host liver lobule as part of normal hepatocytic cords. Transplanted FLEP cells repopulate to comprise 5%-10% of the liver cells after 3 mo.

In this model, stimulation of the regeneration is amplified by two-thirds hepatectomy in addition to DEN administration. One month after administration of DEN, sry-positive cells were observed around the portal areas. At 2 mo, positive cells were detected in the liver lobes, indicative of FLEP cell migration into the hepatic parenchyma. At 3 mo, there was extensive proliferation and liver repopulation with FLEP cells in DEN-treated mice. The persistent liver damage made by DEN administration is important for the proliferation and differentiation of FLEP cells, and the number of cells migrating into the damaged liver is related to improvement of the liver function and suppression of liver fibrosis. These results suggest that FLEP cells themselves can functionally rescue the recipients by directly substituting the damaged cells.

More interestingly, we demonstrate that transplanted FLEP cells also can differentiate into mature bile duct epithelial cells. Our studies provide direct evidence for differentiation of FLEP cells with dual markers into hepatocytes and bile duct epithelial cells after transplantation into the regenerating liver of DEN-treated mice. This is consistent with other reports that bipotential cells of FLEP cells can proliferate and differentiate into hepatocytes and bile ducts[19,26].

In conclusion, we report that transplanted FLEP cells engraft, proliferate, and differentiate into hepatocytes and bile duct epithelial cells with high repopulation capacity in the liver fibrosis induced by DEN. FLEP cell transplantation restores liver function and reduces liver fibrosis effectively, which rescues the damaged liver by substituting the damaged cells directly. These results suggest that FLEP cell transplantation is an attractive method for chronic liver diseases.

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*CLINICAL RESEARCH*

# Endoscopic biliary drainage for patients with unresectable pancreatic cancer with obstructive jaundice who are to undergo gemcitabine chemotherapy

Osamu Takasawa, Naotaka Fujita, Go Kobayashi, Yutaka Noda, Kei Ito, Jun Horaguchi

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## Abstract

**AIM:** To assess optimum endoscopic biliary drainage (EBD) in cases with unresectable pancreatic cancer in the era of gemcitabine (GEM).

**METHODS:** Thirty patients with unresectable pancreatic cancer, who presented with jaundice and underwent chemotherapy using GEM after EBD were included in this study (GEM group). Fifteen cases with the same clinical manifestation and stage of pancreatic cancer treated with EBD alone were also included as controls. A covered metallic stent (CMS) or a plastic stent (PS) was used for EBD. The mean survival time (MST) in each group, risk factors of survival time, type of stent used and associated survival time, occlusion rate of stent, patency period of stent, and risk factors of stent occlusion were evaluated.

**RESULTS:** MST in the GEM group was longer than that in the control (9.9 mo *vs* 6.2 mo). In the GEM group, the survival time was not different between those who underwent metallic stenting and those who underwent plastic stenting. Stent occlusion occurred in 60% of the PS group and 7% of the CMS group. The median stent patency in the PS-GEM group and the CMS-GEM group was 5 mo and 7.5 mo, respectively. Use of a PS was the only risk factor of stent occlusion.

**CONCLUSION:** A CMS is recommended in cases presenting with jaundice due to unresectable pancreatic cancer, since the use of a CMS makes it possible to continue chemotherapy using GEM without repetition of stent replacement.



**Key words:** Gemcitabine chemotherapy; Endoscopic biliary drainage; Covered metallic stent; Pancreatic cancer; Unresectable pancreatic cancer; Obstructive jaundice

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## INTRODUCTION

Pancreatic cancer is one of the most intractable malignancies of the digestive tract and has a dismal prognosis. Such cancer in most patients is in an advanced stage when they first visit medical facilities, and management of obstructive jaundice caused by the tumor is of great concern in the treatment. Endoscopic biliary drainage (EBD) is the mainstay in the control of jaundice[1-5]. Due to a relatively short survival time after establishment of the diagnosis, placement of a plastic stent is considered to be reasonable from the viewpoint of cost-effectiveness[2,3,6-8]. The development of anticancer agents, however, has necessitated reassessment of the optimal choice of stents in this patient group. We evaluated the optimum EBD in cases with unresectable pancreatic cancer in the era of gemcitabine (GEM) chemotherapy.

## MATERIALS AND METHODS

### *Subjects*

One hundred and twelve patients with unresectable pancreatic cancer presented with obstructive jaundice at our medical center during the period from May 2001 to March 2005. Of those, 30 patients (age, 80 years or less; Karnofsky performance status, 60% or greater; stage Ⅳ or greater) who achieved successful decompression of the biliary tree by EBD and underwent chemotherapy with GEM were included in this study (GEM group). Fifteen cases that fulfilled the same criteria and were treated by EBD and the best possible supportive care without chemotherapy during an earlier period (May 1997 to November 2000) were selected as a control. The demographics of the two groups are shown in Table 1.

**Table 1 Patients characteristics**

| | GEM group $n$ = 30 | Control group $n$ = 15 | $P$ |
|---|---|---|---|
| Age (yr) | 61 ± 10 | 67 ± 8 | 0.06 |
| Male/Female | 21/9 | 7/8 | 0.13 |
| Stage (Ⅳa/Ⅳb) | 22/8 | 7/8 | 0.08 |
| PS/CMS | [1]15/15 | 11/4 | 0.32 |

GEM: gemcitabine; PS: plastic stent; CMS: covered expandable metallic stent. [1]The PS was exchanged to CMS after occulusion in 4 patients.

**Table 2 Prognostic factors of survival by univariate analysis**

| | OR | 95% CI | $P$ |
|---|---|---|---|
| Age | 1.03 | 1.00-1.06 | 0.08 |
| Gender (Female) | 1.56 | 0.81-2.99 | 0.18 |
| Stage (Ⅳb) | 2.36 | 1.15-4.84 | 0.02 |
| Liver metastasis (+) | 2.55 | 1.24-5.22 | 0.01 |
| Gemcitabine (-) | 2.72 | 1.35-5.48 | 0.01 |

### Methods

GEM was administered in a standard manner, i.e., a drip infusion of 1000 mg/m$^2$ at a stable speed was given once per week for three consecutive weeks with an interval of one week between courses. After confirming a decrease in the serum total bilirubin level of 20 mg/L or less, administration of GEM was started. When some adverse events occurred, administration of GEM was postponed or its dose was reduced. Adverse effects were evaluated following National Cancer Institute Common Terminology Criteria for Adverse Events ver.3.0 (CTCAE)[9]. The stents used were covered expandable metallic stents 10 mm in caliber (covered biliary Wallstent, Boston Scientific Co.) (CMS) and plastic stents 10 Fr in diameter (Double Layer Biliary Stent, Olympus Co.) (PS). In the GEM group, CMS was placed in 15 patients (CMS-GEM group) and PS was applied in the remaining 15 (PS-GEM group). In the control group, CMS was deployed in 4 patients (CMS-alone group), while PS was applied in the remaining 11 (PS-alone group). The period of stent patency was measured from the day of stenting to the day when the initial occlusion was diagnosed. Stent occlusion was defined as development of cholangitis or jaundice with abnormalities in laboratory tests indicating bile stasis. When a PS has clogged and was replaced with a CMS, the duration from placement of PS to its occlusion was regarded as the patency period of PS. As for survival time, the total period was counted as PS in such cases. In the CMS group, when the stent was occluded and was replaced with a PS, the duration between the initial stenting and the second stenting was measured as the patency period of CMS, and the total time until death was regarded as the survival time for this group. The median survival time (MST) was calculated based on the definition of survival time as the time from the day of hospitalization to that of death or the last date of confirmed survival. The following were evaluated: (1) MST of the GEM group and the control, (2) prognostic factors, (3) correlation between the stent applied and survival time, (4) occlusion rate and patency of stents, (5) risk factors of stent occlusion, and (6) complications of stenting and adverse events of GEM.

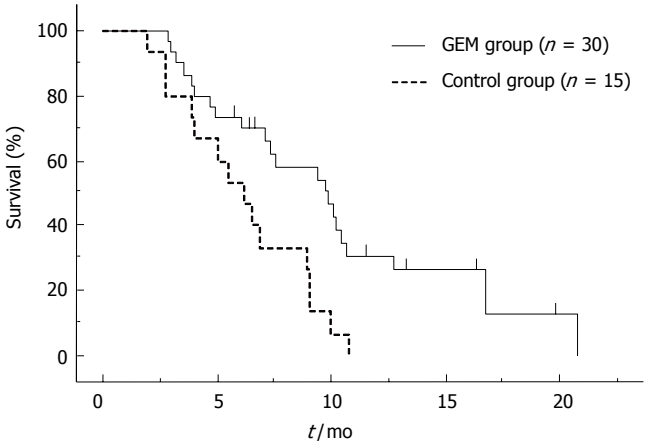


**Figure 1** Survival time in GEM and control groups (9.87 ± 0.33 *vs* 6.23 ± 0.93, $P$ = 0.004).

### Statistical analysis

Stat View v5.0 was used for statistical analysis. Comparison of the two groups was carried out using the Chi-square test, the unpaired $t$ test (Student's $t$- test), or the Mann-Whitney $U$ test, MST and the median patency time of stents were evaluated by the Mantel-Cox log rank test of the Kaplan-Meier curves. For the analysis of the risk factors of stent patency and survival, univariate analysis by Cox's proportional hazard model followed by multivariate analysis was performed.

## RESULTS

The mean survival time in the GEM group and the control was 9.9 mo and 6.2 mo, respectively (9.87 ± 0.33 *vs* 6.23 ± 0.93, $P$ = 0.004, Figure 1). The following were listed as prognostic factors of survival by univariate analysis: clinical stage of pancreatic cancer (Ⅳa *vs* Ⅳb, $P$ = 0.02; OR = 2.36; 95% CI, 1.15-4.84), liver metastasis (+ *vs* -, $P$ = 0.01; OR = 2.55; 95% CI, 1.24-5.22), and GEM treatment (+ *vs* -, $P$ = 0.01, OR = 2.72; 95% CI, 1.35-5.48) (Table 2). Multivariate analysis, however, revealed that none of these prognostic factors had reached the level of significance. The survival curves of the PS group and the CMS group with/without GEM are shown in Figures 2 and 3. There were significant difference between the PS-alone group and the PS-GEM group (5.53 ± 0.94 *vs* 10.1 ± 2.70, $P$ = 0.02), as well as between the PS alone group and the CMS-GEM group (5.53 ± 0.94 *vs* 9.87 ± 0.43, $P$ = 0.001). The difference between the CMS-GEM group and the PS-GEM group was not significant (9.87 ± 0.43 *vs* 10.1 ± 2.70, $P$ = 0.26, Figure 4). Stent obstruction was observed more frequently in the PS group than in the CMS group (PS *vs* CMS, $P$ = 0.0002; OR = 26.4; 95% CI, 11.4-14.6). Only one patient developed stent occlusion (1/15, 7%), which was due to overgrowth of the tumor in the CMS-GEM group; this was treated by additional PS stenting. The stent patency in the PS-GEM group and PS-alone group was 5.0 mo and

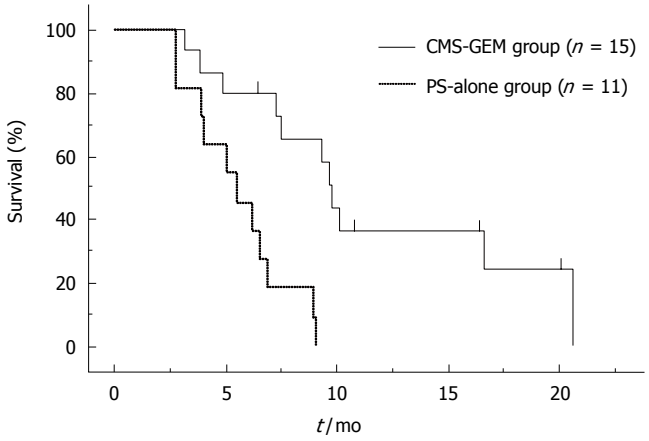


**Figure 2** Survival curves of CMS-GEM and PS-alone groups (9.87 ± 0.43 *vs* 5.53 ± 0.94, *P* = 0.001).

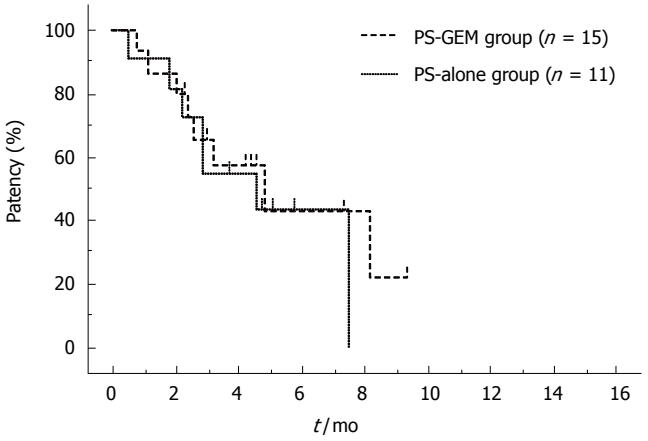


**Figure 5** Stent patency in PS-GEM and PS-alone groups (5.00 ± 2.26 *vs* 4.53 ± 1.31, *P* = 0.59).

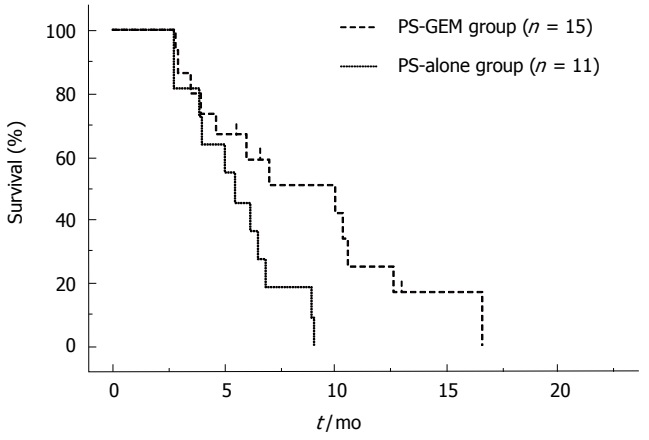


**Figure 3** Survival curves of PS-GEM and PS-alone groups (10.1 ± 2.70 *vs* 5.53 ± 0.94, *P* = 0.02).

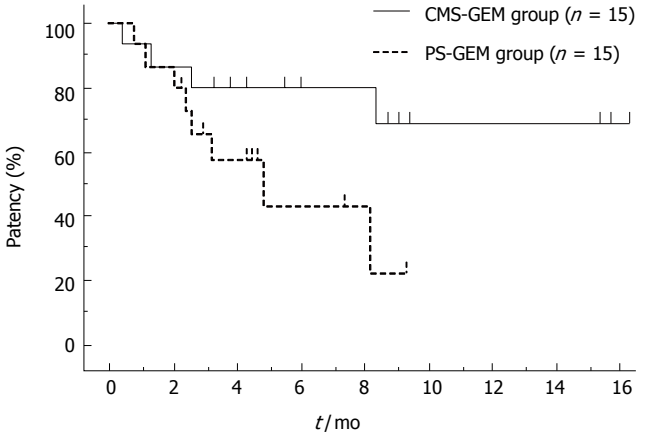


**Figure 6** Stent patency in CMS-GEM and PS-GEM groups (7.49 ± 0.82 *vs* 5.0 ± 2.26, *P* = 0.03).

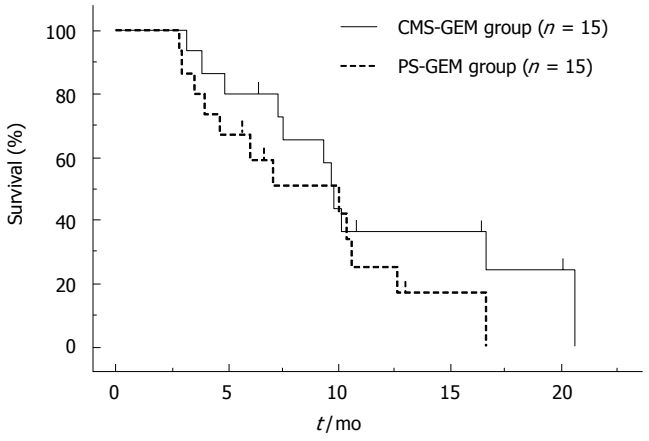


**Figure 4** Survival curves of CMS-GEM and PS-GEM groups (9.87 ± 0.43 *vs* 10.1 ± 2.70, *P* = 0.26).

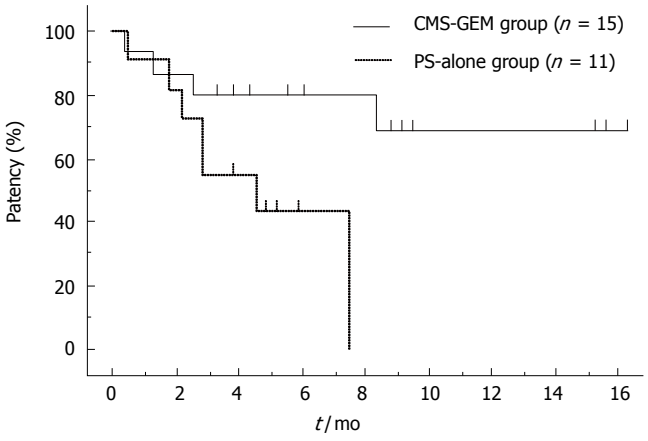


**Figure 7** Stent patency in CMS-GEM and PS-alone groups (7.49 ± 0.82 *vs* 4.53 ± 1.31, *P* = 0.01).

4.5 mo, respectively (5.00 ± 2.26 *vs* 4.53 ± 1.31, *P* = 0.59, Figure 5). Compared with these two groups, the CMS-GEM group showed a patency of 7.5 mo (not reached) (5.00 ± 2.26 *vs* 7.49 ± 0.82, *P* = 0.03 and 5.00 ± 4.53 *vs* 1.31 ± 0.82, *P* = 0.01, respectively, Figures 6 and 7). Univariate analysis revealed use of a plastic stent to be the only risk factor of stent occlusion (*P* = 0.01; OR = 4.7; 95% CI, 1.48-15.4, Table 3). One patient each in the CMS-GEM and the CMS-alone groups developed acute cholecystitis. Either dislodgement of a stent or development of acute

**Table 3 Risk factors of stent occulusion by univariate analysis**

| | OR | 95% CI | *P* |
|---|---|---|---|
| Age | 1.00 | 0.95-1.05 | 1.00 |
| Gender (Male) | 2.01 | 0.72-5.60 | 0.18 |
| Stage (IVb) | 1.30 | 0.57-3.31 | 0.59 |
| Liver metastasis (+) | 1.46 | 0.57-3.74 | 0.43 |
| Stent (PS) | 4.76 | 1.48-15.4 | 0.01 |
| Gemcitabine (-) | 1.30 | 0.51-3.33 | 0.59 |

**Table 4 Adverse effects of GEM chemotherapy *n* (%)**

| | Grade | | |
|---|---|---|---|
| | 1 | 2 | 3 |
| Bone marrow 24 (89) (*n* = 27) | | | |
| Leukocytes 17 (71) | 5 (21) | 10 (42) | 2 (8) |
| Hemoglobin 17 (71) | 10 (42) | 7 (29) | |
| Platelets 6 (25) | 3 (13) | 1 (4) | 2 (8) |
| Gastrointestinal 6 (20) (*n* = 30) | | | |
| Anorexia 3 (9) | | 1 (3) | 2 (6) |
| Nausea 5 (15) | 2 (6) | 2 (6) | 1 (3) |

Administration of gemcitabine (GEM) was abandoned due to adverse effects in 4 patients.

pancreatitis after stent placement was observed. In the GEM group, 24 patients (89%) showed adverse effects of GEM on the bone marrow system, these effects benign grade 3 or greater in 4 patients. Reduction of the dose of GEM was necessary in 3 patients due to bone marrow suppression and in 7 due to digestive symptoms such as nausea and anorexia. Finally, administration of GEM was abandoned due to adverse effects in 4 patients (Table 4).

## DISCUSSION

Before starting chemotherapy, biliary drainage is mandatory in patients with unresectable pancreatic cancer who present with obstructive jaundice. Endoscopic biliary drainage (EBD) is now widely used for such purpose. Many studies have been published on the selection of stents in EBD[1-5]. With regard to PS, development of Teflon stents[4,10] has not been able to prolong the patency period much longer than that of polyethylene stents[5,11-13]. Many a study has revealed the patency of metallic stents to be longer than that of PS[1-3]. The reported patency is 3-5 mo for PS[1-3,5,7,14] and nearly 9 mo for metallic stents[1-3,14]. In current clinical practice, stent selection is based on the stage of malignancy and expected prognosis. Distant metastasis, tumor size, and local extension are considered to be prognostic factors[15-17]. In cases with short life expectancy (4-6 mo or less), such as in advanced pancreatic cancer, occlusion of PS usually does not occur before death, and application of PS is considered to be adequate from the viewpoint of cost-effectiveness as well[2,3,6-8]. Thus far, chemotherapy, radiation and chemoradiotherapy have been performed for patients with unresectable pancreatic cancer, which necessitates hospitalization. However, the results have been quite unsatisfactory. Development of GEM has had an impact on the treatment. Rothenburg *et al*[18] reported significant prolongation of survival with GEM in their phase Ⅱ study of patients in whom 5-FU was not effective. Burris *et al*[19] carried out a randomized controlled trial on the comparison of GEM with 5-FU in cases without previous chemotherapy, and reported that although mass reduction was observed in only 5.4% of the cases, alleviation of symptoms was achieved in 23.8% with a significant difference. The survival time was also significantly prolonged in the patient group treated with GEM compared with that in the 5-FU group. GEM is given to patient by drip infusion at a stable speed that requires a relatively short time without critical side effects, enabling its administration on an outpatient basis. Shortening of the period of hospitalization is quite meaningful for patients with poor prognoses. In this study, prolongation of MST by GEM in patients with stage Ⅳ pancreatic cancer was confirmed. With respect to MST, there was no difference between the CMS-GEM group and the PS-GEM group. However, stent occlusion developed more frequently in the PS-GEM group than in the CMS-GEM group (60% *vs* 7%). Stent occlusion may necessitate postponement of administration of GEM and requires additional intervention or hospitalization, which leads to deterioration of the patient's QOL. Possible influences of GEM on the patency of stents are prolongation of patency by controlling the tumor mass and shortening of patency by clogging subsequent to biliary infection induced by bone marrow suppression. As the comparison of stent patency in the PS-GEM group and that in the PS-alone group showed no difference, GEM may have no or only a subtle effect on the stent patency. Acute cholecystitis, which was managed conservatively, was the only complication relevant to stenting. Discontinuance of GEM due to adverse effects was necessary in only 16% of the patients. When compared to that of the PS group, the stent patency of the CMS-GEM group was longer. It is expected that those patients who are to undergo GEM will have a longer survival than that before GEM was available. The significance of the maintenance of stent patency is much greater than before as it can eliminate readmission due to stent occlusion and postponement of GEM. Development of effective chemotherapy, including a combination of some agents, will further extend the significance of longer stent patency further. The selection of stents should be reassessed from this point of view. Based on the data shown here, we suggest that the use of CMS should be considered in patients with unresectable pancreatic cancer presenting with jaundice that are to undergo GEM. Further studies including cost-benefit assessment and a randomized, prospective comparison trial with metallic and plastic stents are necessary.

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*CLINICAL RESEARCH*

# Colonoscopic evaluation of hematochezia in low and average risk patients for colorectal cancer: A prospective study

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## Abstract

**AIM:** To relate the endoscopic findings in patients with hematochezia with regard to age in "low and average risk" for colorectal cancer (CRC) and to localize significant lesions in order to identify patients who need sigmoidoscopy or total colonoscopy.

**METHODS:** This prospective study was performed in an open access GI endoscopy unit. Out of 4322 consecutive patients undergoing colonoscopy, 918 reported hematochezia. The final study group comprized 180 patients aged below 45 and 237 over 45. Main exclusion criteria were a 1st-degree family history of colorectal carcinoma, patients reporting blood mixed with stools and/or progressive colonic symptoms, or patients who had undergone colon surgery for neoplastic lesions.

**RESULTS:** Total colonoscopy could be performed in 96% of patients. Abnormal findings were observed in 34.3% of the younger and in 65.7% of the older ones. Findings were the presence of polyps in the distal colon ($n$ = 2) and IBD in the proximal colon ($n$ = 29) in the group of the younger patients, and polyps ($n$ = 15), IBD ($n$ = 13), and carcinoma ($n$ = 6, 4 of the lesions were located proximal to the splenic flexure) in the elderly. Our findings suggest that the diagnostic potential of total colonoscopy in patients younger than 45 referring scant hematochezia, is not mandatory. By exploring only the distal tract of the colon we have misdiagnosed two cases of IBD located in the ascending colon. In this group of patients additional risk factors must be identified before performing a total colonoscopy. Regarding the patients older than 45 yr, the exploration of the distal colon would have led to our overlooking a carcinoma, two neoplastic polyps and one IBD located in the proximal colon.

**CONCLUSION:** Young patients with scant hematochezia but without risk factors for neoplasia do not need a total colonoscopy, whereas is mandatory performing a total colonoscopy in older patients even in the presence of anal pathology.



**Key words:** Hematochezia; Colonoscopy; Neoplasia; Colonic neoplasia

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## INTRODUCTION

Hematochezia defined as a chronic intermittent passage of a small amount of bright red blood from the rectum is a clinical problem frequently found in adults of all ages. Its prevalence in the apparently healthy general population is between 9% and 19%[1-5] and in general practice this means 6 consultations every 1000 over 45-year-olds a month[6]. Almost 20% of colonoscopies are prompted by rectal bleeding[7,8].

In addition to its high prevalence, hematochezia is a symptom to be considered carefully, since it is known to be associated also with neoplastic diseases (adenoma, cancer). Studies showed that 7%-10% of patients with chronic overt rectal bleeding did in fact have a colorectal cancer[5-7].

Since neoplastic and non neoplastic diseases may also coexist, a complete diagnostic work-up is indicated including clinical evaluation (clinical history, physical examination of the anal region, digital anorectal exploration), anoscopy, and endoscopic evaluation of the colon. It is a source of controversy as to whether scant hematochezia necessitates total colonoscopy as a first-line procedure or a 60 cm flexible sigmoidoscopy[9-18].

The aims of this study were: (1) to identify the type and prevalence of endoscopic findings in two groups of patients with hematochezia alone aged under *vs* over 45 years old; (2) to ascertain the distal (rectum, sigmoid, descending colon) or proximal (transverse and ascending colon) location of "significant lesions" in order to establish which patients need total colonoscopy.

## MATERIALS AND METHODS

The study was performed prospectively on 4322 consecutive out-patients undergoing colonoscopy during a two-year period (November 2004-October 2005) at the "open access" Unit of Gastroenterology and Digestive Endoscopy at University of Catania, in eastern Sicily-Italy (Figure 1).

Before colonoscopy all patients were asked about the colour of the expelled blood, the frequency and duration of the bleeding episode, and how the blood appeared (on stools, on toilet paper, on the toilet, or mixed with stools).

Exclusion criteria were age below 18 years, positive 1$^{st}$-degree family history of CRC, patients reporting blood mixed with stools, and those whose bleeding was severe enough to require transfusion or hospitalization, patients who reported progressive colonic symptoms, those who had undergone colon surgery for neoplastic lesions, and those who had already had a colonoscopy within the previous year.

After clinical evaluation, all patients underwent anal inspection and anoscopy. Regardless of any anal pathologies detected, all patients underwent total colonoscopy. Endoscopy was performed by 5 expert endoscopists (> 5000 colonoscopies) in patients after the ingestion of 4 litres of polyethylene glycol solution.

The endoscopic evaluation was considered complete when the entire colonic mucosa was visualized. Patients whose colon had been inadequately cleansed and cases in which anatomical or technical problems had prevented a complete exploration of the colon were excluded. Endoscopic findings were classified as normal, inflammatory bowel disease (IBD), neoplastic disease (adenoma and carcinoma), diverticulosis, or angiodysplasia.

Neoplastic polyps > 10 mm, colorectal carcinoma, and colitis were defined as "significant lesions". The pathologies identified were classified as proximal (situated in the transverse and/or ascending colon) and distal (between the rectum and the splenic flexure). The numerical values obtained are expressed as a percentage and the two groups of values were compared using the chi-square test and the Fisher's exact test; a value of $P < 0.05$ was considered statistically significant.

## RESULTS

### *Endoscopic diagnosis*

From 4322 patients who underwent colonoscopy over a two-year period, the indication was hematochezia in 918 cases (21.2%), and other lower digestive tract symptoms in 3404 cases (78.8%).

From 918 patients with hematochezia, 417 (45.4%) met the inclusion criteria (Figure 1). This study group was composed of 244 males (mean age 50.5 ± 14.6 years, range 19-93) and 173 females (mean age 51 ± 14.6 years, range 18-96) yielding a male/female ratio of 1.4:1. 43.2% of these patients (180/417) were between 18 and 45 years old, while 56.8% (237/417) were older than 45 (Table 1).

Proctological examination revealed anal lesions potentially responsible for the bleeding in 265 cases (63.5%). Endoscopy was performed up to the cecum in 96.0% of the patients (417/430) with no complications worthy of note. The 13 incomplete explorations were due to poor colon cleaning ($n$ = 7), abnormal sigmoid-descending colon length ($n$ = 4), and low patient compliance ($n$ = 2). The most common pathologies detected were polyps ($n$ = 77, 18.5%), diverticular disease ($n$ = 44, 10.5%), IBD ($n$ = 42, 10%), and carcinoma ($n$ = 6, 1.4%) (Table 2).

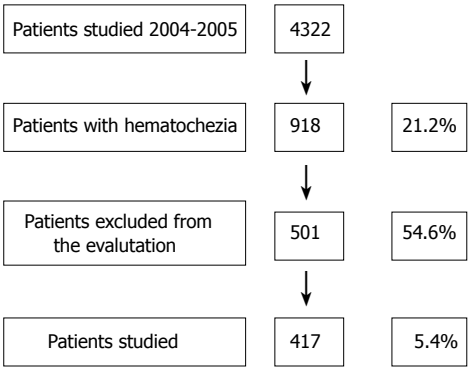


**Figure 1** Study profile.

**Table 1 Demographic data of patients with hematochezia**

| | |
|---|---|
| Patients ($n$) | 417 |
| Men/women | 244/173 |
| Mean age (yr) | 50.5 ± 14.6 |
| Range | 18-96 |
| < 45 years old | 180 (43.2%) |
| > 45 years old | 237 (56.8%) |

**Table 2 Diagnosis ($n$ = 493) in patients with hematochezia ($n$ = 417)**

| Diagnosis | $n$ | % |
|---|---|---|
| Anal pathology | 265 | 63.54 |
| Polyps | 77 | 18.46 |
| (< 10 mm) | 60 | 14.38 |
| (> 10 mm) | 17 | 4.07 |
| Diverticular diseases | 44 | 10.55 |
| IBD | 42 | 10.07 |
| Normal finding | 55 | 13.18 |
| Carcinomas | 6 | 1.43 |
| Angiodysplasia | 4 | 0.95 |
| Total | 493 | |

### *Endoscopic diagnosis with regard to age*

Table 3 shows the endoscopic diagnoses with regard to the patients' age. Anal lesions potentially responsible for hematochezia were seen in 57.2% of the patients (103/180) younger than 45 and in 68.4% (162/237) of the patients older than 45. This difference did not reach statistical significance ($P < 0.84$).

Total colonoscopy revealed pathologies in 45.6% (82/180) of the younger, and in 61.6% (146/237) of the older patients.

None of the younger patients had diverticular disease,

**Table 3 Endoscopic diagnoses in patients with hematochezia**

| Diagnosis | Age group (yr) | | | | *P* |
|---|---|---|---|---|---|
| | < 45 (*n* = 180) | | > 45 (*n* = 237) | | |
| | *n* | % | *n* | % | |
| Anal pathology | 103 | 57.2 | 162 | 68.4 | < 0.05 |
| Polyps | 15 | 8.3 | 62 | 26.2 | < 0.0001 |
| Diverticular diseases | 0 | - | 44 | 18.6 | < 0.0001 |
| IBD | 29 | 6.1 | 13 | 5.5 | < 0.001 |
| Normal finding | 35 | 19.4 | 20 | 8.4 | < 0.01 |
| Carcinomas | 0 | - | 6 | 2.5 | < 0.05 |
| Angiodysplasia | 3 | 1.6 | 1 | 0.4 | NS |

**Table 4 Significant lesions in relationship to age and proximal or distal location of lesions in patients with hematochezia**

| Significant lesions | Age group (yr) | | | | *P* | Location | | Tot. |
|---|---|---|---|---|---|---|---|---|
| | < 45 (*n* = 180) | | > 45 (*n* = 237) | | | Proximal | Distal | |
| | *n* | % | *n* | % | | *n* | *n* | |
| Polyps | 2 | 1.1 | 15 | 6.3 | < 0.05 | 1 | 16 | 17 |
| IBD | 29 | 16.1 | 13 | 5.5 | < 0.001 | 5 | 37 | 42 |
| Carcinomas | 0 | - | 6 | 2.5 | < 0.05 | 1 | 5 | 6 |
| Total | 31 | | 34 | | | 7 | 58 | |

while this was found in 18.6% (44/237) of the older patients. The presence of neoplastic polyps and carcinomas was also much greater in the latter, with a statistically significant difference, particularly for carcinoma ($P < 0.0002$). Rectal bleeding (which has predictive value for the diagnosis of CRC) increased with age: it was 1.0 (95% CI 0.12-3.64) among patients younger than 45 *vs* 6.2 (95% CI 1.30-17.19) for those over 45. No carcinoma was diagnosed among the younger patients, but they had a higher prevalence of IBD (29/180, 16.1%) than the older younger ones (13/237, 5.5%)

Among the 417 patients considered, 65 "significant lesions" were diagnosed in 64 patients (15.3%) (one patient had 2 lesions) including IBD ($n = 42$), polyps with a diameter > 10 mm ($n = 17$), and carcinoma ($n = 6$). Six of these lesions (3 cases of IBD, 2 polyps and 1 carcinoma) were located in the proximal tract of the colon and 59 (39 IBD, 15 polyps and 5 carcinomas) in the distal tract.

As for the age-related prevalence of significant lesions, the patients younger than 45 had 2 polyps > 10 mm and 29 cases (16.1%) of IBD, while the patients over 45 had 15 polyps, 13 cases (5.5%) of IBD, and 6 carcinomas (Table 4).

### *Age-related distribution of "significant lesions" on the colon mucosa*

Figure 2 correlates the patients' ages with the prevalence of "significant lesions" in the proximal and distal colon.

Thirty-one of the 64 patients (48.4%) with "significant lesions" were up to 45 years old and none had carcinoma. Two patients were diagnosed with polyps > 10 mm, both located in the distal colon. Twenty-nine were cases of IBD, two in the proximal colon and 27 in the left colon. Two (non neoplastic) significant lesions would have gone undiagnosed in this younger group if only flexible sigmoidoscopy had been carried out.

Thirty-four of the 64 patients (53.1%) with significant lesions were over 45 years old and included all 6 cases of carcinoma, 15 polyps > 10 mm and 13 cases of IBD. Four of these lesions (1 carcinoma, 2 polyps > 10 mm and 1 IBD) were located proximal to the left flexure and were consequently beyond the reach of flexible sigmoidoscopy (Table 4).

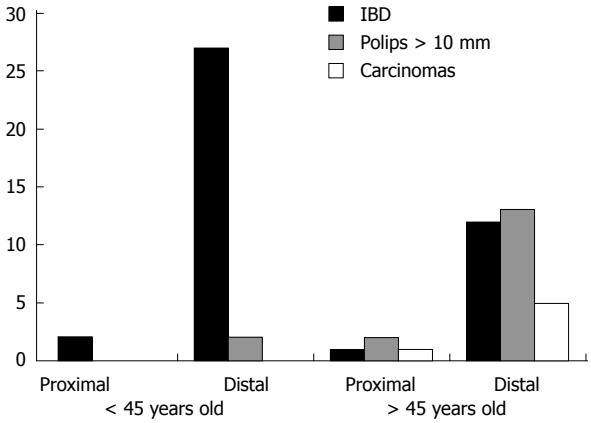


**Figure 2** Age-related location of "significant lesions" on the colonic mucosa.

## DISCUSSION

The passage of blood through the anus is a common symptom in adults and coincides with anal pathologies in 27%-74% of cases[16,19]. In our sample, the prevalence of anal lesions was 63.5%, with no statistically significant difference between the two age groups considered (under/over 45 years old: 57.2% *vs* 68.4%).

Since a large proportion of these patients only bleed from the anus, performing colonoscopy in all cases would only burden the endoscopy unit with an unjustified increase in costs. That is why numerous studies have been performed in an attempt to stratify patients at low and high risk of severe proximal colon according to their bleeding symptoms, but such analyses have produced contradictory results.

In 3 studies[16,20,21], haemorrhoid-type blood loss has rarely been associated with significant pathologies at proximal colon level, so the authors concluded that anal inspection with the rectosigmoidoscope suffices in such situations. Different conclusions were reached in three other papers finding no correlation between anal bleeding symptoms and the severity or location of colonic lesions[14,22].

Similar results were reported in a large prospective assessment that found no correlation between anal bleeding and colonic lesions: the authors concluded that total colonoscopy with a rectosigmoidoscope is always a safe, effective choice, and is also less expensive. This paper also stressed that 3 carcinomas were diagnosed in proximal colon segments among 45 patients under 45 years old[18].

Contrasting opinions are also expressed in the guidelines prepared by the American Society for Gastrointestinal Endoscopy (ASGE) and the European Panel for Appropriateness of Gastrointestinal Endoscopy (EPAGE): while the former specify that middle-aged or older individuals must always undergo a total colonoscopy, even in the presence of an anal lesion that could justify

the hematochezia[23], the latter consider total colonoscopy inappropriate when the source of bleeding has been ascertained by ano- or sigmoido-scopy[24].

When anal lesions causing bleeding are detected, the choice of investigation method should be based on the evaluation of two variables: the bleeding symptoms and the patient's age. For hematochezia patients up to 45 years old, we believe that exploring the colon up to the left flexure is sufficient, whereas it is best to examine all of the mucosal surface in older patients[19,25-30]

It is important to remember, however, that this approach is valid for diagnosing carcinoma, but could overbook up to 7% of other, equally significant pathologies[27]. Our findings suggest that the diagnostic potential of total colonoscopy is low in population up to 45-year-old referring scant hematochezia. By exploring only the distal tract of the colon, we have misdiagnosed two case of IBD located in the ascending colon. In patients under 40 years old, additional risk factors must be identified before performing a total colonoscopy i.e. chronicity (even if it is an intermittent bleeding), the discharge of blood mixed with stools, and a 1st-degree family history of colorectal cancer. Concerning this last point, the *American Cancer Society* (ACS) guidelines suggest that individuals with a positive 1st-degree family history of colorectal carcinoma must undergo total colonoscopy if they are 45 or older, irrespective of any presence or absence of lower digestive tract symptoms[31]. As regard as patients over 45 years endoscopic exploration of the whole colon is indicated even is a distal cause of the bleeding was ascertained.

In the older group of patients, the exploration of the distal colon alone would have led to our overlooking a carcinoma and a neoplastic polyp (> 10 mm) both located in the hepatic flexure. In conclusion, the diagnostic algorithm to adopt in the case of hematochezia can be as follows:

- total colonoscopy is always warranted for patients older than 45 years;

- for patients younger than 45 with no risk factors (e.g. discharge of blood mixed with stools, positive 1st-degree family history of colorectal carcinoma, or clinical history of progressive colorectal disease), rectosigmoidoscopy is initially sufficient. The work-up should be completed with a total colonoscopy in cases of haemorrhage during follow-up despite the site of bleeding having been identified and a suitable therapy implemented.

Such guidelines are justified by the fact that significant lesions can occur, albeit rarely, even in the proximal colon of young adults with a negative family history for colorectal cancer. Failing to consider such a hypothesis has devastating clinical consequences for the patient and also exposes the family physician and specialist to medico-legal consequences.

In the USA, the majority of lawsuits for malpractice have to do with the misdiagnosis of colorectal carcinomas[5,9].

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*RAPID COMMUNICATION*

# Effect of music on patients undergoing outpatient colonoscopy

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## Abstract

**AIM:** To evaluate the effect of relaxing music during colonoscopy under low-dose conscious sedation, on patient satisfaction, scope insertion time and procedure duration, medication doses, and the perceived adequacy of sedation and scope insertion difficulty on the part of the endoscopist.

**METHODS:** One hundred and sixty-seven consecutive adult outpatients presenting for routine colonoscopy under low-dose conscious sedation were randomized to undergo their procedures either with music played during the procedure or no music played.

**RESULTS:** There were no statistical differences between the two groups in terms of meperidine dose, midazolam dose, time to reach the cecum, total procedure time, endoscopist assessment of scope insertion difficulty, endoscopist assessment of adequacy of sedation, or the pain experience of the patients during their procedure. The music group did report significantly better overall procedure satisfaction as compared to the non music group on two of our three different scales.

**CONCLUSION:** While music does not result in shortened procedure times, lower doses of sedative medications or perceived patient pain, the patients who have music playing during their procedures report modestly greater satisfaction with their procedures.



**Key words:** Colonoscopy; Gastrointestinal endoscopy; Music; Music therapy; Relaxation music

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## INTRODUCTION

Colonoscopy plays an important role in the prevention of colon cancer through the diagnosis and removal of premalignant polyps. It is also the most accurate diagnostic tool for the detection of inflammatory bowel disease and many other structural lesions of the colon. Patient satisfaction with a particular medical procedure, including colonoscopy, reflects a number of variables, including the amount of anxiety prior to the procedure and the amount of anxiety and discomfort during it[1]. Anxiety about a procedure and fear of pain are also the reasons why some patients refuse endoscopic procedures for colon cancer screening[2].

The therapeutic uses of music have involved a number of disciplines of medicine, including cardiology, radiology and pulmonary medicine. For instance, studies have shown that music may decrease the anxiety of patients admitted to coronary intensive care units following cardiac surgery, and of patients undergoing stressful procedures like magnetic resonance imaging and bronchoscopy[3-6]. Studies of patients undergoing gastrointestinal endoscopic procedures have also shown that music tends to decrease patient anxiety[7-9]. However, the effects of music have been much more inconsistent when one looks at other outcomes such as pain, procedure time, doses of sedative medications, and patient vital signs. Some studies have reported shorter procedure times[10] and a reduced need for sedative medications[10-12] in colonoscopy patients who listen to music.

We conducted a randomized controlled trial of the use of relaxing music during outpatient colonoscopy done under low-dose conscious sedation, to examine its effects on patient satisfaction and pain, and to see if music has any beneficial effects on endoscopic parameters including colonoscope insertion time, medication doses, and insertion difficulty and adequacy of sedation from the standpoint of the endoscopist.

## MATERIALS AND METHODS

The study was approved by the Institutional Review Board of the University of Missouri-Columbia. One-hundred and sixty-seven consecutive patients presenting for elective outpatient colonoscopy were offered entry into this prospective study. Patients were only told that we were collecting information to assess their attitudes regarding colonoscopy before and after the procedure. They were not told that they were participating in a study to assess the beneficial effects of music. Exclusion criteria were: patients

with a history of prior colon resection, patients scheduled to undergo same-day esophagogastroduodenoscopy (EGD) and colonoscopy, and inability to give informed consent. One patient declined to participate in the study.

The study was single-blinded (from the standpoint of patients). Patients agreeing to participate in the study were randomized to either a music group or a non music group. Randomization was carried out by the use of opaque envelopes, half of which containing a piece of paper that said "music," and the other half containing a piece of paper that said "no music." In the music group, a CD player was playing relaxing music upon the entrance of the patient into the procedure room. We played the same music for all patients in the music group: "Watermark" by Enya (Reprise Records, a Time Warner Company, 1988), which contains 12 tracks (ranging from 1:59 to 4:25 in length). The CD was set on repeat.

Before starting any procedures, patients were given a written questionnaire, administered by one of the investigators (RAP). Patients rated their anxiety about having the colonoscopy on a visual linear analog (VLA) scale[13], in which the patient was asked to place a mark, representing anxiety, on a 100-mm long line, that was scored from 0 to 100, with 0 representing "not anxious at all" and 100 representing "extremely anxious".

All procedures were performed by one of the four experienced gastroenterologists utilizing Olympus colonoscopes (Olympus America Inc, Melville, NY). Trainees were not involved in any of the procedures. Patients were sedated with meperidine and midazolam. Procedures were started after the patient received 50 mg of meperidine and 1 or 2 mg of midazolam. Additional medication was given at the discretion of the endoscopist.

Procedures were timed by one of the investigators (RAP) who observed all the procedures quietly in the room. This investigator recorded the time required to reach the cecal base (insertion time) and the total procedure time.

After each procedure, the endoscopist graded the procedure difficulty on two scales. The "insertion difficulty Ⅰ scale" is a 100-mm VLA scale where 0 represents "very easy" and 100 represents "very difficult". The "insertion difficulty Ⅱ scale" is a five-point scale: 1 = very easy; 2 = relatively easy; 3 = average; 4 = somewhat difficult; and 5 = very difficult. The endoscopist also graded the adequacy of sedation on a four-point scale: 1 = satisfactory; 2 = anxious; 3 = agitated; and 4 = combative.

Following all procedures and just prior to discharge, the patients were given a written post-procedure questionnaire by the same investigator to assess their experience/satisfaction. The "experience Ⅰ scale" is a four-point scale which includes the following responses: 1 = pleasant; 2 = tolerable; 3 = difficult; and 4 = unacceptable. The "experience Ⅱ scale" is a five-point scale which includes the following responses: 1 = much better than I expected; 2 = somewhat better than I expected; 3 = about what I expected; 4 = somewhat worse than I expected; and 5 = much worse than I expected. The "experience Ⅲ scale" is a 100-mm VLA scale where 0 represents "pleasant", and 100 represents "worst experience I ever have". The "pain experience scale" is a 100-mm VLA scale where 0 represents "not painful at all" and 100 represents "unbearable". Patients were also asked if they would like to have "relaxing music" played at their next colonoscopy.

**Table 1 Population demographics and results of pre-procedure patient questionnaires**

| | Music ($n$ = 85) | No music ($n$ = 81) | $P$ |
|---|---|---|---|
| Age (yr) | 58.5 | 54.1 | 0.036 |
| Gender (% females) | 51.8 | 48.1 | 0.64 |
| Prior colonoscopy (%) | 36.5 | 30.9 | 0.45 |
| Pre-procedure anxiety (mm) | 36.3 | 45.1 | 0.053 |

The sample size of our study was estimated based on the data obtained during previous trials we performed on colonoscopy, so that we could detect a difference in the mean procedure time of 3 min between the two groups. Descriptive statistics were used to summarize baseline and outcome variables of the two groups. Mean was used to summarize normally or approximately normally distributed continuous variables. Median was used to summarize non-normally distributed ordinal variables. Proportion was used to summarize nominal variables. Chi-square test and Fisher's exact test were used to compare categorical variables. Both parametric ($t$-test) and non-parametric (Wilcoxon rank-sum test) tests were used to compare continuous variables. $P < 0.05$ was considered statistically significant.

## RESULTS

Table 1 summarizes the population demographics and results of the pre-procedure patient questionnaires for the music and non-music groups. They were similar in terms of gender (51.8% females for the music group *vs* 48.1% for the non-music group, $P = 0.64$) and history of prior colonoscopy (36.5% *vs* 30.9%, $P = 0.45$). The music group was slightly older (58.5 years *vs* 54.1 years, $P = 0.036$), and reported less pre-procedural anxiety (36.3-mm *vs* 45.1-mm, borderline significance at $P = 0.053$).

The indications for colonoscopy were colorectal cancer screening in 24, rectal bleeding in 67, positive fecal occult blood test in 13, anemia in 8, polyp follow-up in 24, chronic diarrhea in 15, change in bowel habits in 9, abdominal pain in 4, and ulcerative colitis surveillance in 2. The distribution of indications in the two groups was similar.

The results of various colonoscopy outcomes and the results of post-procedure questionnaires given to endoscopists are given in Table 2. There was no difference between the two groups in terms of doses of sedative medications, time to reach the cecum, total procedure time, perceived colonoscope insertion difficulty, or perceived adequacy of sedation.

The results of post-procedure patient questionnaires are given in Table 3. Patients reported a better overall experience on the three experience scales, though the difference only attained a statistical significance on the experience Ⅰ and Ⅲ scales ($P = 0.045$ and $P = 0.037$ respectively, compared to $P = 0.080$ for the experience

**Table 2 Procedure outcomes and results of the questionnaire given to endoscopists**

| Outcome | Music ($n$ = 85) | No music ($n$ = 81) | $P$ |
|---|---|---|---|
| Meperidine dose (mg)[1] | 57.0 | 54.6 | 0.68 |
| Midazolam dose (mg)[1] | 1.92 | 1.85 | 0.46 |
| Time to reach cecum (min)[1] | 10.4 | 9.2 | 0.46 |
| Total procedure time (min)[1] | 20.7 | 21.0 | 0.84 |
| Insertion difficulty Ⅰ scale (mm)[1] | 40.9 | 36.5 | 0.47 |
| Insertion difficulty Ⅱ scale (1-5)[2] | 3 | 3 | 0.31 |
| Adequacy of sedation scale (1-4)[2] | 1 | 1 | 0.093 |

[1] Mean value; [2] Median value.

**Table 3 Post-procedure patient questionnaire results**

| Outcome | Music ($n$ = 85) | No music ($n$ = 81) | $P$ |
|---|---|---|---|
| Experience Ⅰ scale (1-4)[2] | 2 | 2 | 0.045 |
| Experience Ⅱ scale (1-5)[2] | 1 | 2 | 0.080 |
| Experience Ⅲ scale (mm)[1] | 22.5 | 28.1 | 0.037 |
| Pain experience (mm)[1] | 25.3 | 25.4 | 0.8 |
| Want music at next colonoscopy (%) | 96.3 | 56.1 | < 0.0001 |

[1] Mean value; [2] Median value.

Ⅱ scale). The perception of pain in the two groups was similar when compared using unpaired $t$-test ($P$ = 0.8). More patients in the music group requested music at the next colonoscopy ($P$ < 0.0001).

## DISCUSSION

Music has been proposed as a useful adjunct for patients undergoing a variety of medical experiences and procedures, including gastrointestinal endoscopy. Music trials relating to gastrointestinal (GI) endoscopic procedures, can be categorized as those that have examined flexible proctosigmoidoscopy[7,8], colonoscopy[9-12], and a mix of upper endoscopy and colonoscopy[14-16]. The one consistent effect that therapeutic music seems to have beneficial effects in the setting of GI endoscopy is to decrease procedure-related anxiety[7-9,16]. Consistent with the anxiolytic effects of music is the observation that music may also decrease heart rates and blood pressure values in patients undergoing lower GI endoscopic procedures[7,12].

However, the effects of music on other outcomes besides anxiety have been much less consistent. Two small studies, one involving 50 flexible sigmoidoscopy patients[7] and the other involving 32 colonoscopy patients[12], showed that the reduction of heart rate and blood pressure values is associated with music. However, no difference has been seen in music-associated vital signs in a trial of 198 patients undergoing upper endoscopy and colonoscopy[16]. Three music trials in colonoscopy patients found that the need for sedative medications is reduced in patients listening to music[10-12], while a trial of music in upper endoscopy and colonoscopy patients has found no difference[5]. A small trial of patients undergoing flexible proctosigmoidoscopy[8] reported that abdominal pain is less severe in patients listening to music. However, other GI endoscopic trials have not found any difference in pain scores[11,15].

Our randomized controlled trial examined the use of music in the outpatient colonoscopy setting in adult patients receiving relatively light conscious sedation (the mean dose of meperidine and midazolam in our patient population was approximately 50 mg and 2 mg respectively). We observed a modest improvement in the overall satisfaction with the colonoscopy experience in patients who listened to music. The music group also expressed a strong preference for having music played again if they would undergo another colonoscopy in the future. However, we found no difference in patients' perception of pain, colonoscope insertion time, total procedure time, doses of sedative medications, or endoscopists' ratings of the difficulty of scope insertion and adequacy of sedation.

Our study has several limitations and weaknesses. First, our results are only applicable to colonoscopy patients who receive light conscious sedation. In many, even most practice settings in America at the present time, patients undergoing colonoscopy are sedated substantially heavier than our patients were sedated in this study.

Second, the benefits of music we observed are modest. We employed just one selection of music for all patients. It is possible that giving patients many more selections and letting them select the music they want would result in greater benefits. This would also be made easier by letting patients use headphones.

Third, the baseline characteristics of the two groups are different. The music group was somewhat older and expressed less pre-procedure anxiety as compared to the non music group. However, since the music group was older and less anxious than the non music group, one might have anticipated that medication administration should have been much less for the music group, assuming that music decreases medication administration. To the contrary, sedative medication doses were not statistically different between the two groups. Therefore, the amount of sedative medication was not benefited from the use of music during colonoscopy.

In conclusion, our study demonstrates that the use of relaxing music during colonoscopy performed under light conscious sedation can modestly improve the overall patient satisfaction. However, in contrast to some other published trials, we did not find a benefit in terms of shortening scope insertion or procedure times, reducing sedative medication doses, or decreasing patients' perceptions of pain. Nonetheless, the observation that music can improve patient satisfaction provides a rationale for its use as a safe adjunct during colonoscopy procedures when light conscious sedation is used. Specific areas in need of further investigation include the role of patient-selected music, the best way to deliver music, the role of music before and during recovery from procedures, and the use of combined audio-visual stimulation in the improvement of patient satisfaction at colonoscopy.

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## COMMENTS

### *Background*

Over the years, music has been used as a therapeutic modality in numerous disciplines of medicine to help reduce anxiety. Though studies of the use of music as an adjunct to GI endoscopic procedures are limited, it has been suggested that relaxing music might reduce anxiety, shorten procedure time, and reduce the doses of sedative medications needed.

### *Research frontiers*

Further research is needed to better define how music can be used to improve patient satisfaction and other outcomes associated with GI endoscopy. Specific areas in need of further investigation include the role of patient selected music, the best way to deliver music, the role of music before and during recovery from procedures, and the use of combined audio-visual stimulation.

### *Innovations and breakthroughs*

Our study demonstrates that the use of relaxing music during colonoscopy performed under light conscious sedation can modestly improve the overall patient satisfaction. However, in contrast to some other published trials, we did not find a benefit in terms of shortening scope insertion or procedure time, reducing sedative medication doses, or decreasing patients' perceptions of pain. Nonetheless, the observation that music can improve patient satisfaction provides a rationale for its use as a safe adjunct during colonoscopy procedures, particularly when light conscious sedation is used.

### *Applications*

The results of our study and others published in the field support the use of relaxing music during colonoscopy done under light conscious sedation as a way of improving patient satisfaction and possibly decreasing patient anxiety. The results also provide impetus for the study of relaxing music in patients being prepared for and awaiting their procedures, and during their post-procedure recovery.

### *Peer review*

The paper provides support for the practice of using relaxing music during colonoscopy procedures done under light conscious sedation.

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*RAPID COMMUNICATION*

# Non-compliance in surveillance for patients with previous resection of large (≥ 1 cm) colorectal adenomas

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## Abstract

**AIM:** To assess the extent and reasons of non-compliance in surveillance for patients undergoing polypectomy of large (≥ 1 cm) colorectal adenomas.

**METHODS:** Between 1995 and 2002, colorectal adenomas ≥ 1 cm were diagnosed in 210 patients and subsequently documented at the Erlangen Registry of Colorectal Polyps. One hundred and fifty-eight patients (75.2%) could be contacted by telephone and agreed to be interviewed. Additionally, records were obtained from the treating physicians.

**RESULTS:** Fifty-four out of 158 patients (34.2%) neglected any surveillance. Reasons for non-compliance included lack of knowledge concerning surveillance intervals (45.8%), no symptoms (29.2%), fear of examination (18.8%) or old age/severe illness (6.3%). In a multivariate analysis, the factors including female gender ($P$ = 0.036) and age > 62 years ($P$ = 0.016) proved to be significantly associated with non-compliance in surveillance.

**CONCLUSION:** Efforts to increase compliance in surveillance are of utmost importance. This applies particularly to women's compliance. Effective strategies for avoiding metachronous colorectal adenoma and cancer should focus on both the improvement in awareness and knowledge of patients and information about physicians for surveillance.



**Key words:** Colorectal adenoma; Colorectal cancer; Surveillance colonoscopy; Non-compliance

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## INTRODUCTION

Regular surveillance is strongly recommended for patients who have undergone an intestinal polypectomy due to the high adenoma recurrence rate and elevated risk of subsequent colorectal cancer (CRC)[1-4]. In particular, according to the guidelines of the Germany Society of Digestion and Metabolism [Deutsche Gesellschaft für Verdauung und Stoffwechsel (DGVS)], a surveillance colonoscopy should be performed in Germany not longer than 3 years after the initial polypectomy, regardless whether a single or multiple adenoma is resected[4]. When no metachronous adenoma is found at the 3-year surveillance colonoscopy, the next surveillance colonoscopy should be performed no later than 5 years. Clinically important colorectal adenomas include large (≥ 1 cm) lesions, those with a high degree of dysplasia, and those with villous histology. In particular, adenoma diameter is considered an important marker of malignant potential due to the fact that a larger adenoma at baseline is associated with a higher risk of CRC[5,6]. In persons who have been found to have a large colorectal adenoma, the colon cancer incidence rate is approximately 4 times higher than the normally expected incidence rate[7-10]. In a study by Gandhi *et al*[11], the 5-year recurrence rate after polypectomy of a colorectal adenoma was 40.93% (1651/4046) while the malignancy rate was 2.17% (88/4046). Although the length and schedules of surveillance programs are not clarified, a follow-up interval of no longer than 3 years in high-risk patients seems justified[4,12].

However, only 11%-51% of all participants with a resected polyp (≥ 1 cm in diameter) have ever returned for a follow-up colonoscopy[2,3,13,14]. Little is known about the reasons of non-compliance in those high-risk patients. However, awareness of the underlying reasons is the cornerstone for changing the prevailing attitude towards colonoscopy surveillance. Therefore, the purpose of

this retrospective study was to evaluate the surveillance behavior in patients undergone resection of a clinically relevant (≥ 1 cm) colorectal adenoma.

## MATERIALS AND METHODS

### Patients and survey methods

The Erlangen Registry of Colorectal Polyps (ERCRP) was established in 1978. Since then all clinico-pathological features concerning resected colorectal carcinomas have been prospectively recorded in this database. In addition, all colorectal polyps removed by endoscopy in the Department of Medicine or in the Department of Surgery of Erlangen University Hospital were prospectively documented and histologically categorized by the Department of Pathology in accordance with the WHO classification[15]. The size of all removed adenomas was accurately measured in the Department of Pathology. Patients whose adenomas were proved to contain invasive carcinomas at the initial examination and patients with inflammatory bowel disease were excluded from this survey. According to the guidelines of the DGVS, a surveillance colonoscopy should be performed after polypectomy at a time interval no longer than three years[4]. These recommendations were part of the discharge letter after initial polypectomy. However, automatic reminders for surveillance colonoscopies were usually not sent to the patients. The present study comprised the period from 1995 to 2002, because prior to these period follow-up recommendations have not been specified and a follow-up no longer than 3 years seemed to be adequate.

### Study variables and analysis

From 1995 to 2002, colorectal adenomas (≥ 1 cm in diameter) were diagnosed in 210 patients by complete colonoscopy. These adenomas were subsequently resected by endoscopic polypectomy or by surgery. Whether the death of 47 patients was due to CRC was evaluated according to the data obtained from the corresponding treating physician.

A total of 163 patients were still alive at the time of our study and could be contacted by telephone. Verbal consent was obtained about their willingness to take part in this survey and to obtain additional information from the treating physician before starting the interview. After the exclusion of 5 patients who refused to take part in this survey, 158 patients were interviewed by a trained research assistant.

Our standardized interview consisted of 10 questions. In the case of non-attendance to the follow-up colonoscopy, some items were skipped and the reasons of non-compliance were asked as shown in Appendix 1.

Additionally, the treating physician of the respective patient was addressed by an official letter from our department, in which he/she was asked to answer a standardized interview of 4 questions shown in Appendix 2.

In the case of not answering our survey for six weeks after having posted the letter, the corresponding physician was called by telephone and the interview based on the interview items, was orally performed.

Information on age, gender, localization of the initial adenoma and indication for initial polypectomy were obtained from the Erlangen Registry of Colorectal Polyps (ERCRP). An overview about the study setup is shown in Figure 1.

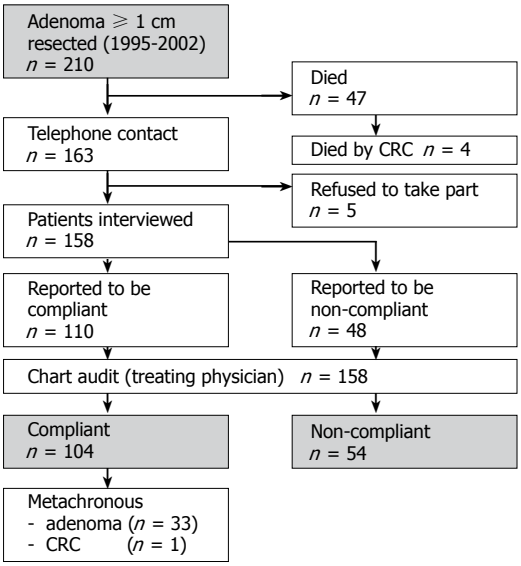


**Figure 1** Flow chart about the study setup.

### Statistical analysis

The significance of differences between the specific proportions was tested by the Fisher's exact test and differences between average values by the non-parametric Mann-Whitney test. Two-sided *P* value less than 0.05 was considered statistically significant. The relative importance of risk factors was assessed by the Cox's stepwise proportional hazard model. Statistical analyses were performed with SPSS version 13 ( SPSS Inc, Chicago, USA).

## RESULTS

A total of 210 patients with a colorectal adenoma (≥ 1 cm in diameter) were diagnosed at Erlangen University Hospital and tested for surveillance. No interview could be carried out with 52 patients, either because they refused to take part in the survey ($n = 5$) or because they died ($n = 47$). According to the corresponding records or to the information given by the treating physician and the civil registry office records, death occurred in 4 (8.5%) of 47 patients at a median period of 9. 2 years after initial colonoscopy due to CRC.

One hundred and fifty-eight patients could be interviewed. The clinico-pathological characteristics of the patients are shown in Table 1. One hundred and four (65.8%) out of the 158 patients underwent regular surveillance colonoscopies. In 33 out of the 104 patients (31.7%) at least one metachronous adenoma was discovered in at least one of the surveillance colonoscopies. The median size of the metachronous adenoma was 1.3 cm (range 0.3 cm to 2.2 cm). A colonic cancer [transverse colon, pT2pN0M0 (UICC I)] was diagnosed in one out of 104 surveillance participants and

**Table 1 Clinico-pathological features of compliance and non-compliance surveillance for the patients**

| | Compliance ($n$ = 110) $n$ (%) | Non-compliance ($n$ = 48) $n$ (%) | $P$[1] |
|---|---|---|---|
| Sex | | | |
| Male | 69 (73.4) | 25 (26.6) | |
| Female | 35 (54.7) | 29 (45.3) | 0.015 |
| Age | | | |
| ⩽ 62 yr | 64 (75.3) | 21 (24.7) | |
| > 62 yr | 40 (54.8) | 33 (45.2) | 0.007 |
| Education | | | |
| < high school degree | 83 (65.9) | 43 (34.1) | |
| ⩾ high school degree | 21 (65.6) | 11 (34.4) | 0.979 |
| Size of adenoma | | | |
| 10 -15 mm | 51 (62.2) | 31 (37.8) | |
| > 15 mm | 53 (69.7) | 23 (30.3) | 0.318 |
| Localisation | | | |
| Rectum | 24 | 13 | |
| Left Colon | 50 | 31 | |
| Right Colon | 28 | 10 | 0.213 |
| Histology | | | |
| Tubulous | 35 (61.4) | 22 (38.6) | |
| Tubulovillous | 61 (66.3) | 31 (33.7) | |
| Villous | 8 (88.9) | 1 (11.1) | 0.268 |
| Dysplasia | | | |
| Mild | 19 (63.3) | 11 | |
| Moderate | 72 (70.6) | 30 (29.4) | |
| Severe | 13 (50.0) | 13 (50.0) | 0.135 |
| Family history of CRC | | | |
| Yes | 14 (73.7) | 5 (26.3) | |
| No | 90 (64.7) | 49 (35.3) | 0.441 |
| NSAID intake | | | |
| Yes | 26 (76.5) | 8 (23.5) | |
| No | 78 (62.9) | 46 (37.1) | 0.140 |

[1]Fisher's exact 2-sided test.

could resected in a curative (R0) manner. An incongruence between the statements of the patients and treating physician concerning the existence of metachronous adenomas was found in 7 (6.7%) out of the 104 patients. Of the 7 patients, 3 wrongly assumed that they had a metachronous colorectal adenoma, and 4 assumed that they had no metachronous adenoma in contrast to the records. The size ($P$ = 0.318), localization ($P$ = 0.213) and histology ($P$ = 0.268) of adenoma as well as the grade of dysplasia ($P$ = 0.135) were not significantly associated with compliance. Furthermore, education ($P$ = 0.979), daily intake of nonsteroidal anti-inflammatory drugs (NSAID) ($P$ = 0.140) and a family history of CRC ($P$ = 0.441) were not significantly related to compliance.

Fifty-four (34.2%) out of 158 patients did not participate in a current surveillance program. Forty-eight (88.9%) out of 54 patients communicated this fact during the telephone interview, and discrepancies between the statements of the patients and the treating physician were found in 6 patients. The main reasons why the 48 patients did not report compliance included no knowledge about the surveillance intervals ($n$ = 22, 45.8%), no symptoms ($n$ = 14, 29.2%), fear of examination ($n$ = 9, 18.8%) or old age/severe illness ($n$ = 3, 6.3%). Difference in gender was also the reason for non-compliance. Male participants reported mainly that they had no symptoms while female patients explained that they had no knowledge about the surveillance intervals. However, there was a trend towards significance in this gender specific difference ($P$ = 0.071) (Figure 2).

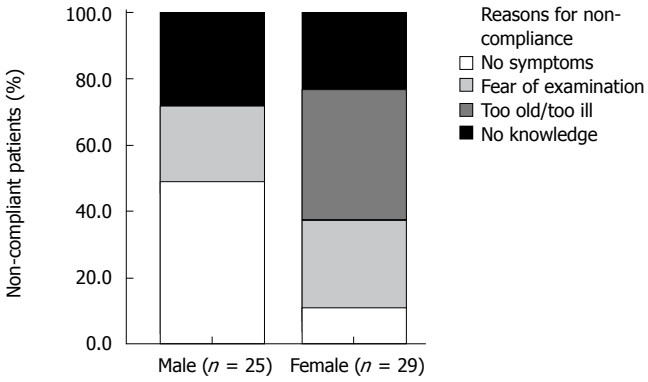


**Figure 2** Gender specific reasons for incompliance in 48 patients with large colorectal adenomas ($P$ = 0.071).

**Table 2 Multivariate analysis of factors significantly associated with non-compliance in univariate analysis**

| | hazard ratio | 95% CI | $P$[1] |
|---|---|---|---|
| Sex (female *vs* male) | 2.089 | 1.051-4.157 | 0.036 |
| Age (< 60 yr *vs* > 60 yr) | 2.326 | 1.171-4.621 | 0.016 |

[1]Cox's stepwise proportional hazard model.

Stratifying various clinico-pathological features between compliance and non-compliance showed that the factors including age > 62 years ($P$ = 0.011) and female gender ($P$ = 0.021) were significantly associated with non-compliance. A multivariate analysis revealed that both were proved to be independent factors for non-compliance (Table 2).

## DISCUSSION

Our findings show that more attention must be paid to the evaluation of CRC screening and the improvement of screening compliance. More than one third (34.1%) of the patients undergone a polypectomy are still alive, and never have any of the recommended controls. Taking into account that most of the patients who died did not attend any follow-up colonoscopy, up to 48% had non-compliance, which is in concordance with several studies reporting that only 11%-51% of all participants with resected polyp have returned for a follow-up colonoscopy[2,3,13,14]. However, a recently study reported that 91% patients underwent follow-up colonoscopy[6], indicating that accurate recall data of testing are required, especially by the treating physician who is responsible for the follow-up of patients. Indeed, most patients in our study with current surveillance were regularly informed by their treating physician and 93.3% of them had knowledge

about the results of the follow-up colonoscopies.

Who are the patients with non-compliance and what is their motivation? Unexpectedly, a significantly higher rate of compliance was found in men than in women during surveillance colonoscopies in our study. In particular, female sex was independently associated with non-compliance. This is *prima facie* surprising and in contrast to data on cancer screening for breast or cervical cancer, according to which 77% of women reported that they have undergone a mammogram and a papanicolaou smear within the past 2 years[16]. However, the participation rates seem to be different in CRC screening where procedures are involved, which may be perceived as disgusting or embarrassing[17]. In particular, only 27% of women undergoing regular breast and cervical cancer screening reported that they have undergone sigmoidoscopy in the preceding 5 years[16]. Furthermore, numerous studies indicate that the participation rates for prophylactic CRC screening are significantly higher in men than in women[18-21]. Although the risk of CRC is similar in men and women, CRC is considered a man's disease[22].

To our knowledge, no data on gender effects are available so far concerning surveillance colonoscopies after resection of a relevant colorectal adenoma. Therefore, in order to understand and improve the compliance rate, it is most important to know the reasons for refusing follow-up strategies. Furthermore, identifying the reasons for the failure to obtain surveillance is an important public health issue, as early detection of recurrences decreases CRC occurrence and societal cost[23,24].

Not having knowledge about surveillance intervals is the reason for non-compliance most frequently mentioned. In particular, 45% of the non-compliant patients and 50% of the female patients considered that they were not informed of the screening procedures by their physicians. These data show the essential importance of the primary care doctors in colorectal surveillance and are somewhat comparable with data from a study by Mandelson *et al*[25], who reported that lack of recommendation by their primary care doctor as a reason for not undergoing initial colorectal cancer screening in older women. However, in contrast to our data, probably all patients undergoing polypectomy in their study were told by the endoscopist to return for a surveillance examination. In contrast to USA, automatic reminders are not usually sent out by the specialists in Germany. Therefore, until now, it is the responsibility of patients and their primary care doctors to arrange surveillance colonoscopies.

Interestingly, the non-compliant patients in our study were not more interested in further information on colorectal adenomas and screening recommendations than patients under current surveillance. In particular, only 9 (16.7%) out of 54 patients without any follow-up coloncoscopy were interested in further information. One explanation might be the fact that most of the non-compliant patients were not aware of the importance of follow-up and that they were not sufficiently informed about running an increased risk of recurrent adenomas and CRC in contrast to the whole population. Another possible explanation might be the fact that these persons wished to avoid unfavorable health information[26]. In addition, flexible sigmoidoscopy trial from the United Kingdom showed that interest in information on colorectal screening is significantly associated with attendance at colorectal screening[20], which is in accordance with our data. Having no symptoms is a reason for incompliance. In our study, 45% of the male patients and only 15% of the female patients brought forward this argument. Furthermore, it is a widespread misbelief, especially in male patients, that bowel cancer development is associated with pain and gastrointestinal symptoms at an early stage.

Some limitations should be considered in interpreting our results. Since the study population pertains to a single university hospital, it remains unclear whether our findings are applicable to other populations in other medical settings. Future studies are obviously needed to corroborate our findings and address this potential limitation. Other considerations in generalizing our findings are the limited number of incompliant patients, the amount and reasons of non-compliance. This assumption is supported by the fact that most of the patients had no interest in further information although they were incompliant.

Of the 210 patients in the present study, 5 (2.4%) developed CRC during the follow-up, 4 died of it, and 33 had a diagnosed metachronous adenoma. This is in accordance with data recently published by us and others, showing that patients with clinically relevant adenomas ($\geqslant$ 1 cm) run an increased risk of recurrence and CRC[3,6,27]. Therefore, careful and frequent total colonoscopies (at intervals not longer than 3 years as long as metachronous adenomas are detected) for patients have to be warranted. Special recommendations have been proposed for patients with a family history of colorectal adenoma or CRC, who ran an increased risk of developing CRC[12].

Family history of CRC was stated by 19 (12.0%) of 158 patients in our study. However, only 14 (73.7%) of the 19 (73.7%) patients with a burden of familial CRC underwent follow-up colonoscopies after a relevant colorectal adenoma was removed. This lack of compliance is consistent with a study by Pho *et al*[28], who evaluated the communication about familial CRC risk in patients with newly diagnosed colorectal adenomas, and demonstrated evidence of poor communication, as only 41% of the patients were aware of the fact that their first-degree relatives run an increased risk of CRC, and stated the need for novel strategies to promote awareness and facilitate screening.

In conclusion, efforts have to be made to further increase CRC surveillance, especially in patients who have undergone resection of a clinically relevant adenoma. Particularly in women and patients with a family history of CRC or adenoma, effective strategies improving the awareness of recurrence and increased CRC risks are needed. Additionally, a thoughtful design of data management systems that document the available data and inform the treating physicians of screening and surveying patients, is necessary to reduce CRC morbidity and mortality.

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## APPENDIX 1

The standardized interview with the patients contained the following questions and information.

(1) The currently treating physician (name and address);

(2) The question of whether a surveillance colonoscopy was performed not longer than three years after the initial polypectomy (yes/no);

(3) The surveillance intervals and the issue of whether the patient was informed of the pending surveillance colonoscopy;

(4) The main reason for a follow-up colonoscopy (What was the main reason for you to have a follow-up?"

□ Routine

□ Having symptoms

(5) The recurrence of metachronous colorectal adenoma ("Has an additional adenoma been discovered at follow-up?" (Yes/no).

In the case of non-attendance to the follow-up colonoscopy, items 3-5 were skipped and the patients were asked for

(6) The reasons of non-compliance ("What was the main reason for you not having a follow-up colonoscopy to date?". The possible answers were:

□ Not knowing about follow-up intervals

□ Having no symptoms

□ Having fear about the endoscopic examination

□ Old age / severe illness for surveillance.

All patients were asked for

(7) Known cases of colorectal cancer in the family history (yes/no)

(8) A daily intake of non-steroidal anti-inflammatory drugs (e.g. aspirin®) (yes/no)

(9) The attendance to the last grade of high school (which approximately corresponds to the German term "Abitur") (yes/no)

(10) Their interest in additional information on surveillance after polypectomy (yes/no).

## APPENDIX 2

The standardized interview with the treating physicians contained the following questions.

(1) Whether the named patient was under his/her treatment (yes/no)

(2) Whether a family history of previous colorectal cancer incidents was known in the patient concerned (yes/no)

(3) Whether a follow-up colonoscopy was performed

□ Yes

□ No

□ No information

(4) In case of a follow-up colonoscopy, whether a metachronous colorectal adenoma or a carcinoma was ever found (yes/no).

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*RAPID COMMUNICATION*

# Role of immunosuppression and tumor differentiation in predicting recurrence after liver transplantation for hepatocellular carcinoma: A multicenter study of 412 patients

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## Abstract

**AIM:** To assess pre-orthotopic liver transplantation (OLT) factors that could be evaluated pre-operatively or controlled post-operatively associated with hepatocellular carcinoma (HCC) recurrence and disease-free survival after liver transplantation (LT).

**METHODS:** Four hundred and twelve patients transplanted for HCC between 1988 and 1998 in 14 French centers, who survived the postoperative period were studied. Kaplan Meier estimates were calculated for 24 variables potentially associated with recurrence of HCC. Uni- and multivariate analyses were conducted to identify independent predictors of recurrence.

**RESULTS:** Overall 5-year disease-free survival was 57.1%. By univariate analysis, variables associated with disease-free survival were: presence of cirrhosis ($P$ = 0.001), etiology of liver disease ($P$ = 0.03), $\alpha$ fetoprotein level (< 200, 200 to 2000, or > 2000; $P$ < 0.0001), $\gamma$-GT activity (N, N to 2N or > 2N; $P$ = 0.02), the number of nodules (1, 2-3 or $\geqslant$ 4; $P$ = 0.02), maximal diameter of the largest nodule (< 3 cm, 3 to 5 cm or > 5 cm; $P$ < 0.0001), the sum of the diameter of the nodules (< 3 cm, 3 to 5 cm, 5 to 10 cm or >10 cm; $P$ < 0.0001), bilobar location ($P$ = 0.01), preoperative portal thrombosis ($P$ < 0.0001), peri-operative treatment of the tumor ($P$ = 0.002) and chemoembolization ($P$ = 0.03), tumor differentiation ($P$ = 0.01), initial type of calcineurin inhibitor ($P$ = 0.003), the use of antilymphocyte antibodies ($P$ = 0.02), rejection episodes ($P$ = 0.003) and period of LT ($P$ < 0.0001). By multivariate analysis, 6 variables were independently associated with HCC recurrence: maximal diameter of the largest nodule ($P$ < 0.0001), time of LT ($P$ < 0.0001), tumor differentiation ($P$ < 0.0001), use of anti-lymphocyte antibody (ATG) or anti-CD3 antibody (OKT3) ($P$ = 0.005), preoperative portal thrombosis ($P$ = 0.06) and the number of nodules ($P$ = 0.06).

**CONCLUSION:** This study identifies immunosuppression, through the use of ATG or OKT3, as a predictive factor of tumor recurrence, and confirms the prognostic value of tumor differentiation.



**Key words:** Immunosuppression; Hepatocellular carcinoma; Tumor differentiation; Liver transplantation

Decaens T, Roudot-Thoraval F, Bresson-Hadni S, Meyer C, Gugenheim J, Durand F, Bernard PH, Boillot O, Compagnon P, Calmus Y, Hardwigsen J, Ducerf C, Pageaux GP, Dharancy S,

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## INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common neoplasms and its incidence is currently rising worldwide[1-3]. HCC usually occurs in cirrhotic livers and less than 30% of patients presenting with HCC are considered candidates for resection[4]. When liver transplantation (LT) was first developed, it was hoped that it would provide a cure for patients with unresectable HCC. However, it became apparent that both intra- and extra-hepatic post-transplant recurrence were frequent, accounting for poor mid-term survival[5-9]. Subsequently, some predictors of recurrence were identified[10-13] in order to select those who may benefit from LT, among patients with HCC.

During the past decade, liver transplantation criteria for HCC were refined and are currently based on the number and size of tumors[10,13]. Other predictors of recurrence have been more recently proposed[14-20]. Some of them, such as micro-vascular involvement[14-18] cannot be assessed prior to LT. Others, such as bi-lobar distribution of HCC[14,18] the sum of diameters of all tumors[19] or tumor differentiation[15-17,19-20] could be evaluated preoperatively and used in clinical practice. In addition, the impact of post transplant factors on HCC recurrence, such as the intensity and type of immunosuppression, have been little studied[21].

The aim of this study was, therefore, to assess in a large series of patients transplanted for HCC, the prognostic value of recurrence criteria, including those more recently proposed, as well as variables that could be evaluated pre-operatively or controlled post-operatively.

## MATERIALS AND METHODS

### Patients

Patients transplanted for HCC between 1988 and 1998 in 14 French liver transplant centers were studied. Patients with incidental HCC, which were defined as tumors diagnosed on the explanted liver but not prior to transplant, were excluded.

Based on these criteria, a cohort of 467 patients was identified. Fifty-five (11.8%) patients who died postoperatively and were not exposed to the risk of recurrence were excluded from the analysis of factors associated with recurrence. Therefore, the final study population consisted of 412 patients who had been transplanted for HCC and were suitable for analysis of recurrence and tumor-free survival. The features of these 412 patients are shown in Table 1.

**Table 1 Clinical features of the study population**

| | |
|---|---|
| Age (yr) | 52.7 ± 9.2 |
| Sex (M/F), *n* (%) | 339 (82.3)/73( 17.7) |
| Etiology: Alc./Vir./Metab./Miscel[1], *n* (%) | 136 (33.2)/236 (57.3)/ 8(2.0)/32 (7.5) |
| Cirrhosis (yes/no), *n* (%) | 377 (91.5)/35 (8.5) |
| AFP (μg/L) | 26.9 (ext: 0.5-245.240) |
| Number of nodules | 1.8 ± 1.3 (median: 1, range: 1-11) |
| Maximum diameter (cm) | 4.1 ± 3.1 (median: 3.4, range: 0.6-25) |
| Bilobar location (yes/no), *n* (%) | 105 (25.5)/307 (74.5) |
| Portal or hepatic vein obstruction (yes/no) (*n*) | 33 (8.1)/379 (91.9) |
| Milan Criteria (yes/no), *n* (%) | 263 (63.8)/149(36.2) |
| Peri-operative treatment (yes/no), *n* (%) | 275 (66.8)/137(33.2) |
| Transarterial chemoembolization (yes/no) | 161 (39.2)/251 (60.8) |
| Pre-transplant surgery (yes/no) | 42 (10.2)/370(89.8) |
| Pre-transplant ethanol injection (yes/no) | 65 (15.8)/347 (84.2) |
| Post-transplant chemotherapy (yes/no) | 57 (13.9)/355 (86.1) |
| Tumor differentiation (Well/moderate/poor), *n* (%) | 248 (69.3)/90 (25.4)/20 (5.3) |
| Time on waiting list (mo) | 4.4 ± 4.0 (median: 3.3, range: 0.1-32.3) |
| Initial immunosuppression Calcineurine inhibitors/ATG-OKT3[2], *n* (%) | 357 (86.6)/55 (13.4) |
| Steroid-treated rejection episodes, *n* (%) | 125 (30.5) |
| Maintenance immunosuppression | |
| Cyclosporine A/tacrolimus, *n* (%) | 284 (68.9)/128 (31.1) |

[1]Alc: alcohol; Vir: Viral; Metab: Metabolic; Miscel: Miscellaneous; [2]ATG: anti-lymphocyte ab; OKT3: anti-CD3 ab.

### Methods

Charts of the 412 patients were individually reviewed and the following data were collected.

**Pretransplant data:** Demographics, etiology and severity of liver disease, liver biochemical tests and prothrombin time, α fetoprotein level, Karnofsky index[22], pretransplant treatments of HCC during the waiting time and time from evaluation to LT (months) were noted. Cirrhotic patients were classified according to Child-Pugh classification[23].

Morphological features and preoperative staging of HCC were evaluated from medical reports of abdominal imaging including ultrasonography, angiography, dynamic computed tomography, and contrast-enhanced magnetic resonance imaging, when available. The number, size, and locations of the tumors as well as the presence of a vascular obstruction were assessed. When the number and size of the nodules differed between the different imaging techniques, the technique that showed the largest number of nodules and the largest nodule was taken into account. From their preoperative characteristics, HCCs were classified according to the modified TNM staging classification for liver transplantation[24] (Figure 1) and according to the Milan Criteria[13] (Table 1).

Treatments of HCC during the waiting time were also assessed [pre-operative treatment: surgery, transarterial chemoembolization (TACE) or percutaneous ethanol injection (PEI)], as well as the immediate post-operative treatments (Table 1).

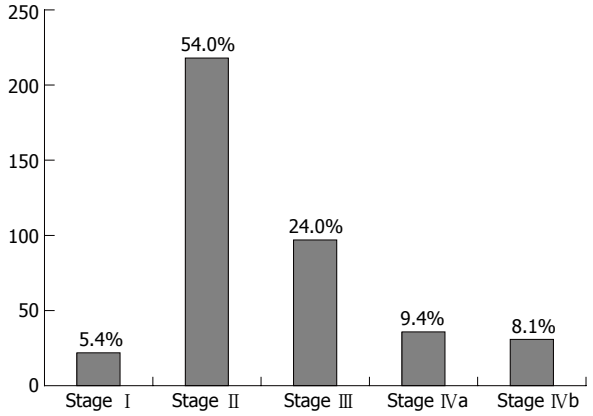


**Figure 1** Staging of tumors according to the modified TNM classification for liver transplantation[24]. T0: Tumor not found; T1: 1 nodule < 1.9 cm; T2: 1 nodule 2.0-5 cm; 2 or 3 nodules, all <3 cm; T3: 1 nodule > 5.0 cm; 2 or 3 nodules, at least 1 > 3.0 cm; T4a: 4 or more nodules, any size; T4b: T2, T3, T4a plus gross intrahepatic portal or hepatic vein involvement as indicated by US, CT or MRI; N1: Involvement of regional (porta hepatitis) nodes; N2: Metastatic disease, including extrahepatic portal or hepatic vein involvement.

**Pathological study of the tumors:** Results of pretransplant tumor biopsies were available in 79 patients. Tumor differentiation was therefore determined by reviewing the pathological reports of the explanted livers, to obtain a larger set of data. Tumor differentiation was graded in 3 stages (grade 1: well-differentiated tumors, grade 2: moderately differentiated tumors and grade 3: poorly differentiated tumors) according to the modified Edmondson Criteria[25]. In case of several tumors showing different stages of differentiation on the explanted liver, the worst grade of differentiation was arbitrarily chosen. Tumor differentiation was obtained in 358 patients (85.4%). It could not be obtained in 40 patients because of total necrosis of tumor nodules due to pre-LT treatment (transarterial chemoembolization or percutaneous ethanol injection), and was not available in 14 patients. Other pathological features which could not be assessed pre-operatively, such as microvascular invasion, number and size of tumor nodules on the explanted liver were also available but were deliberately not taken into account in the present study.

**Posttransplant data:** The type of immunosuppressive drugs (calcineurin inhibitors *vs* antilymphocyte antibodies) (Table 1) and the presence of a histologically proven acute rejection and its treatment were noted (methyl-prednisone pulses, antilymphocyte antibodies). Postoperative death was defined as death occurring during the first 3 mo post-LT. Causes of death (deaths due to HCC recurrence and other late causes of deaths), HCC recurrence, length of follow-up from listing on the waiting list to transplantation and from transplantation to death, HCC recurrence or the most recent information, were determined. Data on immunosuppression and adjuvant postoperative treatment of HCC were also collected. As previously described, patients were screened for tumor recurrence by AFP assay and thoracic and abdominal CT every 3 mo for the first two years and/or when clinically indicated. Additional imaging techniques (bone scan, MRI) were used if necessary.

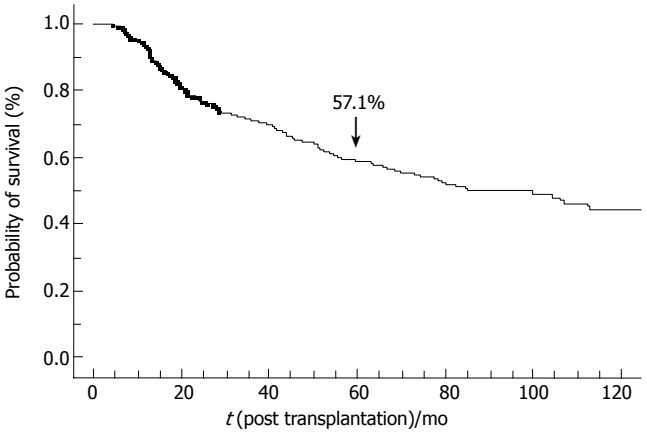


**Figure 2** Overall 5-year disease-free survivals.

### *Statistical analysis*

The proportion of missed data ranged from 0.0% to 14.6%, from one variable to another. In the case of missing data, no extrapolation of the missed values was done for the purpose of statistical analysis. Baseline patient characteristics and other continuous variables were reported as means ± SD or median and range when appropriate. Distributions of categorical variables are expressed as percentages. The Kaplan-Meier method was used to evaluate the probability of survival. Kaplan Meier estimates were calculated for 24 variables with potential prognostic significance and compared by the Logrank test. Factors associated with tumor-free survival at a P level of 0.1 in univariate analysis were entered in a multivariate analysis, using a Cox proportional hazards model to identify independent predictors of recurrence.

## RESULTS

The median waiting time from evaluation to LT was 3.3 mo (range: 0.1-32.3 mo). Median post-operative follow-up period was 52.0 mo (range: 3.2-186.3 mo). Tumor recurrence occurred in 131 cases (31.8%), after a median of 11.8 mo (1-125 mo). Recurrence involved a single site in 55.1% of the cases and multiple locations in 44.9% of the cases. Recurrence involved the liver graft, lungs, bones, brain, skin and other sites in 49.1%, 44.9%, 34.7%, 6.7%, 5.9% and 14.4% of the cases, respectively. The median time from recurrence to death was 5.6 mo (0.2-62.7 mo). One hundred and ninety-seven patients died (47.8%) during follow-up beyond the postoperative phase. Causes of death were HCC recurrence in 121 cases (61.4%), infections in 14 cases (7.1%), cardiovascular events in 11 cases (5.6%), recurrence of underlying liver disease in 8 cases (4.1%), and *de novo* cancers in 9 cases (4.5%). By the end of follow-up, only 10 patients with recurrence were alive.

Overall 5-year tumor-free survival was 57.1% (Figure 2). Five-year overall survival was 57.9% ± 2.5% since the vast majority of patients with recurrence died within 6 mo following recurrence. When restricted to the 330 patients transplanted after 1991, 5-year overall survival was 65%

**Table 2 Factors associated with recurrence-free survival (Univariate analysis)**

| Variables (*n*) | 5-yr recurrence free survival (%) | *P* |
|---|---|---|
| Age | | |
| < 50 (151)/> 50 (261) | 58.2/56.4 | 0.58 |
| Sex | | |
| Male (339)/female(73) | 56.8/58.3 | 0.46 |
| Liver disease etiology | | |
| Alcohol (122)/virus (210)/ Alcohol+virus (41)/others (39) | 64.8/55.1/58.1/40.2 | 0.03 |
| Cirrhosis | | |
| Present (377)/absent (35) | 58.7/35.7 | 0.001 |
| AFP (μg/L) | | |
| < 200 (281)/200-2000 (73)/> 2000 (33) | 62.9/47.8/26.4 | < 0.0001 |
| ASAT | | |
| Normal (139)/N-2N (129)/> 2N (112) | 59.2/60.5/51.1 | 0.3 |
| ALAT | | |
| Normal (178)/N-2N (116)/> 2N (86) | 56.4/63.5/60.6 | 0.3 |
| Alkaline phosphatase | | |
| Normal (170)/high (200) | 60.9/61.1 | 0.2 |
| G-GT | | |
| Normal (107)/N-2N (113)/> 2N (145) | 66.9/59.9/49.7 | 0.02 |
| Child-Pugh classification | | |
| A (219)/B (125)/C (56) | 58.7/49.6/71.1 | 0.07 |
| Karnofsky index | | |
| > 80% (251)/< 80% (155) | 58.2/55.7 | 0.78 |
| Number of nodules, pre- transplant | | |
| 1 (228)/2 or 3 (142)/> 4 (33) | 58.4/58.4/48.5 | 0.02 |
| Maximum diameter of the largest nodule, pre-transplant | | |
| < 3 cm (126)/3-5 cm (191)/> 5 cm (82) | 69.3/61.4/32.9 | < 0.0001 |
| Sum of tumor diameter (cm) | | |
| < 3 cm (123)/3-5 cm (121)/ 5-10 cm (92)/> 10 cm (34) | 72.1/61.1/50.3/19.5 | < 0.0001 |
| Tumor location, pre- transplant | | |
| Uni-lobar (303)/Bi-lobar (104) | 60.4/48.3 | 0.01 |
| Portal or hepatic vein obstruction, pre-transplant | | |
| Absent (373)/Present (33) | 60.2/27.3 | < 0.0001 |
| Pre or post transplant adjuvant treatment | | |
| Present (274)/absent (136) | 61.1/47.9 | 0.002 |
| Pre LT arterial chemoembolization | | |
| Present (241)/absent (162) | 62.3/53.0 | 0.03 |
| Tumor differentiation | | |
| Well (248)/moderate (91)/poor (19) | 58.3/47.1/42.1 | 0.01 |
| Period of transplantation | | |
| < 91 (82)/91-93 (91)/94-96 (118)/97-98 (121) | 26.8/56.0/61.6/73.3 | < 0.0001 |
| Waiting time | | |
| < 6 mo (318)/6-12 mo (71)/> 12 mo (23) | 55.4/67.1/52.2 | 0.18 |
| Maintenance immunosuppression | | |
| Cyclosporine A (264)/tacrolimus (119) | 52.5/70.8 | 0.003 |
| Steroid-treated rejection | | |
| Yes (125)/no (284) | 48.5/60.8 | 0.003 |
| Use of mono or polyclonal antilymphocyte antibodies | | |
| Present (55)/absent (356) | 45.4/58.8 | 0.02 |

± 2.7%. However, if the database was restricted to these 330 patients, statistical analysis of factors associated with recurrence-free survival did not change.

Among the 24 variables that were tested, 16 variables were associated with tumor-free survival by univariate analysis (Table 2): (1) period of LT (< 1991, 1991 to 1993, 1994 to 1996 and >1996; $P < 0.0001$). (2) pre-operatively: presence of cirrhosis ($P = 0.001$), etiology of liver disease ($P = 0.03$), α fetoprotein level (< 200, 200 to 2000 or > 2000; $P < 0.0001$), G-GT activity (N, N to 2N or > 2N; $P = 0.02$), the number of nodules (1, 2-3 or ⩾ 4; $P = 0.02$), maximal diameter of the largest nodule (< 3 cm, 3 to 5 cm or > 5 cm; $P < 0.0001$), the sum of the diameter of the nodules (< 3 cm, 3 to 5 cm, 5 to 10 cm or > 10 cm; $P < 0.0001$), bi-lobar location ($P = 0.01$), preoperative portal or hepatic vein obstruction ($P < 0.0001$), use of a peri-operative adjuvant treatment ($P = 0.002$), use of pre-operative transarterial chemoembolization ($P = 0.03$), (3) pathological data: tumor differentiation (well, moderate or poorly; $P = 0.01$), (4) post-operatively: the initial type of calcineurin inhibitor (cyclosporine A *vs* tacrolimus, $P = 0.003$), the use of antilymphocyte antibodies (mono- and polyclonal) ($P = 0.02$) and rejection episodes requiring steroid pulses ($P = 0.003$). It is noteworthy that calcineurin tacrolimus was more recently used in recent cases and that ATG-OKT3 was not routinely used recently.

**Table 3 Factors associated with recurrence-free survival (Multivariate analysis)**

| | Relative risk | 95% CI | *P* |
|---|---|---|---|
| Use of anti-lymphocyte antibodies | 1.8 | 1.2-2.6 | 0.005 |
| Tumor differentiation | 1.6 | 1.24-2.06 | 0.0006 |
| Maximum diameter of the largest nodule | 1.12 | 1.08-1.17 | < 0.0001 |
| Portal/hepatic vein obstruction | 1.6 | 1.01-2.72 | 0.06 |
| Number of nodules | 1.13 | 1-1.28 | 0.06 |
| Recent period of transplantation | 0.66 | 0.54-0.82 | 0.0001 |

By multivariate analysis (Table 3), 6 variables were independently associated with HCC recurrence: maximal diameter of the largest nodule ($P < 0.0001$; RR = 1.12), period of LT ($P < 0.0001$; RR = 0.66), tumor differentiation ($P < 0.0001$; RR = 1.6), the use of anti-lymphocyte antibody (ATG) or anti-CD3 antibody(OKT3) ($P = 0.005$; RR = 1.8), preoperative portal or hepatic vein thrombosis ($P = 0.06$; RR = 1.65) and the number of nodules ($P = 0.06$; RR = 1.13). Figure 3 shows the influence of the use of anti-lymphocyte antibodies, tumor size and number of nodules on tumor-free survival.

## DISCUSSION

This multicenter retrospective study reports one of the largest numbers of patients transplanted for HCC. In this work, we chose to focus on data that could be assessed preoperatively or monitored postoperatively, because such parameters are the only one of clinical relevance in predicting recurrence. On this basis, first, we show that the immunosuppressive regimen, through the use of anti-lymphocyte antibodies, have an independent negative impact on HCC recurrence. Secondly, we confirm that tumor differentiation, a feature that could be assessed preoperatively, has an independent prognostic value. Thirdly, we show that, among the more recently proposed prognostic factors, bi-lobar distribution of the tumor and

the sum of diameter of the tumors are not independent predictors or recurrence. Lastly, we obviously re-identify the prognostic value of the number and size of the nodules as well as that of tumorous macrovascular invasion.

The negative impact of anti-lymphocyte antibodies on tumor recurrence has not been reported previously. A negative role of immunosuppression on recurrence had been suggested by Pittsburgh's group by comparing the doubling time of tumor recurrence after LT or surgical resection[26]. This negative effect was also suggested in a model of tumor recurrence in immunosuppressed, liver transplanted rats[27] and in a model of liver tumor in cyclosporine A-treated rats[28]. More recently it has also been proposed in a clinical study that tumor recurrence could be favored by the intensity of immunosuppression[21]. In this study, the risk of recurrence was proportional to cyclosporine A blood levels. Anti-lymphocyte antibodies are currently the most powerful immunosuppressive agents and could further impair the anti-tumoral mechanisms required to control tumor cell spreading after LT for HCC. This was suggested by the faster recurrence observed during the first 2 years postoperatively in anti-lymphocyte antibodies-treated patients (Figure 3). Caution is therefore mandatory in using anti-lymphocyte antibodies after LT for HCC. This finding also suggests that the clinical studies carried out in the early 1990s, when the criteria for transplantation of hepatocellular carcinoma were introduced, may have been influenced by the immunosuppressive regimens adopted at that time. Since the current trend is to use less powerful immunosuppressive regimens than a decade ago, it raises the possibility that the impact of the current selection criteria on recurrence, based on tumor staging, might have changed over time and should be re-evaluated on a prospective basis[21].

In our study, tumor differentiation had a strong independent value in predicting recurrence: the poorer the differentiation, the higher (Table 3) and faster (Figure 3) the recurrence. This result is in agreement with the results of 3 other groups[15-17,19], which have found an association between tumor differentiation and the risk of recurrence. The negative impact of dedifferentiation could be explained by the relationship between poorly differentiated tumor cells and microvascular invasion, especially in large tumors[17]. In the present study, tumor differentiation was assessed on the explanted liver in order to make use of a larger set of data since the result of preoperative tumor biopsies were available in only 79 cases, and concerned mainly with well-differentiated tumors. These results suggest, however, that grading tumor differentiation could be useful in improving the selection of liver transplant candidates for HCC, provided there is a relationship between tumor grading on preoperative needle biopsy and postoperative grading on the explanted liver. This could be particularly important in the case of large tumors, exceeding 5 cm[17], for which an expansion of the classical criteria of LT has been recently proposed[19]. The presence of poorly differentiated tumor cells in preoperative biopsy samples would argue against LT in such patients, but LT could be considered in a group of patients suffering from

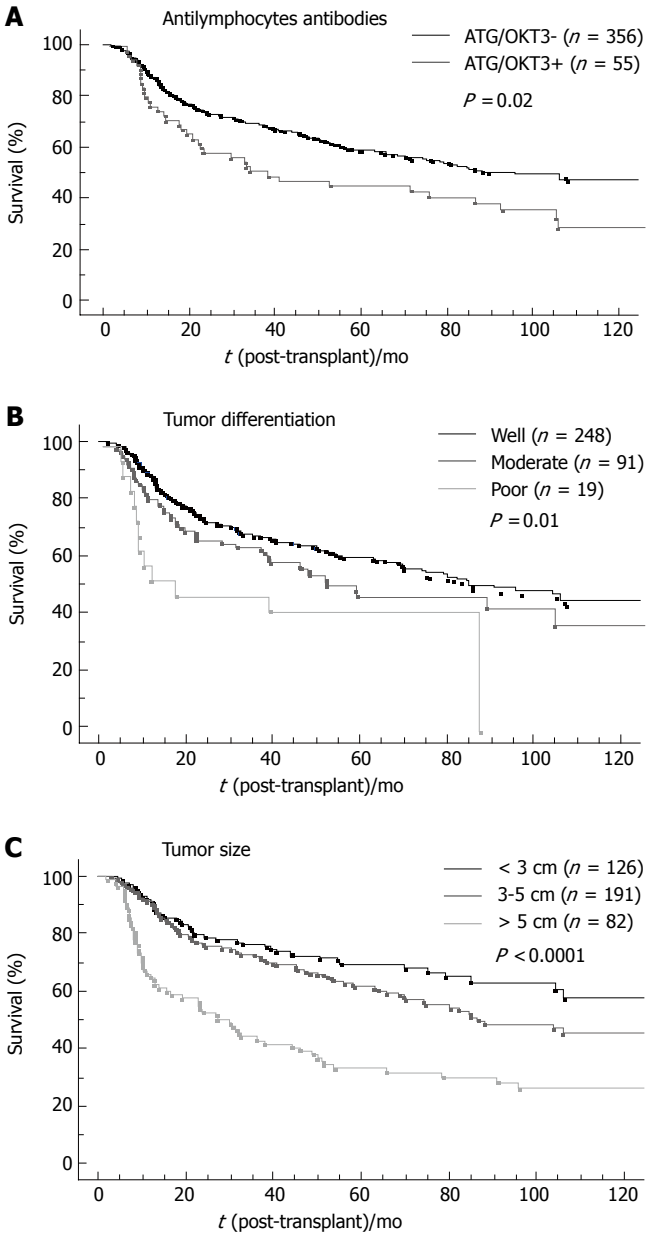


**Figure 3** Influence of immunosuppession (**A**), tumor differentiation (**B**) and size (**C**) on tumor-recurrence free survival.

well-differentiated tumors. Such an approach is supported by the results of a recent Italian study[29] reporting a 6% rate of recurrence after LT for HCC in patients selected on the basis of a preoperative tumor biopsy showing no poorly differentiated cells and including 38% patients who did not meet the Milan Criteria. Such a strategy would mean a change of current protocols, because pre-transplantation biopsies are generally not obtained as a result of the lack of an obvious benefit and the occasional report of needle-tract seeding[30,31].

The number and size of HCC have been identified a decade ago as 2 major predictors of recurrence. Based on this finding, Mazzafero *et al*[13] proposed to restrict LT indications to patients with a single nodule < 5 cm in diameter or with < 3 nodules with a maximum diameter of 3 cm to minimize post-LT recurrence. More recently, bi-lobar distribution of the tumor and the sum of diameter of the tumors, 2 factors that take into account

size and number, have been proposed as predictors of recurrence[14,18,19]. In the present series, we found that these two last factors had no independent predictive value. With respect to this point, we must point out that in our multivariate analysis, the number of tumors was the factor that had the lowest power in predicting recurrence with a *P* value at 0.06; the lack of independent value of bi-lobar distribution, which can be considered a peculiar form of multifocal HCC, is therefore not unexpected. In addition, our results indicate that the size of the largest nodule is more closely related with recurrence than the sum of diameters of all tumors, suggesting that the sum of diameters of the nodules is mainly determined by the size of the largest nodule in a majority of cases. According to the poor recurrence-free survival of patients with a maximal diameter of the largest nodule exceeding 5 cm, we actually consider that liver transplantation should be avoided in such cases.

Finally, we found that a recent period of LT was independently associated with tumor-free survival. This suggests that other factors than those identified in the present study that may have varied over time, such as maintenance immunosuppression levels[21], or improvements in surgical techniques, limiting the peri-operative risk of tumor cell spreading, might also influence recurrence.

In conclusion, we have identified the use of anti-lymphocyte antibodies as a new predictive factor of tumor recurrence after LT for HCC. We also confirmed the independent prognostic value of tumor differentiation and did not confirm the independent prognostic value of either bi-lobar distribution of the tumor or the sum of diameters of the tumors. Based on these results we propose to avoid the use of anti-lymphocyte antibodies after transplantation for HCC, a finding that also raises the issue of a change in the impact of tumor staging over time on recurrence, due to parallel changes in immunosuppression policies. We also propose to test prospectively the usefulness of a pre-transplant tumor biopsy in order to evaluate the importance of tumor differentiation in the decision-making algorithm of candidates for transplantation in HCC.

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*RAPID COMMUNICATION*

# Metastatic tumors to the stomach: Clinical and endoscopic features

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## Abstract

**AIM:** To evaluate the clinical and endoscopic patterns in a large series of patients with metastatic tumors in the stomach.

**METHODS:** A total of 64 patients with gastric metastases from solid malignant tumors were retrospectively examined between 1990 and 2005. The clinicopathological findings were reviewed along with tumor characteristics such as endoscopic pattern, location, size and origin of the primary sites.

**RESULTS:** Common indications for endoscopy were anemia, bleeding and epigastric pain. Metastases presented as solitary (62.5%) or multiple (37.5%) tumors were mainly located in the middle or upper third of stomach. The main primary metastatic tumors were from breast and lung cancer and malignant melanoma.

**CONCLUSION:** As the prognosis of cancer patients has been improving gradually, gastrointestinal (GI) metastases will be encountered more often. Endoscopic examinations should be conducted carefully in patients with malignancies, and endoscopic biopsies and information on the patient's clinical history are useful for correct diagnosis of gastric metastases.



**Key words**: Stomach; Metastatic tumors; Clinical findings; Pathology; Endoscopy

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## INTRODUCTION

The stomach is an unusual site for metastasis. There have been several published reports on metastatic lesions in the stomach. However, information on gastric duodenal metastases is generally limited to single case reports. The present study examined the clinopathological features in a large series of patients with metastatic tumors in the stomach from distant sites.

## MATERIALS AND METHODS

We conducted a chart review of patients with metastatic tumors in the stomach detected endoscopically from January 1990 to December 2003. Patients with malignant lymphoma involving the stomach, or with direct invasion from neighboring organs, were excluded from this study.

Analysis of data included indication for endoscopy, endoscopic appearance, location, primary site and time interval between the diagnosis of primary tumors and diagnosis of metastatic lesions.

## RESULTS

The present study included 64 patients, including 36 men and 28 women, with a mean age of 56 years (range 28-82 years). The common indications for endoscopy were anemia or bleeding (19 cases, 10 of which had active bleeding), dyspepsia (15 cases) and epigastric pain (14 cases). Endoscopic findings mainly included small nodules with or without central ulceration, polypoid mass and ulcers. The other findings included peptic ulcer scar and small black spots, which were metastatic tumors from malignant melanoma. Solitary metastases (40 cases, 62.5%) were more common than multiple metastases (24 cases, 37.5%). The solitary lesions were mainly located in the middle third (43%) or the upper third (45%) of the stomach, and the proportion of solitary lesions was similar to that of multiple lesions. Forty percent of the solitary lesions and 35% of the multiple lesions were located on

**Table 1 Endoscopic appearance and location of metastatic lesions *n* (%)**

| | |
|---|---|
| Small nodules | 28 (43.7) |
| Polypoid mass | 18 (28.1) |
| Ulcers | 8 (12.5) |
| Small black spots | 6 (9.3) |
| Peptic ulcer scar | 4 (6.2) |
| Lower third | 8 (12.5) |
| Middle third | 27 (42.2) |
| Upper third | 29 (45.3) |
| Solitary | 40 (62.5) |
| Multiple | 24 (37.5) |

**Table 2 Site of primary tumor *n* (%)**

| | |
|---|---|
| Breast | 21 (32.8) |
| Lung | 16 (25.0) |
| Malignant melanoma | 14 (21.9) |
| Head/Neck | 4 (6.2) |
| Uterus | 4 (6.2) |
| Colorectum | 3 (4.7) |
| Kidney | 2 (3.1) |
| Soft Tissue | 1 (1.5) |

**Table 3 Time interval between the diagnosis of primary tumors and diagnosis of metastatic lesions**

| Time (yr) | Patients *n* (%) | Primary site | | | |
|---|---|---|---|---|---|
| | | Lung | Breast | Melanoma | Other |
| -1 | 2 (3.1) | 1 | | | 1 |
| 1-2 | 33 (51.5) | 11 | 9 | 7 | 6 |
| 2-3 | 19 (29.6) | 3 | 8 | 4 | 4 |
| > 3 | 10 (15.6) | 1 | 4 | 3 | 2 |
| Total | 64 (100) | 16 | 21 | 14 | 13 |

the greater curvature (Table 1).

The diagnosis of metastatic tumors was confirmed pathologically by endoscopic biopsies in 59 out of 64 cases (92.2%). The remaining 5 cases were diagnosed during surgery for uncontrollable bleeding from the tumor. The primary sites of metastases are shown in Table 2.

The average time interval between the diagnosis of primary tumors and the diagnosis of metastatic lesions was 25.7 mo (1-40 mo), and varied in site of primary tumor (Table 3).

## DISCUSSION

The stomach is an unusual site for metastasis[1-8]. As the prognosis of cancer patients has been improving gradually, gastric metastases are encountered more frequently. In this series, lung, breast, and esophagus were the common primary metastatic sites, and malignant melanoma was associated with the highest rate of metastases. More than half of the metastatic tumors were found within a year of the diagnosis of a primary tumor. In particular, most of the gastric metastases from esophageal and lung cancer were detected within a year, since these cancers progress rapidly. On the other hand, breast cancer extended slowly, and it was sometimes more than three years before gastric metastases were diagnosed after the initial diagnosis of the primary tumor. It was noteworthy that metastatic tumors were detected before diagnosis of the primary tumors in 2 patients[8,9]. Symptoms of metastatic tumors including pain, nausea, vomiting, and signs of bleeding are nonspecific[4-9]. Endoscopy may be useful in the diagnosis and local treatment of gastric metastases, such us endotherapy that was performed successfully in 5 out of 10 patients with active gastrointestinal (GI) bleeding (endoscopic clipping in 4 cases and adrenaline injection in 1 case). In our series, more frequent endoscopic patterns included multiple nodules, bull's eye, extrinsic mass lesions, ulceration and polypoid tumor mass as previously reported[4-8,10-13].

Solitary metastases were more common than multiple metastases. The solitary lesions were mainly located in the middle third (40%) or the upper third (40%) of the stomach, and the proportion of the solitary lesions was similar to that of the multiple lesions. Forty percent of the solitary lesions and 35% of the multiple lesions were located on the greater curvature. About 90% of the metastatic lesions could be confirmed histologically by endoscopic biopsies. Tumor-negative biopsies may be mainly due to extrinsic mass rather than due to sampling error. It should be emphasized that the endoscopist should provide the pathologist with sufficient information about the patient's history to allow an accurate pathological diagnosis.

In conclusion, as the prognosis of cancer patients has been improving gradually, GI metastases will be encountered more often. Endoscopic examinations should be conducted carefully in patients with malignancies, and endoscopic biopsies. Information on the patient's clinical history is useful for the correct diagnosis of gastric metastasis.

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## COMMENTS

### Backgrounds

The stomach is an unusual site for metastasis. There have been several published reports on metastatic lesion in the stomach and information on gastric duodenal metastases is generally limited to single case reports. The present study examined the endoscopic and clinopathological features in a large series of patients with metastatic tumors in the stomach.

### Research frontiers

As the prognosis for cancer patients has been improving gradually, GI metastases will be encountered more often.

### Innovations and breakthroughs

Metastasis to stomach is rare, hence no large series has been published so far. The current study is an improvement in terms of numbers of cases. It reemphasises the same results which have been published earlier.

### Applications

Endoscopic examinations should be conducted carefully in patients with malignancies, and endoscopic biopsies. Information on the patient's clinical history is useful for correct diagnosis of gastric metastasis.

### Peer review

The current paper gives a retrospective analysis of data on metastatic tumors in stomach over a period of 15 years at author's center. Metastasis to stomach is rare, hence no large series has been published so far. Oda et all have published a series of 54 cases of metastatic tumors in stomach in 2001. The current study provides a slight improvement in terms of numbers of cases. Authors have reported in the current article that 19 cases underwent endoscopy due to bleeding and 5 cases were diagnosed with metastatic tumors at the time of surgery due to uncontrolled bleeding.

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*RAPID COMMUNICATION*

# Lactase non-persistence and milk consumption in Estonia

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## Abstract

**AIM:** To define the frequency of the C/T-13910 variant associated with lactase persistence/non-persistence trait and to analyze the milk consumption of lactase non-persistent subjects in Estonia.

**METHODS:** We genotyped 355 Estonians by polymerase chain reaction and direct sequencing. Milk consumption was analyzed by a questionnaire, specially developed to analyze milk consumption and abdominal complaints.

**RESULTS:** The frequency of the genotype of the C/C-13910 (lactase non-persistence) was found to be 24.8% in native Estonians. No other single nucleotide polymorphisms covering the region of 400 bp adjacent to the C/T-13910 variant were found. Lactase non-persistence subjects were found to consume less milk than lactase persistence subjects.

**CONCLUSION:** The frequency of lactase non-persistence defined by the C/C-13910 genotype confirms the results of the previous studies based on indirect methods of determining hypolactasia. Milk consumption of lactase non-persistence subjects is consistent with previously reported figures of adult-type hypolactasia in Estonia. However, lactase non-persistence does not prevent the intake of milk in many adults.



**Key words:** Lactase persistence; Milk; Estonia

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## INTRODUCTION

Lactase enzyme hydrolyses lactose to glucose and galactose facilitating their absorption through the gut wall. Adult-type hypolactasia (lactase non-persistence, primary lactose malabsorption) is the most common enzyme deficiency in the world[1]. Clinically, hypolactasia is the main reason of milk intolerance in adults. Individuals with hypolactasia may develop symptoms of abdominal pain, borborygmi, flatulence and diarrhea if they drink milk[1,2]. The symptoms depend on the age of the subject. It has been reported that development of symptoms depends on the quantity of lactose in the diet and individual sensitivity. Individuals with hypolactasia can tolerate moderate quantities of milk without symptoms[3-5]. Many persons but not all with this condition avoid consuming large quantities of milk.

Normally, lactase activity declines after the weaning. However, a mutation has occurred in human history that maintains lactase activity high throughout life[1]. Specifically, a single nucleotide polymorphism (SNP) C/T (rs4988234) residing 13 910 bp upstream from the initiation codon of the lactase gene (LCT) has been shown to be associated with lactase persistence trait in Asian, European and Northern African populations[6-9]. Genotype CC of the C/T-13910 variant defines hypolactasia (lactase activity under 10 U/g per protein) and CT and TT genotypes' lactase persistence (lactase activity over 10 U/g per protein)[10-12]. More recent functional studies have shown that the C/T-13910 variant is associated with the regulation of the LCT gene at transcriptional level[10,11]. It has been shown that the T-13910 allele enhances LCT promoter activity 4 times more than the C-13910 allele[13,14]. A nuclear binding factor OCT-1 has recently been found to bind to the C/T-13910 region, more efficiently to the T- allele than to the C- allele. However, it seems that some other transcription factors are also needed for complete enhancer activity[15]. The excellent correlation of the C/T-13910 variant to the lactase activity[11] has made it a robust marker for routine clinical diagnostics. By using this SNP, hypolactasia can be diagnosed more easily and accurately than by other tests where environmental factors may interfere with and have

an impact on the results[16].

The prevalence of adult-type hypolactasia differs between ethnic groups. In Finland, the prevalence of adult-type hypolactasia is found to be 18% and in Sweden 10%[6,17]. Previously, the reported prevalence of adult-type hypolactasia in Estonians varies from 23% to 32%[1,18-20] and is 57% among Russians living in Estonia[20].

The aim of this study was to figure out the prevalence of adult-type hypolactasia in Estonians by genotyping of the C/T-13910 polymorphism and to find out if there are differences in milk consumption and intolerance in individuals with different genotypes of the C/T-13910 variant. We also studied if other SNPs exist adjacent to the C/T-13910 variant.

## MATERIALS AND METHODS

### Study population

The study was carried out in Väike-Maarja, Estonia. A random sample ($n$ = 434) of the population aged 25-70 years was drawn, the sample corresponded to the age and gender structure of the general population in Estonia. An informed consent was obtained from the subjects who participated in the study. The study was approved by the Ethics Committee of the Tartu University. Three hundred and sixty-six persons participated in the study (response rate 84%) and gave a blood sample, fulfilled a questionnaire about their health and personal data, including nationality of their grandparents, symptoms of milk intolerance and milk consumption habits. There were 167 males and 199 females, and their average age was 48.9 years. A blood sample was drawn for DNA analysis from each participant. DNA was isolated using the standard procedure. In statistical analysis chi-square test was used.

### Genotyping

The DNA fragment spanning the C/T-13910 variant was amplified by polymerase chain reaction (PCR) and analyzed by direct sequencing. The total volume of PCR was 50 μL containing genomic DNA (100 ng), reverse (5' -GTCACTTTGATATGATGAGAGCA-3') and forward (5'-CCTCGTTAATACCCACTGACCTA-3') primers (20 ng each), dNTPs (200 μmol/L) and 0.5 U of Taq polymerase in a standard buffer (Dynazyme, Finnzymes, Espoo, Finland). The PCR was initiated with denaturation at 95℃ for 10 min (during which enzyme was added), then 35 cycles were carried out in following conditions: denaturation at 94℃ for 30 s, annealing at 53℃ for 30 s, extension at 72℃ for 75 s and a final extension at 72℃ for 10 min. The size of PCR products was verified by 1.5% agarose gel electrophoresis with ethidium bromide. The purification of PCR products was done by 2.5 U of shrimp alkaline phosphatase (USB) and 5 U of exonuclease I (New England Biolabs) at 37℃ for 60 min, after which enzymes were inactivated at 80℃ for 15 min. The cyclic sequencing consisted of BigDye 3.1 terminator (Applied Biosystems) according to the manufacturer's instructions with a total volume of 10 μL. Sequencing reaction was as follows: at 96℃ for 1 min, then 25 cycles at 96℃ for 10 s, at 55℃ for 5 s and at 60℃ for 4 min. To remove unincorporated nucleotides, sequencing reaction products were purified by Millipore Multiscreen plates (Millipore, USA) with Sephadex G-50 superfine sepharose (Amersham Biosciences, Sweden). The sequenced products were at first electrophoresed on an ABI 3730 DNA analyzer (Applied Biosystems) and then Sequencing Analysis 5.2 software (Applied Biosystems) was used for base calling. The obtained sequence was analyzed by Sequencher 4.1.4 software (Gene Codes, USA).

**Table 1 Frequency of the C/T-13910 variant**

| Genotype of C/T-13910 variant | Frequency (%) |
|---|---|
| CC | 24.8 |
| CT | 47.5 |
| TT | 27.7 |

**Table 2 Milk consumption and self-reported milk intolerance in Estonian adult population according to different genotypes of the C/T-13910 variant *n* (%)**

| Genotype | C/C-13910 | C/T-13910 | T/T-13910 | Total | *P* |
|---|---|---|---|---|---|
| Drinking milk (dL/d) | | | | | [a]$P < 0.05$ |
| 0 | 24 (31) | 34 (23) | 21 (24) | 79 (25) | |
| 1-2 | 38 (49) | 63 (42) | 35 (40) | 136 (43) | |
| 3-7 | 15 (19) | 40 (27) | 23 (27) | 78 (25) | |
| 8 and more | 1 (1) | 12 (8) | 8 (9) | 21 (7) | |
| Symptoms from milk | 17 (22) | 7 (5) | 5 (6) | 29 (9) | [b]$P < 0.001$ |

[a]$P < 0.05$ *vs* C/T-13910 and T/T-13910 in drinking milk; [b]$P < 0.001$ *vs* those without milk intolerance.

## RESULTS

The genotype was obtained from 355 subjects. According to their ethnic origin there were 314 Estonians (at least three grandparents being reported to be Estonians) and 41 other nationalities or mixed marriages (mostly Russians, Finns, but also Ukrainians, Swedes, Germans, Polish). In the final frequency analysis, the samples of the abovementioned 314 subjects were included. The frequencies of different C/T-13910 genotypes are presented in Table 1.

We found only two other single nucleotide mutations upstream of the C/T-13910 variant. One was C/T-variant (rs4988233), 101bp upstream of the C/T-13910 variant that was found in four subjects. The other was G/T variant 26bp upstream of the C/T-13910 variant that was found only in one subject. We did not find any polymorphisms downstream of the C/T-13910 variant.

Milk consumption and self-reported milk intolerance in the Estonian adult population with different genotypes are presented in Table 2. The subjects with the C/C-13910 genotype of adult-type hypolactasia drank less milk and reported more often milk intolerance as compared with those with the C/T-13910 or the T/T-13910 genotype.

## DISCUSSION

We found that the frequency of adult-type hypolactasia was 24.8% in Estonia. This confirmed the results of the previous studies based on indirect methods of determining hypolactasia, according to which the frequency of hypolactasia varies from 23% to 32%[1]. Our results may be more accurate because of the direct genotyping used for diagnosis in comparison with previously used lactose tolerance tests[16].

The prevalence of adult-type hypolactasia in Europe increases from west to east and from north to south, and is the lowest in Northern Europe[1]. The frequency of 25% is in agreement with this pattern. We have previously shown that the prevalence of the C/C-13910 genotype is 10% in Sweden and 18% in Finland[6,17].

The binding sites of transcription factors usually extend 100-200 bp. In the present study, we found only few other single nucleotide mutations adjacent to the C/T-13910 variant, indicating that the C/T-13910 variant is the only SNP in Estonian population affecting the binding of transcription factor at this binding site, thus confirming the previous findings that this SNP is the only SNP that affects transcription of the LCT gene in all studied populations.

As expected, we found that the consumption of milk was lower in lactase non-persistent subjects than in lactase persistent subjects, suggesting that lactase non-persistence does not prevent the intake of milk. Indeed, only 2/3 of subjects with lactase non-persistence get symptoms after consuming milk and most of the malabsorbers can consume couple of glasses of milk per day, especially during meals[3,21]. This may explain the fairly high rate of milk consumers among genotype CC-13910. Our results concerning milk consumption of CC-13910 genotype subjects are consistent with previously reported results[22,23]. Still, some individuals with hypolactasia may have severe abdominal complaints after milk consumption and it is of utmost importance to diagnose hypolactasia and give recommendations to restrict their fresh milk intake. The restriction of milk consumption is not indicated for those with hypolactasia but without any symptoms after drinking milk.

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*RAPID COMMUNICATION*

# Long term results of use of azathioprine in patients with ulcerative colitis in India

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## Abstract

**AIM:** To evaluate the role of azathioprine (AZA) in Indian patients with ulcerative colitis over longer duration of time.

**METHODS:** One hundred fifty six patients with ulcerative colitis who were treated with AZA from January 1995 to December 2003 were reviewed. The indications for its use were as follows: (1) steroid dependent and steroid refractory disease; (2) Azathioprine monotherapy for naïve patients with severe disease; and (3) combination therapy (AZA + sulfasalazine or 5-aminosalicylates) for naïve patients with severe disease. The data included patient and disease demographics, efficacy and toxicity profile of AZA. Patients with a minimum duration of 6 mo use of AZA were included in this report.

**RESULTS:** Of a total of 156 patients treated with AZA, 45 were excluded from analysis for the following reasons- (follow up less than 6 mo, *n* = 9; poor follow up, *n* = 18; adverse affects, *n* = 18). In steroid refractory/dependent group the mean number of relapses prior to and post initiation of AZA therapy were 3.28 (± 0.81) and 0.94 (± 0.29) respectively. Discontinuation of steroids could be accomplished in 12 of the 15 steroid dependent patients. The proportion of patients with sustained remission of 1, 2, 3, 4 and 5 years duration were calculated. Eighteen patients experienced adverse effects necessitating withdrawal of AZA (pancreatitis, *n* = 7; hepatitis, *n* = 3; gastrointestinal intolerance, *n* = 2; alopecia, *n* = 2; and hematological, *n* = 4) while 13 patients needed dose reduction or temporary withdrawal of the drug.

**CONCLUSION:** Azathioprine is well tolerated and has therapeutic benefits lasting as long as 4 years. Adverse effects such as pancreatitis, hepatitis, cytopenias and gastrointestinal symptoms do occur but are controlled by drug withdrawal only.



**Key words:** Ulcerative colitis; Azathioprine; Immunosuppressive

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## INTRODUCTION

Immunosuppressive drugs were initially used clinically in the treatment of leukemias and solid organ transplantation. Over time, it was learned that these drugs were capable of long term modulation of the immune system in transplant recipients, a fact that was later extrapolated to change the course of many chronic diseases with presumed immune etiopathogenesis[1]. Based on the results of many controlled studies immunosuppressive drugs have obtained an obvious place in the medical armamentarium of ulcerative colitis in which there is growing evidence of dysregulation of the mucosal immune system[2].

Out of the various immunosuppressive drugs, the purine analogues, azathioprine (AZA) and 6-mercaptopurine (6-MP) have been most extensively used and are now considered as the first line of immunosuppressive agents for the maintenance of remission in patients with ulcerative colitis. Bowen and colleagues in 1966, first gave an encouraging report on the use of AZA in ulcerative colitis, which was followed by a number of uncontrolled observations in a series of patients. Finally, controlled double blind studies were done comparing AZA with placebo and other drugs[3-11]. These studies leave little doubt about the usefulness of AZA as a steroid sparing agent for maintenance of remission in chronic active disease. Its role in severe ulcerative colitis has been documented by our group earlier[11]. However, despite impressive data from clinical trials supporting the use of AZA, many clinicians may still be reluctant to use it

because of fear of drug toxicity in the long term. Often, patients are put on this drug either too late in the course of the disease, i.e. after multiple courses of steroids or not started at all even when unable to taper steroids after many months. Recently, some studies tried to evaluate the long-term benefit and tolerability of AZA in patients with inflammatory bowel disease (IBD)[12-16]. However, the optimal duration of AZA, 6-MP therapy is unclear and has not been studied in ulcerative colitis. We report results of our experience with AZA in patients with ulcerative colitis with particular attention to clinical response and adverse events and this is probably the first such study from India.

## MATERIALS AND METHODS

### Patients and methods

The analysis included 156 patients with ulcerative colitis who were treated with AZA from January 1995 to December 2003. Some of these patients have been the subjects of our previously published reports. The indications for AZA included (1) steroid dependent/steroid refractory disease and frequent relapses, (2) AZA monotherapy in naïve patients with severe disease and (3) combination therapy [AZA + sulfasalazine or 5-aminosalicylates (5-ASA)] in naive patients with severe disease. Steroid-dependence was defined as a requirement for corticosteroids at a prednisolone-equivalent dose of more than 10 mg/d on an average for at least 6 mos to control disease activity after failure of at least one attempt to withdraw corticosteroids. Steroid-resistance was defined as evidence of persistent active IBD despite 6 wk treatment with corticosteroids at a dose $\geqslant$ 30 mg/d during the 6 preceding months. Each patient had been regularly examined as an outpatient and a record had been maintained detailing drug tolerability, course of the disease and side effects, if any. Reasons for modifying the dose or stopping the drug have been recorded. Patients had been continuing other conventional drugs like SLZ or 5-ASA derivatives (oral and/or enema preparations). Each relapse had been diagnosed with routine stool and sigmoidoscopic examination showing evidence of active colitis and had been treated with steroids. Patients with a minimum of 6 mo of AZA treatment were eligible for inclusion in the study. Patients with no follow-up for 1 year were taken as lost to follow-up.

Medical information collected included the following: (1) patient characteristics such as age, gender, age at diagnosis, personal habits such as smoking, family history of IBD and extent of disease etc.; (2) Disease activity assessed at each visit; remission being defined as absence of symptoms of active disease as rectal bleeding and normal bowel frequency and hence no need for steroids for at least 6 mo. Relapse was defined as presence of blood and mucus in stools, increased bowel frequency, active colitis on sigmoidoscopy and need for reintroduction of steroids or the need for colectomy. (3) AZA related data including age at initiation, duration of disease prior to AZA initiation, indication of its use and drug adverse effects which included fever, gastrointestinal intolerance (recurrent nausea, vomiting), pancreatitis, hepatitis, infectious complications, hematological abnormalities and malignancies. Laboratory test results were reviewed for evidence of hepatic dysfunction based on increase in alanine aminotransferase (more than two times upper limit of normal), leukopenia (less than 2500/cumm) and thrombocytopenia (< 1 lac/cumm).

**Table 1 Baseline characteristics of the patients at initiation of azathioprine treatment**

| Characteristic | |
|---|---|
| Age (yr) (mean ± SD) | 34 (34.76 ± 11.70) |
| Sex (M/F) | 56/55 |
| Disease duration (yr) (range) | 0-7.3 |
| Disease extent *n* (%) | |
| Pancolitis | 51 (45.9) |
| Proctosigmoiditis | 29 (26.2) |
| Left sided | 31 (27.9) |
| Indication of use *n* (%) | |
| Steroid dependent/refractory | 54 (48.65) |
| Azathioprine monotherapy for naïve severe disease | 16 (14.41) |
| Combination therapy for naive severe disease | 41 (36.94) |

Recommendations for laboratory monitoring included complete hemogram, alanine aminotransferase at 2, 4 and 8 wk after initiation of treatment and then every 3 mo thereafter. In some cases, noncompliance, had resulted in blood tests being performed at 4-8 mo intervals. When investigation abnormalities were found, the dose reduction or discontinuation was done depending upon the values and the side effects reported.

### Statistical analysis

Data was analyzed as mean, median and range as suitable. Where appropriate, the median values (not the mean) were used to represent the data most accurately, given the limited sample size and survival curve analysis. A paired students t-test and standard deviation were used to compare the pre and post AZA change in the corticosteroid dose. The threshold of significance was set at 0.05. The probabilities of relapse were calculated using life table analysis. The effect of various variables on time to relapse was explored by Kaplan-Meier survival curves and compared using log rank test. Non-parametric Mann-Whitney test was used for comparing the mean relapse rate per year for the neutropenic and non-neutropenic group. The study was approved by the Hospital Drug Trial Ethical Committee.

## RESULTS

A total of 156 patients treated with AZA for ulcerative colitis were enrolled. Forty-five patients were excluded from analysis because of either of the following reasons: (a) follow up period of less than 6 mo, $n$ = 9; (b) poor compliance, $n$ = 18; (c) adverse effects, $n$ = 18. The study sample, thus, had 111 patients; 56 were males. The Median age of the patients was 34 years (range 11-73). The disease was pancolonic in 40%, left sided in 32% and proctosigmoiditis in 28%. The characteristics of the study patients are summarized in Table 1. The mean follow up

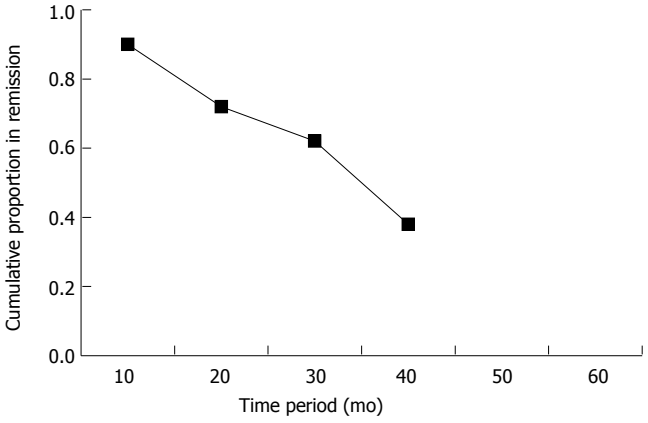


**Figure 1** Cumulative proportion of patients with ulcerative colitis in remission with azathioprine

period of patients on AZA was 18.70 ± 13.78 mo with a range of 6-73 mo.

In the steroid refractory steroid dependent and frequent relapsers group, the mean numbers of relapses prior and post initiation of AZA therapy were 3.28 (± 0.81) and 0.94 (± 0.29) respectively ($P < 0.01$). Discontinuation of steroids could be accomplished in 12 of the 15 patients who were on steroids for long time; in 1 patient the dose was decreased to half while the other two patients did not improve and underwent proctocolectomy.

Our analysis revealed that the proportion of patients in remission at 12, 24, 36, 48 and 60 mo were 0.91, 0.88, 0.76, 0.53 and 0.38 respectively. Life table analysis in our study showed that maintenance AZA treatment is effective for at least until 42 mo of treatment. Figure 1. As the number of patients on AZA beyond 42 mo is small we cannot comment on the efficacy beyond 42 mo. Various parameters, namely age, gender, disease activity at onset, extent of disease and indication of treatment were evaluated for predicting response by Kaplan Meier survival analysis. None of the above mentioned factors emerged as predictors of maintenance of remission. Thirteen patients became neutropenic (i.e. neutrophil count $\leqslant 2.5 \times 10^9$) while taking AZA. The mean number of yearly relapses for the neutropenic group was 0.2023 (± 0.3930) as compared to 0.1742 (± 0.3886) for the non-neutropenic group; the difference not being statistically significant ($P > 0.59$).

All patients necessitating drug withdrawal had experienced side effects within 6 mos of initial therapy. Hematological toxicity requiring drug withdrawal was seen in 4 patients; 3 had bicytopenia, 1 had thrombocytopenia. Dose reduction was required in 5 patients for bicytopenia and 5 for leukopenia. Persistent upper respiratory tract infection in 2 patients and pneumonia in 1 resulted in temporary discontinuation of the drug. Five patients had to undergo surgery, the indications being, unsatisfactory response resulting in chronic active disease, $n = 2$; malignancy, $n = 1$ and fulminant colitis, $n = 2$. The reasons for cessation of therapy included acute pancreatitis, $n = 7$; alanine aminotransferase, hepatotoxicity, in 3 gastrointestinal intolerance such as nausea, recurrent vomiting in 2 patients and alopecia in 2 patients.

## DISCUSSION

Ulcerative colitis treatment has undergone a significant change in the last 20 years. In particular, the use of immunomodulatory drugs like AZA and 6-MP has become widespread, as increasing familiarity and clinical trials have led to a clearer understanding of their benefits and potential risks. The results of our analysis show that AZA is efficacious and well tolerated in most patients with ulcerative colitis. Although ours is an analysis of results of all patients where AZA was used without randomization, it perhaps provides the 'best available' and the 'only' evidence of its use from our country to date. The scarcity of data on its use from our country is possibly related to fear of drug toxicity as AZA has been commonly viewed as a 'cancer drug' with high incidence of bone marrow toxicity, increased predisposition to infections, teratogenicity and malignancies, especially myeloproliferative disorders. However, it needs to be realized that this perception is derived in part from the side effect profile when the drug was originally used in preventing transplant rejection and in treatment of leukemia where significantly higher dosages were used.

Our analysis revealed that AZA is well tolerated as only 16.22% (18/111) experienced side effects requiring discontinuation of the drug. This frequency is comparable to the previously reported rate of approximately 15%. Pancreatitis, hepatitis, alopecia and gastrointestinal intolerance were the reasons for drug withdrawal. Nevertheless, all these adverse effects responded promptly to just drug withdrawal. Infections occurred in 3 cases resulting in temporary withdrawal of the drug; no patient died due to severe sepsis. In a large series by Connell *et al*[16] asymptomatic leukopenia was reported in 5% of patients and associated infections in only 1% patients with severe leukopenia. Fear of neoplasia, both intestinal and extraintestinal, has been cited as a major barrier for physicians choosing to use this drug in IBD[17,18]. We did come across one patient with rectal carcinoma who had been on AZA for 17 mo. However, an important confounding factor in the evaluation of the drugs oncogenic potential was the predisposition towards spontaneous neoplasm in ulcerative colitis. This patient had disease duration of 7 years and finally underwent surgery.

The spectrum of adverse events associated with AZA provides the most cogent argument for optimizing and individualizing drug dosing by determination of TPMT activity (phenotype or genotype) prior to initiation of therapy[19]. However, the validity of this recommendation still remains a debated topic. Nevertheless, careful regular clinical follow-up and monitoring of laboratory tests are fundamental to prevent and control adverse effects.

The effectiveness of AZA has been demonstrated by both uncontrolled and controlled trials in patients with ulcerative colitis. Our data corroborates previous clinical studies suggesting efficacy of AZA in maintenance of remission in steroid dependent and steroid refractory cases. Discontinuation of the steroids could be accomplished in 12 of the 15 patients who were taking low

doses of steroids as a maintenance dose; in one patient steroid requirement decreased and two patients who did not respond underwent proctocolectomy. There was a significant decline in the mean number of relapses pre and post initiation of AZA therapy. The most convincing controlled trial to date evaluating the maintenance benefits of AZA showed a 12 mo relapse rate of 36% for patients on AZA versus 59% for patients randomized to placebo. In a metaanalysis, AZA was found to be useful for maintenance of remission in ulcerative colitis, the pooled odd's ratio (OR) being 2.26 (95% CI: 1.27-4.01). The number needed to treat (NNT) to prevent one recurrence was 6 patients. For induction of remission, the pooled OR of the response to AZA therapy compared with placebo in active ulcerative colitis was 1.45 (95% CI: 0.68-3.08)[20]. In all these trials most patients were maintained on additional aminosalicylate therapy. However, the role of concurrent use of 5-ASA in patients effectively maintained on AZA has been questioned[21]. We reported earlier the role of AZA as monotherapy versus sulfasalazine monotherapy in the maintenance of disease remission. Although relapse rates were comparable in both groups, a trend towards earlier treatment failure was seen in patients on AZA monotherapy[22]. We have tried to shift the paradigm from 'step up' approach to combination therapy in naïve patients with severe ulcerative colitis[11]. Unfortunately the published experience with combination therapy in naïve patients is not large but we expect future studies to comment on this. It might be intuitive that early use of AZA may reset the immunostat of the body and influence the course of the disease. The results of our earlier study have demonstrated a trend in favor of combination therapy in preventing relapses but more information needs to be accrued over a longer follow-up. Life table analysis in our present study shows that AZA treatment is effective for as long as 42 mo of treatment. Although there is a gradual but variable decline in the proportion of patients who remained relapse free over the follow-up time period, it does not suggest that the effectiveness 'wears out' after this period. However, a recent retrospective study has suggested that the efficacy of AZA declines with time in patients with ulcerative colitis[20].

Over the past few years, great interest has surfaced in the potential therapeutic levels of leukopenia and neutropenia in achieving remission. Anecdotal reports have suggested that these may now be desirable end points of AZA therapy[23,24]. However, the cumulative remission percent determined by Kaplan Meier survival analysis showed no difference between neutropenic and non-neutropenic groups by log rank analysis in our study, suggesting that AZA dose titration to achieve neutropenia is not necessary for optimizing the dose.

Although a large array of therapeutic options exists, we have analyzed the therapeutic effects and toxicity of this not very new drug among patients with ulcerative colitis in the Indian population. We are of the opinion that the data is suggestive of the effectiveness of AZA in maintenance of remission and the drug, if indicated, should not be withheld for fear of toxicity.

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*RAPID COMMUNICATION*

# Safety and efficacy of hepatitis A vaccine in children with chronic liver disease

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## Abstract

**AIM:** To study the safety and efficacy of hepatitis A vaccine (HAV) in children with chronic liver disease of various etiologies.

**METHODS:** Eleven children with chronic liver disease and thirteen age- and sex-matched controls negative for HAV antibodies were vaccinated against hepatitis A after they gave their informed consent. Children with uncontrolled coagulopathy or signs of hepatic decompensation were excluded. The vaccine (Havrix: 720 ELISA units in 0.5 mL, from GlaxoSmithKline Biologicals) was given intramuscularly in the deltoid in 2 doses 6 mo apart. Children were tested for HAV antibodies one and six months after the 1st dose and one month after the 2nd dose. Total serum bilirubin, alanine aminotransferase (ALT), and aspartate aminotransferase (AST) were determined immediately before and after one month of the 1st dose of the vaccine.

**RESULTS:** Only 7 out of the 11 patients were positive for HAV antibodies after the 1st dose of the vaccine, as compared to 100% of the controls. One month after the 2nd dose, all patients tested were positive for HAV antibodies. No deterioration in liver functions of patients was noted after vaccination. No adverse events, immediate or late, were reported by the mothers after each dose of the vaccine.

**CONCLUSION:** Hepatitis A vaccine is both safe and effective in this small studied group of children with chronic liver disease. Given the high seroconversion rate, post-vaccination testing for HAV antibodies is not needed.



**Key words:** Children; Chronic liver disease; Hepatitis A; Hepatitis A vaccine

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## INTRODUCTION

Hepatitis A virus (HAV) infection is common. In general, hepatitis A is a self-limited illness with a recovery time measured in months[1]. Young children are often asymptomatic, whereas adults are more likely to be symptomatic and may present with jaundice. Although most patients have a relatively quick recovery without long-term sequelae, about 15% may have a prolonged cholestatic syndrome or a relapsing course over 6-9 mo. A smaller subset will progress to fulminant hepatic failure and death or transplantation, with estimated case fatality rates ranging from 0.01%-0.03% to 0.2% of cases among hospitalized patients[2,3]. Two major risk factors have been identified in patients who developed fulminant hepatic failure: age over 40 years and the presence of underlying chronic liver disease (CLD)[2-4].

Apart from the growing evidence that acute hepatitis A has a more severe clinical course and a higher death rate in individuals with chronic hepatitis B[4-6], and that there is an increased risk of fulminant hepatitis A in patients with chronic hepatitis C[7], patients with other chronic liver diseases also appear to be at an increased risk of developing more severe disease[4,5,8,9].

As the pool of patients with CLD grows, and acute viral hepatitis continues to occur with only a slightly reduced incidence, it is inevitable that a greater number of individuals with CLD will be at risk of developing superimposed acute and chronic hepatitis[1]. To minimize the occurrence of acute hepatitis in patients with CLD, a variety of organizations have recommended hepatitis A and B vaccination in these patients[10,11].

Given the growing evidence that hepatitis A super-infection is probably worse in at least some and probably most patients with CLD, vaccination seems reasonable.

In evaluating HAV vaccination in patients with CLD, an effective strategy should include choice of vaccines, in addition to issues related to safety and efficacy of the vaccine[1].

The issue of pre-vaccination screening for hepatitis A in children with chronic liver disease has been discussed in details in a previous report[12]. The aim of the present study was to evaluate the safety and efficacy of hepatitis A vaccine (Havrix, GlaxoSmithKline Biologicals) in a group of Egyptian children suffering from CLD of various etiologies.

## MATERIALS AND METHODS

### Patients

We included 172 children: 101 children having CLD and 71 healthy age- and sex- matched brothers, sisters and contacts of the patients as a control group. Their age ranged between 2 and 18 years, with a mean of 7.8 ± 4 years. Parental consent was obtained for medical examination, venepuncture and vaccination. The etiological diagnoses of the 101 children with CLD included autoimmune hepatitis (14 children, 14%), cholestatic disorders of infancy (16 children, 16%), hepatitis B virus (HBV) infection (4 children, 4%) and hepatitis C virus (HCV) infection (9 children, 9%). The remaining 58 children (57%) had miscellaneous causes for their CLD.

Inclusion criteria of children with CLD were willingness to participate in the study, any CLD regardless of etiology, no previous history of vaccination against hepatitis A, children of both sexes. Exclusion criteria were children with uncontrolled coagulopathy, children with decompensated liver disease (hepatic coma, massive ascites, frequent bleeders from esophageal varices, repeated spontaneous bacterial peritonitis), children with known immunological deficiency, and infants below 2 years of age (those who will not be vaccinated).

All children were tested for anti-HAV antibodies by a competitive enzyme immunoassay (ELISA) using commercially available kits (Dia. Pro. Diagnostic Bioprobes Srl., Milano, Italy).

The test results were calculated by means of a cut-off value determined by the following formula: Cut-off = (NC + PC)/3 (NC: negative control; PC: positive control), and interpreted as a ratio of cut-off value and optical density ($A_{450\ nm}$) of the sample (or Co/S) (Table 1).

Out of the 101 children with CLD, only 15 cases were negative for hepatitis A (15%) and 11 consented to be vaccinated. Among the controls, only 16 were negative for hepatitis A (22.5%) and 13 consented to receive the vaccine. Among the 11 cases, 4 were diagnosed as cholestatic disorder of infancy, 3 as HCV infection, 2 as glycogen storage disease, 1 as HBV infection and 1 as Wilson's disease.

Two doses of the vaccine (Havrix: 720 ELISA units in 0.5 mL, from GlaxoSmithKline Biologicals) given intramuscularly in the deltoid 6 mo apart. The mothers were asked to report any immediate or late reactions after the vaccine was given.

Total serum bilirubin, alanine aminotransferase (ALT), and aspartate aminotransferase (AST) were determined immediately before and one month after the 1st dose of the vaccine. Testing for anti-HAV antibodies was done at 3 occasions: one and six months after the 1st dose of the vaccine and one month after the 2nd dose of the vaccine.

**Table 1 Ratio of cut-off value and optical density ($A_{450\ nm}$) of the sample (or Co/S)**

| Co/S | Interpretation |
|---|---|
| < 0.9 | Negative |
| 0.9-1.1 | Equivocal |
| > 1.1 | Positive |

**Table 2 Rate of seroconversion in patients and controls 1 and 6 mo after 1st dose and 1 mo after 2nd dose of HA vaccine**

| Time | Patients ($n$ = 11) $n$ (%) | Controls ($n$ = 13) $n$ (%) | $P$ |
|---|---|---|---|
| 1 mo after the 1st dose of HA vaccine | | | |
| Positive | 7 (63.6) | 13 (100) | 0.07 |
| Equivocal | 2 (18.2) | 0 | |
| Negative | 2 (18.2) | 0 | |
| 6 mo after the 1st dose of HA vaccine | | | |
| Positive | 7 (63.6) | 13 (100) | 0.09 |
| Equivocal | 3 (27.3) | 0 | |
| Negative | 1 (9.1) | 0 | |
| 1 mo after the 2nd (booster) dose of HA vaccine | | | |
| Positive | 11 (100) | 13 (100) | 0.63 |
| Equivocal | 0 | 0 | |
| Negative | 0 | 0 | |

HA: Hepatitis A.

### Statistical analysis

All patients' data were collected, tabulated and processed using SPSS 12.0 for Windows XP. Categorical variables were compared using Fischer's exact test. Continuous data were expressed as mean ± SD, range and median if appropriate. Repeated measures for HAV IgG were followed in the same group using Freidman test. These variables were compared in both cases and controls by analysis of variance of repeated measures using the general linear model. Paired variables before and after the vaccine was given were compared using Wilcoxon-Signed Rank test, while unpaired data were compared by Mann-Whitney $U$ test. In all tests, $P < 0.05$ was considered statistically significant.

## RESULTS

Testing for HAV antibodies in children with CLD, revealed that only 7 out of the 11 children with CLD were positive for HAV antibodies while 100% of the controls were positive for HAV antibodies one and six months after the 1st dose of the vaccine ($P$ = 0.07 and 0.09 respectively) (Table 2). However, all the children with CLD were positive for HAV antibodies one month after the second dose (booster).

**Table 3 Comparison of the mean value of seroconversion (Co/S) between patients and controls**

| Time | Patients ($n$ = 11) | | Controls ($n$ = 13) | |
|---|---|---|---|---|
| | Range | Mean ± SD | Range | Mean ± SD |
| 1 mo after the 1st dose of HA vaccine | 0.6-1.6 | 1.1 ± 0.4 | 1.5-3.6 | 2.5 ± 0.97 |
| 6 mo after the 1st dose of HA vaccine | 0.9-4.8 | 3 ± 1.3 | 2.3-6.9 | 4.7 ± 1.8 |
| 1 mo after the 2nd (booster) dose of HA vaccine | 3.2-5.3 | 4.6 ± 0.8 | 4.2-5.7 | 4.9 ± 0.7 |
| $P$ | [b]$P$ = 0.003 | | [a]$P$ = 0.02 | |

[a]$P$ = 0.02 within controls by Friedman test; [b]$P$ = 0.003 within patients by Friedman test.

**Table 4 Comparison of liver function tests before and after vaccination**

| | Before vaccination | | After vaccination | | $P$ |
|---|---|---|---|---|---|
| | Range | Median | Range | Median | |
| Patients | | | | | |
| Total Serum Bilirubin (mg/L) | 4-210 | 7 | 3-250 | 7.5 | 9.5 |
| ALT (IU/L) | 19-163 | 42 | 19-136 | 27.5 | 0.11 |
| AST (IU/L) | 28-299 | 38 | 30-288 | 43 | 0.07 |
| Controls | | | | | |
| Total Serum Bilirubin (mg/L) | 3-6 | 5 | 1-6 | 4 | 4.8 |
| ALT (IU/L) | 9-21 | 18 | 10-27 | 17 | 0.93 |
| AST (IU/L) | 21-39 | 27 | 21-57 | 31 | 0.01[b] |

[b]$P$ < 0.01 *vs* controls before and after vaccination (Wilcoxon Signed Ranks Test). ALT: alanine aminotransferase; AST: aspartate aminotransferase.

According to the interpretation of results in Table 1, a significant rise in HAV antibodies (Co/S) was noted both in the children with CLD and in the controls over the 3 readings ($P$ = 0.003 and 0.02 respectively) (Table 3).

No changes in liver function were noted after vaccination except for an insignificant rise in AST of the children with CLD ($P$ = 0.07) and a significant rise in AST of the controls ($P$ = 0.01), although which was within the normal AST range. No adverse events, immediate or late, were reported by the mothers after each dose of the vaccine (Table 4).

Comparison of the difference in liver functions before and after vaccination revealed a significant decrease in ALT of the children with CLD after vaccination as compared to the controls (Table 5).

**Table 5 Comparison of differences in liver functions before and after vaccination between patients and controls (mean ± SD)**

| | Bilirubin (mg/L) | ALT (IU/L) | AST (IU/L) |
|---|---|---|---|
| Patients | 0.8 ± 17 | -11.3 ± 26 | 14.8 ± 23.8 |
| Controls | -0.18 ± 0.9 | 0.54 ± 7.3 | 5.1 ± 6.8 |
| $P$ | 0.92 | 0.03[a] | 0.58 |

[a]$P$ < 0.05 *vs* controls before and after vaccination (Mann-Whitney $U$ test). ALT: alanine aminotransferase; AST: aspartate aminotransferase.

## DISCUSSION

HAV vaccine is currently recommended for three groups: communities with endemic HAV infection (> 20/100 000), individuals at an increased risk of HAV exposure, and individuals with an increased risk of developing severe disease[11]. Patients with CLD are considered to be at risk of developing severe disease, and also at an increased risk of HAV exposure. Patients with serological evidence of previous hepatitis A are considered to have probable lifelong immunity. As such, the seroprevalence of HAV markers plays a central role in determining a vaccination strategy[1]. To determine the need of our children with CLD for hepatitis A vaccination, pre-vaccination screening for HAV antibodies was carried out and vaccination was planned for those negative for HAV antibodies. Because of the high seroprevalence of HAV antibodies among our patients and controls, it seems cost-effective to pretest those above 5 years of age, while pre-vaccination screening would be cost-ineffective in those below 5 years of age[12]. In general, targeted vaccination strategies have been found to be the most cost-effective[13]. Even at the lowest estimated anti-HAV be seroprevalenced rates, selective or deferred vaccination is the most cost-effective strategy in patients with CLD, if the recommended HAV vaccination schedule is to be used[14]. The prevalence of anti-HAV must decrease to below 12% before universal vaccination becomes a more cost-effective strategy[1].

Among our studied children with CLD and controls, no immediate or late adverse effects after vaccination were noticed by the mothers. Liver functions were assessed immediately before and one month after the 1st dose of the vaccine. No significant changes were noted in the children with CLD apart from a significant decrease in ALT as assessed by comparing the mean difference (Table 5). Patients with CLD can receive HAV vaccination with little worry about the vaccine-related adverse effects. Since 1980s, hepatitis A vaccine has been studied extensively in individuals of all ages, and has been known to be safe[15-18]. No significant adverse events have been found to be associated with the use of hepatitis A vaccine in patients with CLD[19].

Only 7 out of 11 children with CLD were positive for HAV antibodies while 100% of the controls were positive for HAV antibodies one and six months after the 1st dose of the vaccine. However, one month after the booster dose all the children with CLD (100%) were positive for HAV antibodies. A rise in antibody level as calculated by Co/S was noted in both the children with CLD and the controls over the 3 readings. Similar seroconversion rates have been reported in children with chronic hepatitis B and C[20] and other chronic liver diseases[18]. A booster dose could induce seroprotection in all children[20]. Keeffe and co-workers[19] reported that the mean geometric titer of anti-HAV is significantly lower in patients with CLD than in healthy controls. However, seroconversion to an immune state has been found in approximately 95% of patients with CLD and 98% of healthy controls (seronegative patients can achieve anti-HAV titers of ⩾ 30 kIU/L as

previously defined). A smaller trial in Chinese patients also noted that the seroconversion rates are similar in 65 chronic HBV infection patients given HAV vaccine and healthy controls, although the anti-HAV titer is lower in patients with CLD[21]. Seroconversion in all patients after the 1st dose of the vaccine has been reported by Giacchino *et al*[22] in a group of children with a variety of metabolic liver diseases.

Although studies have demonstrated the immu-nogenicity of HAV vaccine in mild and moderate CLD, the efficacy of HAV vaccines in advanced or decompensated liver disease has not been thoroughly investigated. Smallwood *et al*[23] found that seroconversion can be achieved in only 48.6% of patients with end-stage liver disease awaiting liver transplantation following a normal HAV vaccination schedule. Patients with end-stage liver disease with uncontrolled coagulopathy and signs of hepatic decompensation were not included in our study.

In conclusion, hepatitis A vaccine is indicated for patients with CLD who are negative for HAV antibodies, and should be provided as early as possible in the course of CLD, as the immunogenicity is poor in advanced liver disease and after liver transplantation. In view of the high seroconversion rate for patients with mild to moderate disease, post-vaccination testing is not needed. The vaccine is both safe and effective in children with mild to moderate liver disease.

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*RAPID COMMUNICATION*

# Ultrasound guided percutaneous treatment for splenic abscesses: The significance in treatment of critically ill patients

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## Abstract

**AIM:** To analyze the results of ultrasound guided percutaneous needle aspiration (PNA) and percutaneous catheter drainage (PCD) in the treatment of splenic abscess.

**METHODS:** Thirty-six patients (14 females and 22 males, with an average age of 54.1 ± 14.1 years) with splenic abscess were treated with ultrasound guided PNA and/or PCD. Patients with splenic abscess < 50 mm in diameter were initially treated by PNA and those with abscess ⩾ 50 mm and bilocular abscesses were initially treated by an 8-French catheter drainage. The clinical characteristics, underlying diseases, organism spectra, therapeutic methods, and mortality rates were analyzed.

**RESULTS:** Twenty-seven patients had unilocular and 9 bilocular abscess. PNA was performed in 19 patients (52.8%), and 8 of them (42.1%) required PCD because of recurrence of abscess. In 17 patients (47.2%), PCD was performed initially. PCD was performed twice in six patients and three times in two. PNA was definitive treatment for 10 and PCD for 21 patients. One patient with PCD was referred for splenectomy, with successful outcome. In all 4 deceased patients, malignancy was the underlying condition. Twenty-one patients (58.3%) underwent 33 surgical interventions on abdomen before treatment. Cultures were positive in 30 patients (83.3%). Gram-negative bacillus predominated (46.7%). There were no complications related to the procedure.

**CONCLUSION:** Percutaneous treatmnet of splenic abscess is an effective alternative to surgery, allowing preservation of the spleen. This treatment is especially indicative for the patients in critical condition postoperatively. We recommend PNA as primary treatment for splenic abscesses < 50 mm, and PCD for those ⩾ 50 mm in diameter and for bilocular abscesses.



**Key words:** Interventional ultrasound; Needle aspiration; Catheter drainage; Spleen preservation

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## INTRODUCTION

Splenic abscess is an uncommon entity with a reported frequency in autopsy series between 0.14% and 0.7%, and with high mortality rates because of delayed detection and treatment[1-3]. It often presents with either vague or nonspecific signs, thus making clinical diagnosis difficult. Current imaging modalities allow early diagnosis. Furthermore, splenic abscess often occurs in the patients with underlying diseases[1,4-6].

Many surgeons report that splenectomy is the best way for treatment of splenic abscess[2,4,6,7]. Although antibiotics and splenectomy are traditionally considered the treatment of choice, there is wide acceptance of important immunologic support of the spleen[8-10]. Recently, spleen-preserving management using medical treatment and percutaneous imaging-guided drainage were proved to be efficient methods for the treatment of splenic abscess[8-13]. Our study was undertaken to determine the current role of percutaneous ultrasound guided treatment of splenic abscesses as an alternative to surgery, with intention to analyze the role of needle aspiration and continous catheter drainage especially in patients in critical condition postoperatively.

## MATERIALS AND METHODS

### Subjects

From January 1, 1999 to December 31, 2004, all consecutive patients who were admitted to our hospital and subjected to percutaneous treatment of splenic abscess, were included in this study. All patients gave written informed consent. This study was approved by the local ethics committee.

### *Diagnosis and treatment*

The diagnosis of splenic abscess was made on the basis of clinical and imaging findings with ultrasound or computed tomography. Splenic abscess was defined as an intrasplenic pus collection. Case definition required patients to have one or more defects on spleen ultrasound examination or computed tomography along with the identification of pus. The demographic, clinical, and laboratory characteristics, underlying diseases, organism spectra, abscess number and size, and mortality rates were analyzed.

The size of abscess was recorded as the largest diameter in either solitary or bilocular abscess. The decision to select the method for percutaneous treatment between percutaneous needle aspiration (PNA) or percutaneous catheter drainage (PCD) was made on the basis of the longest diameter of abscess and number of abscesses. The patients with splenic abscess shorter than 50 mm in maximal diameter were initially treated with PNA. The reason for this is that the end of an 8-French pigtail catheter is a ring with 25 mm diameter and its insertion in a cavity shorter than its double length (50 mm in its longest diameter) would be difficult. In the case of the recurrence of abscess collection, an 8-French catheter for continous drainage was introduced. Patients with splenic abscess equal or greater than 50 mm in diameter were initially treated with PCD. All bilocular splenic abscess were treated with catheter regardless of the maximal diameter of the abscess. All surviving patients were followed up clinically for six months after percutaneous treatment. Patients' response to treatment in terms of clinical symptoms and laboratory test results were monitored. The criteria for successful treatment were if the infection had subsided clinically and if there had been sonographic evidence of abscess resolution (Figure 1). Patients were followed up in the out-patient clinic biweekly after they discharged from the hospital. Clinical examination, white blood cell count and neutrophillia, CRP and abdominal ultrasonography were performed at each follow-up visit.

**Antibiotics policy:** At present, all patients were treated with intravenous ampicillin 500 mg qid, cefuroxime 750 mg tid, and metronidazole 500 mg tid. The antibiotics were adjusted according to the results of the culture and sensitivity test of pus aspirated at the time of the drainage procedure. Antibiotics adjustment was done immediately when the sensitivity test was available. Patients with negative culture results were continuously treated with combination of ampicillin, cefuroxime, and metronidasole. Intravenous antibiotics were continued for 10 d. If antibiotic therapy was changed according to the results of sensitivity test, new antibiotics were administered for 10 d. Patients were discharged earlier with a percutaneous intravenous catheter inserted for a completion of therapy if fever had subsided for at least 48 h. The patients were then put on appropriate oral antibiotics for a total treatment period of 6 wk.

**Intervention:** All percutaneous interventions were performed under ultrasound guidance with a General Electric Logiq 400 machine and 3.5 MHz curvilinear transducer (General Electric, Chicago, USA). A free-hand technique using an 18 Gauge disposable trocar needle (Boston Scientific, Boston, USA) of varying lengths (10-16 cm) was employed for puncturing of abscesses. A sample of pus was routinely taken and sent for microbiological analyses, including microscopy, culture and antibiotics sensitivity tests.

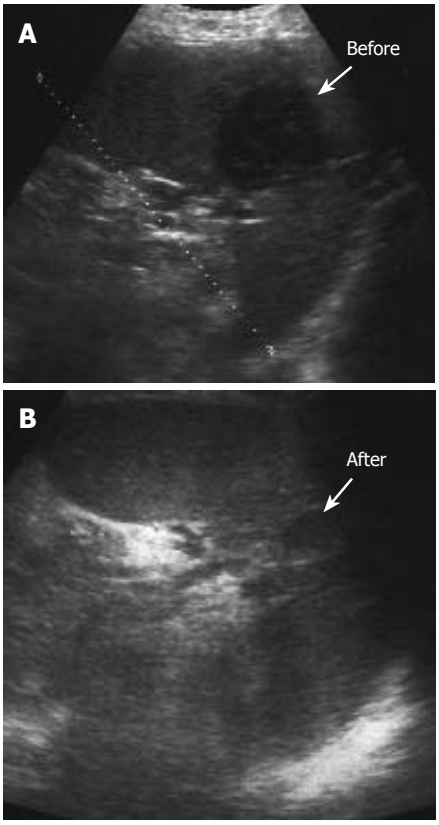


**Figure 1** Ultrasonographic appearance of splenic abscess before and after percutaneous treatment.

**Percutaneous drainage:** The applied drainage technique was the trocar method using an 8F multisidehole pigtail catheter (Boston Scientific, Boston, USA). by this method, reduction of catheter manipulations avoids damage of splenic tissues. Careful localization of the lesion and proper selection of the entry site were required. An optimal route of access must transverse the least possible amount of splenic parenchyma and avoid bowel and pleura.

Aspiration was then performed with the catheter until no more pus could be removed. The catheter was then secured to the skin for continuous external drainage and the patient was sent back to the ward. If after 24 h there was no catheter output, a follow-up sonography was performed. If the abscess cavity was absent, the catheter was removed. If a residual cavity was present, the catheter was flushed with saline and content aspirated until such time that the aspirated content is clear. Residual abscesses were treated with catheter repositioning and aspiration or introducing the new catheter. Subsequent sonography was performed three days later and the catheter was removed if the catheter remained unproductive. Otherwise, the catheter was left *in situ* until it stopped producing any content. Sonography was repeated every 3 d until the cavity either disappeared or showed significant reduction, along with clinical recovery.

**Needle aspiration:** Complete evacuation of pus from each cavity was attempted with 18G disposable trocar

**Table 1 Underlying pathology in patients with splenic abscess**

| Causes | *n* | % |
|---|---|---|
| Pancreatitis | 8 | 22 |
| Trauma | 8 | 22 |
| Stomach surgery | 8 | 22 |
| Deviscerated hydatid cyst | 2 | 6 |
| Nephrectomia | 2 | 6 |
| Liver cirrhosis | 1 | 3 |
| Spleen infarction | 1 | 3 |
| Unknown | 6 | 16 |

needle (Boston Scientific). The needle tip was inserted into the abscess for a complete pus removal. Sonography was performed every 3 d and the size of the abscess was recorded. If there was either no clinical improvement or no reduction in size of the abscess cavity, catheter for continuous drainage was introduced.

### *Statistical analysis*

Statistical analysis was done using statistical software Med Calc v. 8.0. Quantitative variables were compared using two-sample *t* test for independent samples, whereas categorical variables were analyzed by Fischer exact test. Statistical level of $P < 0.05$ was considered as significant for all performed tests.

## RESULTS

Splenic abscess was diagnosed in 41 patients during the study period. Five of them, who died within 6 wk after percutaneous treatment because of underlying diseases, were excluded. Out of the remaining 36 patients with splenic abscess, 14 were females and 22 males, with a mean (± SD) age of 54.1 ± 14.1 (range 7-69) years.

Prior to hospital admission, patients were symptomatic for a mean 16.83 ± 18.20 (range 1-72) d. The symptoms were fever in 33 patients (91.7%), left upper quadrant abdominal pain in 23 patients (63.9%), diffuse abdominal pain in 7 patients (19.4%), and shortness of breath in 6 patients (16.7%). The physical examination revealed splenomegaly in 24 patients (60%), left upper quadrant abdominal tenderness in 21 patients (58.3%), and generalized abdominal tenderness in 8 patients (22.2%). Leucocytosis over 10 000/mm$^3$ was revealed in 32 patients (88.8%), and CRP over 10 (normal up to 3.3) mg/L in 29 patients (55.6%). Among 36 patients, a potential underlying disease for splenic abscess was found in 30 (83.3%). Acute hemorrhagic necrotic pancreatitis, trauma and malignancy were the leading diseases (8 patients affected by each, covering 22.2%) (Table 1). Statistical analysis revealed that the presence of underlying disease did not correlate with the size and number of abscess, age, gender, and species of microorganism.

Frank pus was obtained from the abscesses in all 36 patients. A microbial pathogen was isolated in 30 patients (83.3%). Blood culture was positive in 11 of 30 patients (36.6%) and abscess culture in 23 of 30 patients (83.3%). All patients with both positive blood and abscess cultures had identical pathogens. The microorganisms of positive culture were predominantly gram negative (14 cases, 46.7%), with *K. pneumoniae* as the leading pathogen (20%). Gram positive microorganisms were isolated in 10 patients (33.3%). More than one organism was isolated in 16.7% of patients with positive culture (Table 2). All patients received appropriate antibiotic therapy. Twelve patients after pus culture and sensitivity test were obtained, had antibiotics changed, including imipenem ($n$ = 5), claritromycine ($n$ = 3), ciprofloksacin ($n$ = 2), and gentamycin ($n$ = 2). The mean hospital stay (± SD) was 20.1 ± 14 d. The shortest hospital stay was in the group with PNA (Table 3). The mean hospital stay (± SD) was significantly shorter ($P < 0.001$) in 19 patients of groups PNA + PNA/PCD (12.4 ± 7.7 d) than in 17 patients of PCD groups (28.7 ± 14.7 d).

**Table 2 Bacterial isolates in patients with splenic abscess**

| Microorganism | *n* | Isolated from | | |
|---|---|---|---|---|
| | | Pus | Blood | Pus and blood |
| **Aerobes** | | | | |
| **Gram positive** | | | | |
| *Staphyloccocus aureus (SA)* | 6 | 3 | 1 | 2 |
| *Streptoccocus viridans (SV)* | 2 | 2 | 0 | 0 |
| *Enterococcus species* | 2 | 2 | 0 | 0 |
| **Gram negative** | | | | |
| *Klebsiella pneumoniae (KP)* | 6 | 4 | 1 | 1 |
| *Pseudomonas species* | 5 | 1 | 1 | 3 |
| *Escherichia coli (EC)* | 3 | 2 | 1 | 0 |
| **Gram positive + Gram negative** | | | | |
| *SA + EC* | 3 | 2 | 1 | 0 |
| *SV + KP* | 2 | 2 | 0 | 0 |
| **Anaerobes** | | | | |
| *Bacteroides fragilis* | 1 | 1 | 0 | 0 |
| **Sterile culture** | 6 | 0 | 0 | 0 |
| **Total** | 36 | 19 | 5 | 6 |

The patients had either unilocular (27 cases) or bilocular (9 cases) splenic abscess. Sizes of abscess ranged from 26 to 97 mm, with a mean of 58.9 ± 8.6 mm. Size of abscess ranged from 26 to 49 mm, with a mean 40.2 ± 5.5 mm, in the PNA group and from 57 to 97 mm, with a mean 77.5 ± 11.7 mm, in the PCD group. Twenty-one patients (58.3%) underwent 33 surgical interventions on abdomen before percutaneous treatment. Six patients had two and three patients had three surgical interventions before percutaneous treatment was applied (Table 3).

Needle aspiration was performed in 19 patients (52.8%). In 10 patients, needle aspiration was a definitive and successful treatment, and one patient died 55 d after PNA and repeated surgery due to recurrence of stomach malignancy. Eight of 19 patients (42.1%) had recurrence of abscess collection and required continuous percutaneous drainage and instilled antibiotic therapy through the catheter. In 17 patients (47.2%) with abscess collection greater than 50 mm in diameter or bilocular abscess, percutaneous catheter drainage was initially performed.

Six patients with persistent abscess cavity or poor drainage underwent percutaneous catheter drainage twice

**Table 3 Type of treatment, hospital stay and number of surgeries prior to percutaneous treatment in patients with splenic abscess**

| Treatment method | *n* | Hospital stay (d) | Number of surgeries before percutaneous treatment | | |
|---|---|---|---|---|---|
| | | | One | Two | Three |
| PNA | 11 | 9.4 ± 5.3 | 2 | 2 | 0 |
| PNA/PCD | 8 | 16.6 ± 8.7 | 4 | 0 | 0 |
| PCD x 1 | 9 | 23.9 ± 17 | 5 | 1 | 1 |
| PCD x 2 | 6 | 32.5 ± 10 | 1 | 2 | 1 |
| PCD > 2 | 2 | 39.1 ± 7.1 | 0 | 1 | 1 |
| Total | 36 | 20.1 ± 14 | 12 | 6 | 3 |

PNA: Percutaneous needle aspiration; PCD: Percutaneous catheter drainage.

**Table 4 Type of treatment and outcome in patients with splenic abscess**

| Treatment method | *n* | Outcome of treatment | |
|---|---|---|---|
| | | Success | Death |
| PNA | 11 | 10 | 1 |
| PNA/PCD | 8 | 7 | 1 |
| PCD x 1 | 9 | 8[1] | 1 |
| PCD x 2 | 6 | 5 | 1 |
| PCD > 2 | 2 | 2 | 0 |
| Total | 36 | 32 | 4 |

PNA: Percutaneous needle aspiration; PCD: Percutaneous catheter drainage.
[1]One patient underwent surgery after PCD.

and two patients for three times. PCD was a definitive and successful treatmnet for 21 patients, seven received it after PNA and PCD, five after two PCD and two after three PCD. One patient with percutaneous drainage was eventually referred for splenectomy, with a favorable outcome (Table 4). There was no statistically significant difference in the success of percutaneous treatment between unilocular and bilocular splenic abscesses ($P > 0.95$). As for the imaging techniques applied for splenic abscess, all patients underwent chest X-ray. Twenty-two patients (61.1%) had left lower lung infiltration (6 patients, 16.7%) or left pleural effusion (16 patients, 44.4%). The abnormalities revealed by chest X-ray disappeared after successful treatment of the splenic abscess. The mortality rate was 11.1% (Table 4). All 4 deceased patients died of predisposing factors such as malignancy rather than of splenic abscess itself.

## DISCUSSION

In the past, antibiotic therapy and splenectomy were cosidered the treatments of choice for splenic abscess[2,14]. Because of the increased number of immunocompromised patients within the general population, the incidence of splenic abscesses has increased over the last decade[15]. The spleen is important for proper immunologic function, and splenectomy carries an increased morbidity rate with the danger of postsplenectomic infections. Current therapeutic strategies established spleen-preserving treatment in cases of trauma and benign lesions. Thus, percutaneous drainage of splenic abscesses is used instead of surgical treatment with good results[9-13], provided that certain conditions were present. Contrary to these opinions, some new studies recommended splenectomy as the treatment of choice for splenic abscesses and splenic pseudocysts[2,16].

Our series revealed that gram negative bacilli were the leading pathogens causing splenic abscess. *K. pneumoniae* was the most frequently found pathogen. Some previous studies had similar[1], but other reports revealed different results (mailnly *Streptococcus* and *Staphylococcus* species)[4,6,7].

Percutaneous treatment is the most convenient when the abscess collection is unilocular or bilocular with a discrete wall and no internal septations, and when its content is liquid enough to be drained. As for the location, better results are obtained at the periphery and at the middle or lower pole of the spleen. The number of collections is another important factor. If there are more than two collections, surgical treatment should be considered[9]. All patients in our study had unilocoluar or bilocular abscess located at the middle or lower pole of the spleen.

Percutaneous treatment is indicated especially when patients are in critical condition postoperatively or when the risks of general anesthesia, surgical drainage, or splenectomy are substantial. Some authors report that percutaneous drainage or needle aspiration should be considered as the first line of treatment, reserving splenectomy for exceptional cases only[11-13]. More than half of patients enrolled in our study had surgical intervention in abdomen or retroperitoneum (9 after malignancy) earlier and percutaneous drainage or needle aspiration was the only available treatment for those patients. Percutaneous treatment was a successful and definitive treatment in almost all of our patients. All deceased patients died of underlying deseases of malignancies. The advantages of percutaneous treatment were compared with surgery, including external drainage without significant risks of intra-abdominal spillage and avoidance of perioperative complications, less time and cost, better acceptance by the patient, and easier nursing care. Also, immunologic disfunction after splenectomy is avoided, which is a desirable outcome, especially in young patients[9, 11-13].

We recommend needle aspiration, primarily in the treatment of splenic abscess with the maximal diameter less than 50 mm, since our experience with those cases showed that needle aspiration was sufficient to solve these splenic abscesses in more than 50% cases. Also, the method is much simpler and less aggressive than percutaneous catheter drainage, allowing shorter hospital stay and lower costs. If needle aspiration was insufficient, we subsequently used the drainage technique by the trocar method using pigtail an 8-French catheters. In patients with splenic abscesses longer or equal to 50 mm in maximal diameter, we initially introduced an 8-French catheter. As for the bilocular abscesses, we always introduced a single 8-French catheter to take the advantage of the hydrostatic pressure gradient created when one of the cavities was drained, thereby causing sometimes spontaneous drainage of other cavities. If persistence of clinical signs was combined with poor drainage, the correct location of the catheter should be verified. The possibility

of placing an additional catheter should always be explored if the abscess continues to exist. Complications associated with percutaneous drainage of splenic abscesses include hemorrhage, pleural empyema, pneumothorax and fistula formation[9,17]. These complications are rarely encountered if the exclusion criteria were carefully followed. In our study, all splenic abscesses were successfully drained without any complications.

In conclusion, ultrasound guided percutaneous treatment of splenic abscess is a safe and effective alternative to surgery, allowing preservation of the spleen, avoiding perioperative complications, ensuring better compliance and easier nursing care. This treatment is indicated especially when patients are in critical condition postoperatively or when the risks of general anesthesia, surgical drainage, or splenectomy are substantial. We recommend needle aspiration primarily in the treatment of splenic abscesses smaller than 50 mm and continuous percutaneous drainage for those larger than 50 mm in the longest diameter as well as for the bilocular abscesses.

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*RAPID COMMUNICATION*

# Effects of huoxiangzhengqi liquid on enteric mucosal immune responses in mice with Bacillus dysenteriae and Salmonella typhimurium induced diarrhea

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## Abstract

**AIM:** To explore effects of huoxiangzhengqi liquid (HXZQ) on enteric mucosal immune responses in mice with Bacillus dysenteriae and Salmonella typhimurium induced diarrhea (BSD).

**METHODS:** BSD was induced in Balb/c mice by oral administration with Bacillus dysenteriae and Salmonella typhimurium. HXZQ was administrated from the day of diarrhea induction at dosages of 5.21 g/kg and 0.52 g/kg, respectively. The onset of diarrhea and lasting time were recorded. Peyer's patches and peripheral lymphocytes were prepared for flow cytometry, and levels of TNF-$\alpha$ in peripheral blood and enteric tissue homogenates were determined with ELISA. Student's *t* test was employed for statistics.

**RESULTS:** Mice in BSD group started showing continuous diarrhea on the day of induction until the fourth day when they were sacrificed. Diarrhea in the mice of HXZQ high and low dose groups lasted for 36 and 54 h, respectively. There were more $CD4^+$ and $CD8^+$ cells in peripheral blood, fewer $CD4^+$ cells in Peyer's patches in BSD mice compared to normal mice. Fewer $CD4^+$ and $CD8^+$ cells was shown in the mice in HXZQ high group compared to BSD mice. In Peyer's patch, there were more $CD8^+$ cells in mice in HXZQ high and low dose groups and more $CD4^+$ in mice in HXZQ high group. Higher levels of TNF-$\alpha$ in peripheral blood and intestinal tissue homogenates in BSD group were observed. Mice in HXZQ high group showed decreased levels of TNF-$\alpha$ in peripheral blood and enteric tissue homogenates.

**CONCLUSION:** The immune regulation of $CD4^+$ and $CD8^+$ cells in Peyer's patch and suppression of TNF-$\alpha$ levels in enteric homogenates may partially explain the effect of HXZQ on improvement of BSD.

**Key words:** Diarrhea; Peyer's patches; TNF-$\alpha$; Huoxiangzhengqi liquid

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## INTRODUCTION

Huoxiangzhengqi liquid (HXZQ), consisting of Agastache rugosa, Ammi majus, areca peel, hoelen, beefsteak plant leaf, aurantii nobilis pericarpium, atractylodes macrocephala, magnolia bark, pinelliae tuber, platycodon root and Glycyrrhiza uralensis, has been used in China for thousands of years for the treatment of various diarrhea[1]. It is one of earliest marketed herbal medicines and is produced by China Tongrentang Pharmaceuticals Corp(GMP certificated), Beijing. Pharmacological research has shown that HXZQ has the activities of improving gastrointestinal dysfunction, anti-inflammation and immune regulation[1-3].

Enteric mucosal responses play an important role in anti-inflammation of the digestive tract[4,5]. However, the effects of HXZQ on enteric mucosal immune responses are largely unknown.

Dysenteriae and Salmonella typhimurium induced diarrhea (BSD) is one of widely used animal models for

human infectious diarrhea study[6,7]. In the present study, we investigated the effects of huoxiangzhengqi liquid on enteric mucosal immune responses in mice with BSD.

## MATERIALS AND METHODS

### Animals

Forty Balb/c mice, female and male in equal number, 8-10 wk old, were purchased from the Research Institute of Experimental Animals, Chinese Academy of Medical Science, Beijing. Mice were randomly divided into four groups with 10 in each group: control group (Control), BSD group, Huoxiangzhengqi liquid treated BSD groups at high dosage and low dosage (HXZQ high, HXZQ low). Mice were housed in a temperature-, humidity- and light-controlled environment with free access to rodent chow and water. The light-dark cycle was 12 h:12 h with the light phase from 06:00 am to 18:00 pm.

### Induction of BSD

BSD were induced by oral administration with sodium ampicillin (Northern China Pharmaceutical Limited Company, Guangzhou, China) at a dosage of 20 g/kg body weight, and 8 h later, were orally administered with Bacillus dysenteriae and Salmonella typhimurium (National Institute for Control of Drugs and Biological Products, Beijing, China) at the proportion of 1:1 with 0.2 mL/mouse (concentration at 1010 CFU/mL). Mice in BSD, HXZQ high, and HXZQ low groups were induced with BSD, and mice in Control group were treated with equal volume physiological saline. The onset of diarrhea and its lasting time were recorded as previously reported[6,7].

### Treatment of BSD with HXZQ

The administrations of HXZQ started from the day of induction of BSD, once a day for three days. The dosage of HXZQ was determined according to our *in vitro* tests, and the doses at 5.21 g (crude herbal drug)/kg body weight and 0.52 g/kg were used for the mice in HXZQ high and HXZQ low groups, respectively, in a volume of 10 mL/kg. The mice in control group and BSD group were administered with the same volume of saline.

### Preparation of Peyer's patch cell suspension

The mice were sacrificed on the fourth day after the treatment. Peyer's patch cells were separately harvested according to Lyscom's method[8]. Briefly, the tissues were mechanically dissociated into phosphate buffered saline (PBS). Lymphocytes were isolated by density centrifugation on Ficoll Hypaque (Lymphoprep, Nycomed, Olso, Norway). The lymphocytes were collected by repeated centrifugation at 463 rpm for 5 min after the tissues were washed twice with PBS and then suspended in PBS at a density of $1 \times 10^6$ cells/mL for flow cytometry.

### Preparation of peripheral lymphocytes

Whole blood samples were collected before the mice were sacrificed, and suspended in tubes containing 25 μL heparin (5000 IU/mL). Erythrocytes were lysed in a buffer containing 0.84% $NH_4Cl$ at pH 7.4 for 3-5 min. The remaining cells were then washed in saline twice, and resuspended for flow cytometry.

### Preparation of enteric tissue homogenates

Colons were washed and put into homogenate medium (dehydrated alcohol and saline at a ratio of 1:4) at 300 g/L. After mixed sufficiently, they were placed at 4℃ for 2 h, and centrifuged at 3500 rpm for 15 min. The supernatant was collected, and stored at 4℃ for ELISA.

### Flow cytometry for $CD4^+$ and $CD8^+$

Flow cytometry was performed on freshly isolated plasma lymphocytes and Peyer's patch cell suspension. Immunofluorescence staining of cell surface markers was performed using R-Phycoerythrin (PE)-conjugated OX-38 (BD Biosciences Pharmingen) for $CD4^+$ staining, Peridinin Chlorophyll-a Protein (PerCP)-conjugated OX-8 (BD Biosciences Pharmingen) for $CD8^+$ staining. Each sample of 0.1 mL peripheral lymphocytes or Peyer's patch cell suspension was incubated for 30 min at 4℃ in the dark, with a solution consisting of 6 μL PBS 0.01 mol/L and an appropriate concentration of antibodies. After washing in saline, the cells were resuspended and fixed. Flow cytometric analysis was performed using an FACS Caliburflow cytometer utilizing CELLQuest software (Becton Dickinson, San Jose, CA, USA). Analysis was carried out on $1 \times 10^4$ cells for each sample.

### ELISA for TNF-α

Detection of TNF-α in peripheral blood and intestinal tissue homogenates was conducted with ELISA kit (Cat. No.88-7340 eBioscience, Inc) according to instructions of the manuals.

### Statistical analysis

Data were expressed as mean ± SD. ANOVA was used to determine significance in the data set. Student-Newman-Keuls test was employed for variables between both groups when equal variances were assumed and Dunnetts's *t* test when equal variances not assumed. $P < 0.05$ was taken as significant.

## RESULTS

### Incidence and lasting time of diarrhea

After induction of BSD, all mice showed diarrhea within 6 h on the day of induction. The diarrhea in the mice in BSD group lasted until the fourth day when the mice were sacrificed. Diarrhea lasting time of the mice in HXZQ high and HXZQ low groups were 36 and 54 h, respectively.

### Changes of $CD4^+$ and $CD8^+$ cells in peripheral blood and Peyer's patch

Figure 1 shows that there were more $CD4^+$ and $CD8^+$ cells in BSD mice in periphery ($P < 0.05$ *vs* control group), less $CD4^+$ and more $CD8^+$ cells in Peyer's patch ($P < 0.05$ *vs* control group). Less $CD4^+$ and $CD8^+$ cells showed in the mice in HXZQ high group ($P < 0.05$ *vs* BSD group), and less $CD8^+$ cells in HXZQ low group ($P < 0.05$ *vs* BSD

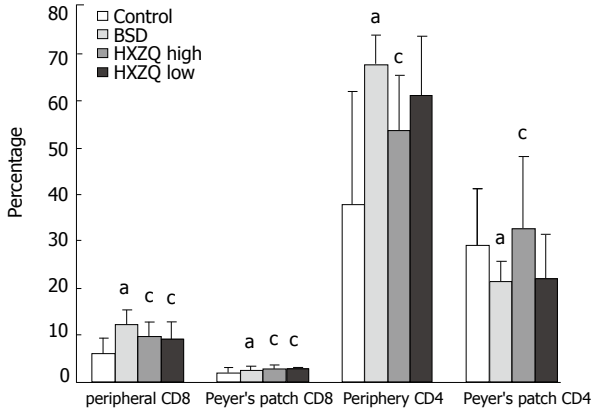


**Figure 1** $CD4^+$ and $CD8^+$ cells in peripheral blood and Peyer's patch were analyzed with flow cytometry. Data represent mean ± SE. $^aP < 0.05$ *vs* control group, $^cP < 0.05$ *vs* BSD group.

group) in periphery. In Peyer's patch, there were more $CD8^+$ cells in mice in HXZQ high and low groups ($P < 0.05$ *vs* BSD group), and more $CD4^+$ in mice in HXZQ high group ($P < 0.05$ *vs* BSD group).

### *TNF-α in peripheral blood and intestinal tissue homogenates*

Table 1 shows that there were higher levels of TNF-α in peripheral blood and intestinal tissue homogenates in BSD group ($P < 0.05$ *vs* control group). Mice in HXZQ high group showed decreased levels of TNF-α in peripheral blood and enteric tissue homogenates ($P < 0.05$ *vs* BSD group), and mice in HXZQ low group showed decreased levels of TNF-α in enteric tissue homogenates ($P < 0.05$ *vs* BSD group).

**Table 1 Changes of TNF-α in peripheral blood and enteric tissue homogenates**

| Group | Dosage (g/kg) | *n* | Peripheral blood (ng/mL) | Enteric tissue homogenates (ng/mL) |
|---|---|---|---|---|
| Control | - | 10 | 2.24 ± 0.26 | 1.83 ± 1 |
| BSD | - | 10 | 2.76 ± 0.26[a] | 4.13 ± 1.7[a] |
| HXZQ high | 5.21 | 10 | 2.31 ± 0.27[c] | 2.00 ± 0.6[c] |
| HXZQ low | 0.521 | 10 | 2.64 ± 0.54 | 2.7 ± 1.53[c] |

Data represent mean ± SE. $^aP < 0.05$ *vs* control group, $^cP < 0.05$ *vs* BSD group. TNF-α in peripheral blood and enteric tissue homogenates was analyzed with ELISA.

## DISCUSSION

HXZQ is one of ancient compound liquids of traditional Chinese herbal preparations. In China, it has been used for the effective treatment of various common gastrointestinal infectious diseases (GID), such as acute gastroenteritis, diarrhea and gastrointestinal type Frigorism[1]. It has been reported that HXZQ can promote gastroenteric motility, ameliorate myoelectricity of enteric smooth muscle and regulate gastrointestinal function[2]. Huoxiangzhengqi liquid has also been shown to have regulatory effects on specific immune responses[3]. Agastache rugosa is the essential component of huoxiangzhengqi liquid, and the extract of Agastache rugosa has been reported to have immune modulatory activities[9-15]. BSD in mice is induced by administration of attacking pathogenic bacteria, and shares similar pathogenesis and symptoms with GID[6,7]. Our study showed that HXZQ at 5.21 g/kg and 0.521 g/kg could significantly ameliorate diarrhea in BSD mice.

Although bacteria are usually regarded as harmful, it is now recognized that the breakdown or a failure of components of the indigenous microflora is responsible for allergic diseases or food enteropathies[8-10]. Intestinal microbial species and its product can partially modulate mucosal immune responses and influence the expansion and structure of lymphoid tissues, the activation of $TH_1$ and $TH_2$ T cells[11,12]. Intestinal bacteria are required for the development of gut-associated lymphoid tissues (GALT), which mediate a variety of host immune functions, such as mucosal immunity and oral tolerance[13]. Peyer's patch lymphocytes, as one important part of GALT, are considered to be the effectors in reacting with intestinal antigens, and mediate immune tolerance[14]. It has been known that some drugs can reestablish the microenvironment of the intestinal tract, thereby enhancing mucosal as well as systemic immune response[15-17]. Intestinal immunity has been evaluated with respect to $CD4^+$ cells armed with secretory intestine-derived IgA, and $CD8^+$ cells armed with IgG[18]. Down-regulation of immune responses by regulatory T cells is one of the major mechanisms involved in the induction of tolerance and self- and allo-antigen[19]. Microflora-associated antigens can drive antigen-specific $CD4^+$ T cells to cause intestinal inflammation. Therefore, the effectors $CD4^+$ T cells seem to regulate the development of intestinal inflammation[4]. Our results showed that there were significant changes in $CD4^+$ and $CD8^+$ cells both in Peyer's patch and peripheral blood in BSD mice, and HXZQ could regulate the proportion of $CD4^+$ to $CD8^+$. These results further supported that dysbacteria might take some active role in immune response in BSD mice, and HXZQ might have potential regulative activities on enteric mucosal immune responses in diarrhea. The results also indicate that $CD4^+$ and $CD8^+$ T cells in Peyer's patch may serve as one of the targets of the immunoregulatory activity of huxiangzhengqi liquid.

TNF-α is a pleiotropic cytokine with strong proinflammatory, immunomodulatory properties, and plays a critical role in inflammation and inflammatory bowel disease[20,21]. Anti-TNF therapy has been proven to be a milestone in the treatment of inflammatory bowel disease, and equally important in other inflammation-mediated conditions, including viral infections and mucosal inflammation[22,23]. Different strategies have been explored aiming at inhibiting TNF. Infliximab, a chimeric antibody to TNF-α, holds much promise for the treatment of Crohn's disease[24]. TNF-α levels in enteric tissue homogenates and peripheral blood in BSD mice were higher in our study, while HXZQ could decrease TNF-α. The results could partially explain the immunosuppressive activities of HXZQ in immune response of mice, and further support that dysbacteria could lead to increased inflammatory response in BSD mice.

In conclusion, enteric immune response might be actively involved in diarrhea and regulation of CD4 and CD8 T cells in Peyer's patch and suppression of TNF-α levels in enteric homogenates might be one of the mechanisms of HXZQ in the treatment of diarrhea.

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*RAPID COMMUNICATION*

# Effects of ketamine on proinflammatory cytokines and nuclear factor kappaB in polymicrobial sepsis rats

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## Abstract

**AIM:** To explore the effects of ketamine on hemodynamics, plasma proinflammatory cytokine (TNF-α and IL-6) levels and nuclear factor kappa B (NF-κB) activation during polymicrobial sepsis.

**METHODS:** Male Sprague-Dawlay rats were subjected to cecal ligation and puncture (CLP) or sham operation. The rats were randomly assigned into four equal groups: sham CLP group, CLP group, ketamine (KT) Ⅰ group and KTⅡ group. Thirty minutes before CLP, ketamine (5 mg/kg per hour and 10 mg/kg per hour, respectively) was infused continuously through the left femoral vein cannula in KTⅠ group or KTⅡ group. Sham CLP group and CLP group received 0.9% saline only (5 mL/kg per hour). The right femoral artery was cannulated to monitor mean arterial pressure (MAP) and heart rates (HR),and draw blood samples. The proinflammatory cytokine (TNF-α and IL-6) levels of plasma were measured using enzyme-linked immunosorbent assays (ELISA). The hepatic NF-κB activation was determined by Western blot and HPIAS 2000 image analysis system. Twenty hours after CLP, the rats were killed by right femoral artery phlebotomization.

**RESULTS:** CLP produced progressive hypotension, and a first increase followed by a decrease in HR. The hypotension was prevented, and the HR was slightly steady in ketamine treated rats. TNF-α levels of plasma reached a peak value at 2 h after CLP. Ketamine (KT Ⅰ group or KTⅡ group) caused a significant decrease compared with CLP group at 2, 5 and 9 h time points after CLP (14.3 ± 1.9 *vs* 4.3 ± 0.9, 9.7 ± 1.4 *vs* 4.3 ± 0.9; 9.3 ± 1.5 *vs* 4.3 ± 0.9, 8.7 ± 1.4 *vs* 4.3 ± 0.9; 6.0 ± 1.5 *vs* 5.0 ± 1.7, 5.3 ± 0.8 *vs* 5.0 ± 1.7; $P < 0.01$, respectively). The IL-6 levels of plasma firstly ascended and then descended in CLP group, and reached a peak value at 9 h after CLP. Ketamine (KTⅠ group or KTⅡ group) caused a significant decrease compared with CLP group at 5, 9 or 20 h after CLP (135.0 ± 52.6 *vs* 60.0 ± 16.3, 112.5 ± 52.6 *vs* 60.0 ± 16.3; 410.0 ± 68.7 *vs* 62.5 ± 12.5, 250.0 ± 28.0 *vs* 62.5 ± 12.5; 320.0 ± 25.9 *vs* 52.5 ± 10.1, 215.0 ± 44.6 *vs* 52.5 ± 10.1; $P < 0.05$, respectively). The IL-6 levels of plasma in KTⅡ group were lower than those of KTⅠ group at 9 h after CLP (250.0 ± 28.0 *vs* 410.0 ± 68.7; $P < 0.05$). In addition, CLP increased hepatic NF-κB expression compared with sham CLP. Ketamine suppressed NF-κB activation in a dose-dependent manner at 4 h after CLP (237.7 ± 3.5 *vs* 246.9 ± 3.1; $P < 0.05$).

**CONCLUSION:** Ketamine stabilizes the hemodynamics, attenuates the proinflammatory cytokine responses, and inhibits hepatic NF-κB activation. These findings suggest that ketamine has protective effects against polymicrobial sepsis in rats.



**Key words**: Ketamine; Sepsis; Hemodynamics; Tumor necrosis factor α; Interleukin 6; Nuclear factor kappa B

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## INTRODUCTION

Sepsis/Septic shock is a complex pathophysiologic process characterized by profound hypotension, progressive metabolic acidosis, systemic inflammatory response syndrome (SIRS), multiple organ dysfunction syndrome (MODS), and even death. Although its pathophysiology is not well defined, that monocytes orchestrate the innate immune response to Gram-positive and Gram-negative bacteria by expressing a variety of inflammatory cytokines, especially tumor necrosis factor-α (TNF-α) is considered to play an important role in the pathogenesis of sepsis/septic shock[1,2].

It is known that nuclear factor kappa B (NF-κB) is an

inducible nuclear transcription factor that plays a central role in regulating the transcription of proinflammatory cytokines[3], such as TNF-α, interleukin 6 (IL-6), adhesion molecules, the other mediators involved in SIRS, severe sepsis, septic shock and MODS.

Recently, several investigators have reported that intravenous anesthetic ketamine has inhibitory effects on lipopolysaccharide (LPS)-induced TNF-α production in endotoxin-shock rats[4,5]. Others have documented that ketamine could suppress proinflammatory cytokine production in human whole blood *in vitro*[6,7]. Thus we hypothesized that ketamine could inhibit proinflammatory cytokine responses and NF-κB activation after cecal ligation and puncture (CLP, a model of polymicrobial sepsis) challenge during polymicrobial sepsis in rats.

## MATERIALS AND METHODS

### Establishment of rat model of polymicrobial sepsis

Polymicrobial sepsis was induced in male Sprague-Dawley (SD) rats (200-250 g) by CLP according to the method of Chaudry[8]. The rats were anesthetized with 20% ethyl carbamate (1 g/kg). Then the cecum was isolated and ligated with a 3-0 silk ligature just distal to the ileocecal valve, punctured twice at the opposite ends with a 9-gauge needle, and returned into the abdominal cavity. Following this, the abdominal incision was closed in two layers and the rats received normal saline solution (3 mL/100 g) subcutaneously.

### Experimental protocols

Rats were randomly assigned into four equal groups: CLP group, sham operation group, KT Ⅰ (ketamine Ⅰ) group and KT Ⅱ (ketamine Ⅱ) group. In CLP group, thirty minutes before CLP rats received 0.9% saline through the left femoral vein cannula. Polymicrobial sepsis was induced by CLP operation. In sham CLP group, rats were subjected to the same surgical procedure except that the cecum was neither ligated nor punctured. In KT Ⅰ and KT Ⅱ groups, ketamine (5 mg/kg per hour and 10 mg/kg per hour, respectively) was infused continuously through the left femoral vein cannula 30 min before CLP. The right femoral artery was cannulated to monitor the mean arterial pressure (MAP), heart rates (HR) and draw blood samples in all groups.

### Plasma cytokine levels

At 2, 5, 9 and 20 h after CLP or sham CLP operation, blood was drawn *via* the right femoral artery (1.0 mL each). The plasma was immediately separated by centrifugation at 3000 rotation per min for 15 min at 4℃, divided into aliquots and stored at -70℃ until assayed. The proinflammatory cytokine levels of plasma were quantified using enzyme-linked immunosorbent assays (ELISA)(American R & D and Bender).

### Preparation of nuclear extracts[9]

Fresh liver tissue of 0.1 g was homogenized in buffer A (10 mmol/L HEPES-NaOH pH 7.9, 1 mmol/L EDTA, 10 mmol/L KCl, 1.5 mmol/L $MgCl_2$, 1 mmol/L PMSF, 1 mmol/L DTT, 1 μg/mL Leupeptin), and the homogenates were incubated on ice (15 min) with gentle agitation. After lysis, nuclei were collected by centrifugation (14 000 rpm for 5 min). Nuclear proteins were extracted by incubation of nuclei on ice (30 min) in hypertonic salt buffer B (20 mmol/L HEPES-NaOH, pH 7.9, 0.2 mmol/L EDTA, 420 mmol/L NaCl, 1.5 mmol/L $MgCl_2$, 25% glycerin 0.5 mmol/L PMSF, 0.5 mmol/L DTT, 1 μg/mL Leupeptin). Extracts were centrifuged and nuclear extract supernatant was harvested and stored at -70℃. Proteins were quantified by the Lowry-Kalckar assays.

### Western blot analysis

The nuclear protein extracts of the liver tissue (20 μg) were boiled in Laemmli sample buffer and subjected to 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis. Before transfer, gels were equilibrated for 15 min in cathode buffer. Proteins were transferred at 0.8 mA/cm for 2 h onto NC membranes, preequilibrated in methanol using a semidry blotting apparatus. Equal loading and transfer were monitored by immersing ponceau S staining of the membranes. Nonspecific binding sites were blocked by immersing the membrane into blocking solution overnight at 4℃. Membranes were washed three times (10 min each) in TBST and incubated in 1:200 dilution of rabbit-antirat NF-κB p65 polyclonal antibody in blocking solution for 1 h at room temperature, followed by extensive washing three times (10 min each) with TBST. Bound antibody was decorated with 1:1000 goat-antirabbit IgG diluted in blocking solution for 30 min at room temperature, after washing four times (10 min each). Immunocomplexes were detected using DAB staining, Western blotting reagents and determined using HPIAS 2000 image analysis system.

### Statistical analysis

Data were presented as mean ± SD. Analysis of variance (ANOVA) was used to evaluate whether values at the same time point were different for the control and ketamine groups and two-way analysis of variance for repeated measurements with multiple comparisons. A significant difference was presumed at a $P$ value < 0.05.

## RESULTS

### Hemodynamics (MAP and HR)

No significant differences were noted in baseline MAP among all groups. CLP challenge decreased MAP in CLP group, KT Ⅰ group and KT Ⅱ group but not in sham CLP group. At 20 h time point after CLP challenge, MAP decreased by 6.8%, 71.7%, 41.5% and 24.7% in sham CLP, CLP, KT Ⅰ, and KT Ⅱ groups, respectively. There were significant differences between CLP group and KT Ⅰ group at 14, 16, 18 and 20 h time points ($P$ < 0.05, respectively). At the same time significant differences in MAP were found between the CLP group and KT Ⅱ group at 8, 10, 12, 14, 16, 18 and 20 h time points after CLP challenge during polymicrobial sepsis ($P$ < 0.05, respectively) (Figure 1) .

There were no significant differences in baseline HR among all groups. First increased followed by a decrease in HR after CLP challenge, HR was slightly steady in

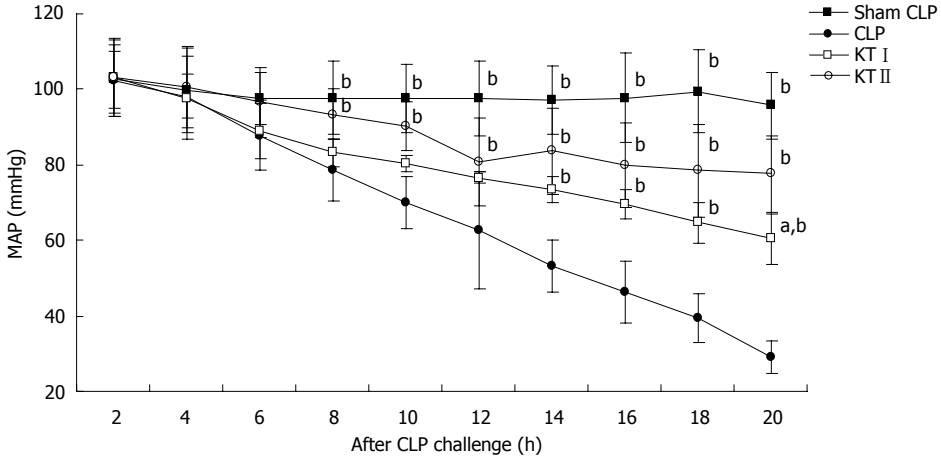


**Figure 1** Changes of MAP (mean ± SD). Closed squares, sham CLP group; Closed circles, CLP group; open squares, ketamine Ⅰ group; open circles, ketamine Ⅱ group, $^{b}P < 0.01$ *vs* CLP group, $^{a}P < 0.05$ *vs* ketamine Ⅱ group.

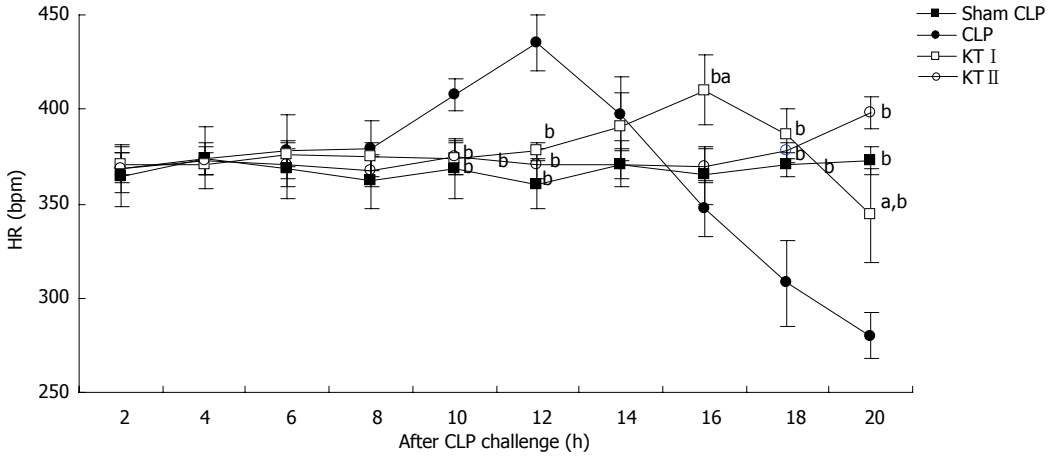


**Figure 2** Changes of plasma TNF-α (mean ± SD). Closed squares, sham CLP group; Closed circles, CLP group; Open squares, ketamine Ⅰ group; Open circles, ketamine Ⅱ group, $^{b}P < 0.01$ *vs* CLP group, $^{a}P < 0.05$ *vs* ketamine Ⅱ group.

**Table 1 Changes of TNF-α, (pg/mL, $n$ = 5, mean ± SD)**

| Group | Time point (after CLP) | | | |
|---|---|---|---|---|
| | 2 h | 5 h | 9 h | 20 h |
| Sham CLP | 4.3 ± 0.9[b] | 4.3 ± 0.9[b] | 5.0 ± 1.7[b] | 4.0 ± 0.9 |
| CLP | 27.3 ± 3.1 | 15.0 ± 2.0 | 10.7 ± 1.9 | 4.3 ± 0.9 |
| KT Ⅰ | 14.3 ± 1.9[a,b] | 9.3 ± 1.5[b] | 6.0 ± 1.5[b] | 4.3 ± 1.5 |
| KT Ⅱ | 9.7 ± 1.4[b] | 8.7 ± 1.4[b] | 5.3 ± 0.8[b] | 3.7 ± 0.8 |

$^{b}P < 0.01$ *vs* CLP group, $^{a}P < 0.05$ *vs* ketamine Ⅱ group.

**Table 2 Changes of IL-6, (pg/mL, $n$ = 5, mean ± SD)**

| Group | Time point (after CLP) | | | |
|---|---|---|---|---|
| | 2 h | 5 h | 9 h | 20 h |
| Sham CLP | 50.0 ± 15.3 | 60.0 ± 16.3[b] | 62.5 ± 12.5[b] | 52.5 ± 10.1[b] |
| CLP | 52.5 ± 10.5 | 217.5 ± 25.9 | 882.5 ± 127.1 | 567.5 ± 144.9 |
| KT Ⅰ | 50.0 ± 8.8 | 135.0 ± 52.6[a] | 410.0 ± 68.7[b,c] | 320.0 ± 25.9[b] |
| KT Ⅱ | 45.0 ± 11.2 | 112.5 ± 52.6[b] | 250.0 ± 28.0[b] | 215.0 ± 44.6[b] |

$^{a}P < 0.05$, $^{b}P < 0.01$ *vs* CLP group; $^{c}P < 0.05$ *vs* ketamine Ⅱ group.

ketamine treated rats. There were significant differences in HR at 10, 12, 16, 18, and 20 h time points between CLP group and KT Ⅰ group ($P < 0.05$, respectively). Significant differences in HR were found between CLP group and KT Ⅱ group at 10, 12, 18 and 20 h time points after CLP challenge during polymicrobial sepsis ($P < 0.05$, respectively) (Figure 2).

### *Plasma cytokine levels of TNF-α and IL-6*

TNF-α levels of plasma were significantly increased after CLP challenge. In CLP group, TNF-α levels reached a peak level at 2 h after CLP operation (27.3 ± 3.1 pg/mL). Ketamine (KT Ⅰ group or KT Ⅱ group) caused a significant decrease compared with CLP group at 2, 5 and 9 h after CLP challenge ($P < 0.05$, respectively). TNF-α levels in KT Ⅱ group were lower than those of KT Ⅰ group at 2 h after CLP challenge ($P < 0.05$) (Table 1).

IL-6 levels of plasma were firstly increased followed by a decrease during polymicrobial sepsis in CLP group, and reached a peak level at 9 h after CLP (883 ± 127 pg/mL). Ketamine (KT Ⅰ group or KT Ⅱ group) caused a significant decrease compared with CLP group at 5, 9 and 20 h after CLP challenge ($P < 0.05$, respectively). IL-6 levels in KT Ⅱ group were lower compared with those of KT Ⅰ group at 9 h after CLP challenge ($P < 0.05$, respectively) (Table 2).

### *Activity of NF-κB p65*

Western blot analysis demonstrated that hepatic NF-κB activity was obviously increased in CLP group (266 ± 7, absorbency) compared with sham CLP group ($P < 0.05$) at 4 h after CLP. NF-κB activation in KT Ⅰ group or KT Ⅱ group was significantly decreased after ketamine treatment

**Table 3 Changes of NF-κB P65 absorbency, ($n$ = 5, mean ± SD)**

| Group | 4 h after CLP |
|---|---|
| Sham group | 215.5 ± 2.7[b] |
| CLP group | 264.5 ± 7.4 |
| KT I group | 246.9 ± 3.1[a,b] |
| KT Ⅱ group | 237.7 ± 3.5[b] |

[a]$P$ < 0.05 *vs* ketamine Ⅱ group; [b]$P$ < 0.01 *vs* CLP group.

($P$ < 0.05, respectively). By contrast, NF-κB activation in KT Ⅱ group was lower than those in KT I group ($P$ < 0.05) (Table 3 and Figure 3).

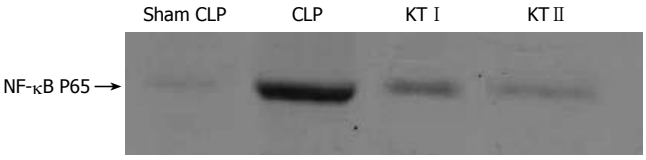


**Figure 3** Hepatic NF-κB activation.

## DISCUSSION

In our study, CLP produced progressive hypotension and obvious increase in plasma cytokine concentrations. Ketamine administration inhibited development of hypotension and increase of cytokine concentrations, which is in accordance with the literature[4-7]. It indicates that a subanesthetic dose of ketamine could inhibit endotoxin-induced expression of TNF-α and other proinflammatory mediators. We found that CLP provoked a transient elevation of TNF-α and IL-6 concentration in the plasma and enhanced the hepatic NF-κB activation in polymicrobial sepsis rats. In contrast, in the presence of ketamine, polymicrobial sepsis had inhibitory effects on the production of cytokines. Moreover, ketamine decreased the hepatic NF-κB activation during polymicrobial sepsis. This inhibitory effect of ketamine was the most important finding of the present study.

Since TNF-α is considered to play a key role in the pathogenesis of sepsis/septic shock, we detected TNF-α concentrations of plasma in rats. In addition, we also examined IL-6 concentrations. In 1994, Takenaka discovered that intravenous anesthetic ketamine had inhibitory effects on LPS-induced TNF-α production in endotoxin-induced shock in mice *in vivo*[3]. Following that more studies discovered that ketamine suppressed TNF-α mRNA expression and proinflammatory cytokine IL-6 and IL-8 production in human whole blood *in vitro*[10]. Our study demonstrated that ketamine had the same inhibitory effects on TNF-α and IL-6 expression during polymicrobial sepsis.

NF-κB is an inducible nuclear transcription factor that plays a central role in regulating the transcription of proinflammatory cytokines[3], including TNF-α, IL-6. However, the present study did not determine the cellular source of the cytokines. Numerous studies have implicated macrophages and other immunocompetent cells as important sources of proinflammatory cytokines in various models of shock and sepsis. Some investigators suggested that the hepatic Kupffer cells are a major source of proinflammatory cytokine release during acute phase of sepsis[11]. We attempted to identify the hepatic NF-κB activation during polymicrobial sepsis.

Until now, the effects of ketamine on NF-κB have been reported limitedly. In 2000, Sakai discovered ketamine inhibited endotoxin-induced NF-κB expression in brain cells *in vivo* and *in vitro*, in a dose-dependent manner[12]. In 2004, Sun discovered that ketamine suppressed endotoxin-induced NF-κB activation and TNF-α expression in the intestine, lung and liver[13]. In 2005, Suliburk found that ketamine attenuated liver injury attributed to endotoxemia: a role of cyclooxygenase-2[14]. In addition, Yang found that large dose ketamine inhibited LPS-induced acute lung injury in rats[15]. Our study indicated ketamine suppressed the hepatic NF-κB activation after CLP challenge during polymicrobial sepsis.

The doses of ketamine used in this study were 5 mg/kg per hour and 10 mg/kg per hour. Compared with the subanesthetic dose or the anesthetic dose, these doses of ketamine were relatively higher. It might be species-dependent. The two doses both inhibited proinflammatory cytokine responses and hepatic NF-κB activation, especially higher dose ketamine treatment group. It indicates that the effects of ketamine might be dose-dependent.

In summary, the current studies show that administration of ketamine has protective effects against polymicrobial sepsis in rats. Ketamine probably inhibits NF-κB activation, and attenuates the proinflammatory cytokine response. However, the precise mechanisms underlying the inhibitory effects are still unknown. Further investigations are needed, such as on toll-like receptors[16,17].

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*RAPID COMMUNICATION*

# Loss of fragile histidine triad protein expression in inflammatory bowel disease

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## Abstract

**AIM:** To investigate the expression of fragile histidine triad (FHIT) protein in 64 patients with ulcerative colitis (UC) and Crohn's disease (CD), and its relation with clinicopathological data.

**METHODS:** Rabbit-anti-FHIT antibody was used to detect FHIT protein expression in 64 formalin-fixed, paraffin-embedded tissue specimens of inflammatory bowel disease (IBD) by citrate-microwave-streptavidin (SP)-HRP immunohistochemical method.

**RESULTS:** The positive FHIT protein expression was 22.79% ± 16.16%, 42.14% ± 16.82% in active and remittent phases of UC, 36.07% ± 19.23% in CD, and 57.05% ± 8.86% in normal colon mucosa. Statistically significant differences in FHIT protein expression were observed between the active and remittent phases of UC, between the active phase of UC and normal colon mucosa, as well as between the remittent phase of UC and normal colon mucosa, and between CD and normal colon mucosa.

**CONCLUSION:** Our results show that FHIT protein expression is completely absent or reduced in IBD, suggesting that the FHIT gene might be associated with the oncogenesis and progression of IBD, an early event from inflammatory conditions to carcinoma in IBD.



**Key words:** Fragile histidine triad protein expression; Ulcerative colitis; Crohn's disease; Inflammatory bowel disease

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## INTRODUCTION

Inflammatory bowel disease (IBD) is a collection of chronic idiopathic inflammatory disorders of the intestine and/or colon, including two independent diseases: ulcerative colitis (UC) and Crohn's disease (CD)[1]. Up to now, the complex etiology and pathogenesis of IBD are not known with certainty, but the growing theories suggest that many factors such as environment, genetic alteration, uncontrolled immune system, *etc*, can result in chronic gut inflammation[2-7]. However, none of the theories provides a sufficient explanation of either disease, indicating that their etiology is multifactorial. It has been well established that colorectal carcinoma is the most serious complication of patients with long-standing IBD who have an increased risk of developing colorectal carcinoma[8-11]. The cumulative risk of carcinoma in IBD patients is estimated to be 10-20 times greater in the small bowel and 4-20 times greater in the large bowel than that in the small and large bowel of general population. The mean duration of colitis before cancer diagnosis ranges 17-20 years. Dysplasia occurring in IBD constitutes a precursor stage of carcinoma[12,13]. Patients with IBD are characterized by recurrent acute mucosa inflammation, mucosal ulceration, epithelial necrosis and regeneration, all of which may result in DNA damage, genetic alterations including enhanced microsatellite instability of mucosa, activation of oncogene, inactivation of tumor suppressor gene, increasing susceptibility to mutagenesis and subsequent neoplastic transformation[8-18].

The fragile histidine triad (FHIT) gene was discovered at human chromosome 3p14.2 in 1996 by Ohta *et al*[19] using the exon trapping method, and has been identified as a candidate tumor-suppressor gene. This gene not only spans the translocation breakpoint of familial renal-cell carcinoma, but also encompasses the most active common human chromosomal fragile region, FRA3B[19,20]. The approximately 1-megabase FHIT gene includes 10 exons, encoding 1.1Kb mRNA transcript, 16.8 kDa, and 147 amino acid proteins[21]. FHIT protein is a member of the recently discovered histidine triad (HIT) family of nucleotide-binding proteins with a high specific hydrolysing activity for diadenosine 5', 5'''-P1, Pn-polyphosphate (ApnA), where $n$ = 3-6. The FHIT protein encoded by the FHIT gene can hydrolyze AP3A

and AP4A to ADP and AMP[20,22]. It was reported that the FHIT gene can facilitate deletions and aberrant transcripts and may play an important role in a variety of human malignancies[23-26], including cancers of lung[27], breast[28], pancreas[29], urinary bladder[30], head and neck[31] and gastrointestinal carcinomas[32-34].

Since the role of FHIT aberrations is unclear and results from different investigators are contradictory, the present study was to evaluate the expression of FHIT protein in patients with IBD and its relation with clinicopathological data.

## MATERIALS AND METHODS

### Patients

Biopsy specimens from 64 IBD patients including 47 UC and 17 CD patients were obtained from several hospitals in Hubei Province from 1990 to 2005. All the patients were diagnosed in the light of clinical, endoscopic, histological and radiological criteria. All IBD patients underwent sigmoidoscopy or colonscopy for routine clinical evaluation. Mucosal inflammation in active UC was classified as mild inflammation: mild-to-moderate small round-cell infiltration with formation of a few crypt abscesses in the lamina propria; severe inflammation: severe small round-cell infiltration with multiple crypt abscesses and partial granulation in the lamina propria. Remission was defined histologically as areas with branched or regenerated irregular crypts without acute inflammation but with chronic mild inflammation. Ten apparently normal colonic tissue sections were obtained. The mean age of 47 patients including 34 men and 13 women at the onset of UC was 36.9 years (averaged 13.4 years). The mean age of the remaining 17 patients including 10 men and 7 women at the onset of CD was 38.0 years (averaged 13.9 years).

### Reagents and antibodies

Rabbit anti-FHIT polyclonal antibody (Zymed Company, USA) was purchased from Beijing Zhongshan Biological Technology CO, Ltd. Immunohistochemical staining S-P kit (Zemed MAXIM, USA) was purchased from Maixin Co, LTD.

### Methods

All the biopsy specimens were fixed in 10% buffered formalin and embedded in paraffin wax. Four-μM thick sections were routinely prepared and stained with haematoxylin and eosin, while other sections were prepared with immunohistochemical SP staining method. In brief, the tissue sections were routinely dewaxed in xylene, rehydrated at graded concentrations of alcohol, and treated with 3% hydrogen peroxide for 10 min to block the endogenous peroxidase. The sections were immersed in citrate buffer (5 mmol/L sodium citrate, pH = 6.0), heated in a microwave oven for 2 min to enhance antigen retrieval, and then placed at room temperature for 10 min. The sections were blocked with normal goat serum for 15 min and incubated with rabbit anti-FHIT polyclonal antibody (1:100 dilution) overnight at 4℃. After washed three times in PBS, the binding of antibodies to their antigenic sites in the tissue sections was further amplified with biotinylated goat anti-rabbit antibody followed by reaction with streptavidin-biotin peroxidase. Antibody localization was detected with diaminobenzidine as a chromogen substrate. Sections prepared by substituting PBS for the primary antibody served as a negative control. The freshly prepared substrate DAB was added for color development. The sections were washed in distilled water and counterstained with haematoxylin and mounted for examination.

### Determination of FHIT expression

Negatively expressed FHIT manifested as blue-stained nuclei while positively expressed FHIT manifested as brown or dark brown cytoplasm and/or cell membrane mainly in epithelial tissues. Expressions of these target proteins were semi-quantitated with automatic image analyzer (Nikon,Japan) and HPIAS-2000 image analyzing program, in which the average value of positive cells in 10 randomly selected high power fields (× 400) for each section was used for the comparison of the target protein expressions.

### Statistical analysis

Quantitative variables were expressed as mean ± SD. Statistical comparisons between groups were made by one-way ANOVA. Differences in results between the two groups were tested with $t$ test. $P < 0.05$ was considered statistically significant. All analyses were performed using the SPSS version 14.0.

## RESULTS

The classification of IBD patients according to their clinicopathological characteristics and their association with FHIT protein expression are shown in Tables 1 and 2.

The pattern of FHIT protein expression was confined to the epithelial cells, especially cytoplasm. As shown in Figure 1A-1D, FHIT protein expression was unequivocal in normal colonic tissue and reduced or absent in UC and CD. The positive FHIT expression was 22.79% ± 16.16%, 42.14% ± 16.82%, respectively in active and remittent phases of UC, 36.07% ± 19.23% in CD, and 57.05% ± 8.86% in normal colon mucosa. Statistically significant differences in FHIT protein expression were observed between the active and remittent phases of UC ($P < 0.05$), between the active phase of UC and normal colon mucosa ($P < 0.01$), as well as, between the remittent phase of UC and normal colon mucosa ($P < 0.01$), and between CD and normal colon mucosa ($P < 0.01$).

## DISCUSSION

Recent studies showed that tumourigenesis is related to inflammatory conditions, such as IBD and pancreatitis, and inflammation has been considered as precancerosis, but the possible link between inflammation and tumourigenes is still unclear[14,35,36]. Chronic inflammatory conditions such as UC are thought as the risk factor for some carcinomas, the incidence of colon carcinoma in UC is estimated to be 5-7 times greater than what we have expected,

**Table 1 Clinicopathological data of patients with inflammatory bowel disease**

| Patient No. | Sex | Age (yr) | Disease extension |
|---|---|---|---|
| Crohn's disease | | | |
| 1 | M | 54 | Terminal ileum |
| 2 | F | 20 | Ileocolonic |
| 3 | M | 40 | Pancolitis |
| 4 | M | 70 | Right-sided |
| 5 | F | 62 | Ileocolonic |
| 6 | M | 27 | Terminal ileum |
| 7 | M | 26 | Terminal ileum |
| 8 | F | 34 | Ileocolonic |
| 9 | M | 51 | Pancolitis |
| 10 | M | 41 | Pancolitis |
| 11 | M | 29 | Right-sided |
| 12 | F | 25 | Left-sided |
| 13 | M | 35 | Terminal ileum |
| 14 | F | 38 | Ileocolonic |
| 15 | F | 27 | Terminal ileum |
| 16 | M | 31 | Right-sided |
| 17 | M | 42 | Ileocolonic |
| Ulcerative colitis-active | | | |
| 18 | M | 37 | Ileocolonic |
| 19 | M | 45 | Rectosigmoid |
| 20 | M | 42 | Rectosigmoid |
| 21 | M | 58 | Rectosigmoid |
| 22 | M | 28 | Rectosigmoid |
| 23 | F | 26 | Rectosigmoid |
| 24 | M | 36 | Rectosigmoid |
| 25 | M | 27 | Rectosigmoid |
| 26 | M | 32 | Rectosigmoid |
| 27 | F | 47 | Rectosigmoid |
| 28 | M | 33 | Rectosigmoid |
| 29 | M | 33 | Rectosigmoid |
| 30 | M | 36 | Left-sided |
| 31 | F | 44 | Rectosigmoid |
| 32 | M | 41 | Rectosigmoid |
| 33 | M | 24 | Left-sided |
| 34 | F | 58 | Rectosigmoid |
| 35 | F | 50 | Rectosigmoid |
| 36 | M | 64 | Rectosigmoid |
| 37 | M | 48 | Rectosigmoid |
| 38 | M | 60 | Pancolitis |
| 39 | M | 35 | Pancolitis |
| 40 | M | 30 | Rectosigmoid |
| 41 | M | 13 | Left-sided |
| 42 | M | 52 | Pancolitis |
| 43 | F | 38 | Pancolitis |
| 44 | M | 17 | Pancolitis |
| 45 | F | 26 | Left-sided |
| 46 | M | 37 | Pancolitis |
| 47 | M | 20 | Rectosigmoid |
| 48 | M | 43 | Pancolitis |
| 49 | F | 51 | Pancolitis |
| 50 | M | 32 | Rectosigmoid |
| Ulcerative colitis-Remittent | | | |
| 51 | M | 8 | Ileocolonic |
| 52 | M | 18 | Rectosigmoid |
| 53 | F | 35 | Rectosigmoid |
| 54 | F | 24 | Rectosigmoid |
| 55 | M | 36 | Rectosigmoid |
| 56 | F | 35 | Rectosigmoid |
| 57 | M | 35 | Pancolitis |
| 58 | F | 41 | Pancolitis |
| 59 | M | 13 | Rectosigmoid |
| 60 | F | 44 | Pancolitis |
| 61 | F | 45 | Pancolitis |
| 62 | M | 60 | Rectosigmoid |
| 63 | M | 23 | Rectosigmoid |
| 64 | M | 56 | Left-sided |

**Table 2 Comparison of FHIT protein immunohistochemical expression in ulcerative colitis, Crohn's disease, and normal colon mucosa (mean ± SD)**

| Groups | *n* | Positive rate of FHIT protein expression (%) |
|---|---|---|
| Normal colon mucosa | 10 | 57.05 ± 8.86 |
| Ulcerative colitis | | |
| Active | 33 | 22.79 ± 16.16 |
| Remittent | 14 | 42.14 ± 16.82 |
| Crohn's disease | 17 | 36.07 ± 19.23 |

and colon carcinoma occurs in 20%-35% patients with IBD. Compared with sporadic colorectal cancers, such as adenomatous polyposis and hereditary non-polyposis colorectal cancer syndrome, the prognosis of UC-associated colorectal cancer is the worst, the 5-year survival rate of patients is the lowest (< 40%)[10,12]. In our study, the positive rate of FHIT expression was 31.88% ± 20.33%, 22.79% ± 16.16%, 42.14% ± 16.82% in initial, active and remittent phases of UC, and 36.07% ± 19.23% in CD, and 57.05% ± 8.86% in normal colon mucosa. Statistically significant differences in FHIT protein expression were observed between the active and remittent phases of UC, between the active phase of UC and normal colon mucosa, as well as between the remittent phase of UC and normal colon mucosa, and between CD and normal colon mucosa. The severer the inflammation (active phase) is, the more reduction the FHIT protein expression is.

The possible interpretations for these results are as follows. IBD is strongly related to environment, and microsatellite instability in 50% UC patients is enhanced during inflammation. Microsatellites are simple repetitive sequences of DNA that are scattered throughout the genome. These sequences are stably inherited, varying from individual to individual, and have a low alteration rate. Instability within these sequences has been recognized as a marker for genome mutations and DNA repair deficiency. Recently, there have been some reports on detecting microsatellite instability in non-neoplastic settings, including inflammatory mucosa of IBD[8]. Inflammation results in an increase in DNA damage, strengthening the ability of cells to repair the damage before replication[37]. Mutations of oncogene and tumor suppressor gene may be the initiating events in tumourigenesis arising from an inflammatory backgroud, and continuous production of mutations may be required for tumor progression[38]. Patients with IBD are characterized by recurrent acute inflammation of the mucosa, mucosal ulceration, and epithelial necrosis and regeneration. Various kinetic analyses have shown increasing epithelial cell proliferation or cell death in crypts and dysplastic glands in IBD, especially in UC[9,36]. Active inflammation and regeneration may increase epithelial cell turnover, susceptibility to mutagenesis, and neoplastic transformation[39,40]. The positive rate of FHIT expression in remission of UC was 42.14% ± 16.82%. The possible theoretical explanation is that the abnormal architecture after active ulceration might impair mucosal function and lead to increased cell turnover. Another possibility is that remission in UC is

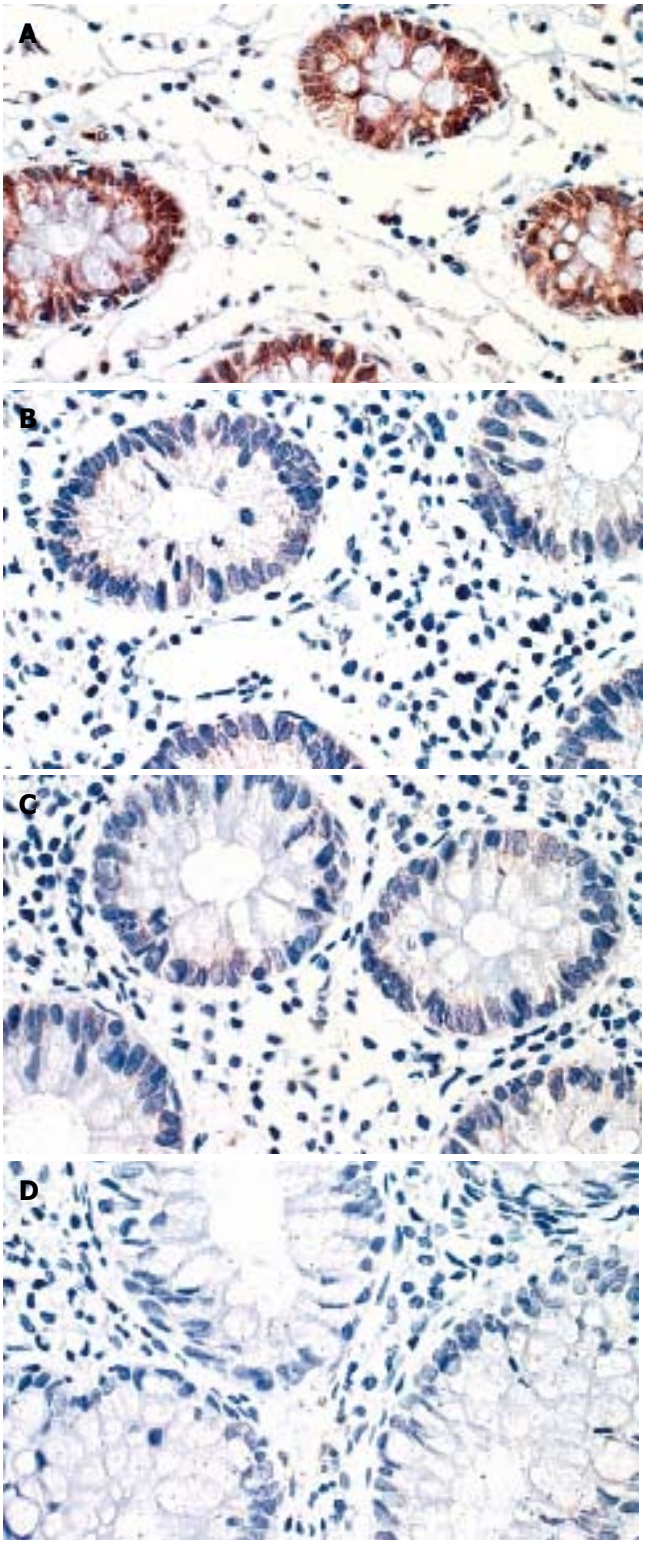


**Figure 1** FHIT protein expression in normal colonic epithelium (**A**), ulcerative colitis (**B**, **C**), and Crohn's disease (**D**) (SP × 400).

only relative with mucosal damage at a subclinical and subhistologic level between the time points of obvious relapse.

Chronic inflammation and epithelial cell damage that characterize IBD result in increased cell proliferation and cell death[41]. At the same time, the accelerated cell turnover prediposes to genetic alterations in mucosa, which is in line with dysplasia and carcinoma in IBD. Nobuyasu Arai *et al*[9] suggested that P53 accumulation and high P21WAF1/CIP1 expression accelerate epithelial cell turnover and may result in an elevated risk of developing dysplasia and carcinoma in patients with IBD.

Chronic inflammation and epithelial damage might predispose to DNA damage in mucosa and accelerate the gene mutation. In the present study, FHIT-protein immunohistochemical expression was absent or reduced in IBD, which might be a precursor from inflammation to carcinoma transformation, suggesting that the alteration of FHIT-protein expression might remain long before any histological morphological change[10]. Skopelitou AS *et al*[42] showed that FHIT protein immunostaining is completely absent or reduced in most *H pylori*-related chronic gastritis, which may play a role in the development and progression of gastric cancer. The reduced or absent FHIT protein expression during inflammation is due to the direct exposure of the gastrointestinal tract to environmental factors that induce FHIT/FRA3B breakage and sustain FHIT damage as an early event or due to DNA damage and FHIT gene mutation induced by chronic inflammation[43].

Reduced or absent FHIT protein expression may play a role in accelerating epithelial cell turnover or carcinoma transformation in IBD. Diadenosine triphosphase is first sequestered and eventually hydrolyzed by FHIT to ADP and AMP, the balance between cellular AP3A level and FHIT enzymatic activity may affect cell death or survival. Reduced or absent FHIT protein expression can inhibit the enzymatic activity of FHIT and increase the cellular AP3A level.AP3A can strengthen the transmission of cellular survival signals and accelerate the epithelial cell turnover. It has been suggested that AP4A can also be hydrolyzed by FHIT to ADP and AMP. AP4A is an intracellular regulatory molecule which may regulate the ability of cells to adapt to metabolic stresses such as oxidation and DNA damage. When the function of FHIT protein is diminished, the normal level of AP4A is deviated, which may result in the inability of cells to adapt to environmental stresses and cause genetic damage. At the same time, the ratio of AP3A to AP4A is increased, which can also increase cellular survival[10,20-23,28,30,32-34]. FHIT protein is involved in the regulation of apoptosis in cell culture systems, which is independent of P53, Bax or Bcl-2 expression and has been considered as an independent mechanism for tumor suppression. FHIT-induced apoptosis comprises signaling processes of FHIT-caspase 8-caspase 9-Bid-PARP, which has two pathways: one is activated by caspase-8, the other involves bypassing mitochondria[31,32]. When FHIT protein is abnormal, apoptosis is disrupted and cellular proliferation is accelerated. FHIT is involved in the regulation of cell cycle and DNA retrieval, FHIT protein can transform damaged DNA into S phase[44]. When FHIT protein expression is reduced or absent, disruption of cell cycle regulation leads to uncontrolled proliferation and formation of tumors.

In conclusion, the high frequency of complete absence and/or reduced immunohistochemical expression of FHIT protein suggests that the FHIT gene might be involved in

most cases of IBD, which might be a precursor from the inflammatory conditions to carcinoma transformation.

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*RAPID COMMUNICATION*

# Molecular forms of trefoil factor 1 in normal gastric mucosa and its expression in normal and abnormal gastric tissues

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## Abstract

**AIM:** To study the molecular forms of trefoil factor 1 (TFF1) in normal gastric mucosa and its expression in normal and abnormal gastric tissues (gastric carcinoma, atypical hyperplasia and intestinalized gastric mucosa) and the role of TFF1 in the carcinogenesis and progression of gastric carcinoma and its molecular biological mechanism underlying gastric mucosa protection.

**METHODS:** The molecular forms of TFF1 in normal gastric mucosa were observed by Western blot. The expression of TFF1 in normal and abnormal gastric tissues (gastric carcinoma, atypical hyperplasia and intestinalized gastric mucosa) was also assayed by immunohistochemical method. The average positive AO was estimated by Motic Images Advanced 3.0 software.

**RESULTS:** Three patterns of TFF1 were found in normal gastric mucosa: monomer, dimmer, and TFF1 compound whose molecular weight is about 21 kDa. The concentration of TFF1 compound was the highest among these three patterns. TFF1 was expressed mainly in epithelial cytoplasm of the mucosa in gastric body and antrum, especially around the nuclei. The closer the TFF1 to the lumen, the higher the expression of TFF1. The expression of TFF1 in peripheral tissue of gastric carcinoma (0.51 ± 0.07) was higher than that in normal gastric mucosa (0.44 ± 0.06, $P < 0.001$). The expression of TFF1 in gastric adenocarcinoma was positively related to the differentiation of adenocarcinoma. The lower the differentiation of adenocarcinoma was, the weaker the expression of TFF1. No TFF1 was expressed in poorly-differentiated adenocarcinoma. The expression of TFF1 in moderately-well differentiated adenocarcinoma (0.45 ± 0.07) was a little lower than that in normal mucosa ($P > 0.05$). The expression of TFF1 in gastric mucosa with atypical hyperplasia (0.57 ± 0.03) was significantly higher than that in normal gastric mucosa ($P < 0.001$). No TFF1 was expressed in intestinalized gastric mucosa. There was no statistically significant difference between the expressions of TFF1 in gastric mucosa around the intestinalized tissue (0.45 ± 0.07) and normal gastric mucosa ($P > 0.05$).

**CONCLUSION:** TFF1 is expressed mainly in epithelial cytoplasm of the mucosa in gastric body and antrum. Its main pattern is TFF1 compound, which may have a greater biological activity than monomer and dimer. The expression of TFF1 in peripheral mucosa of gastric ulcer is higher than that in mucosa 5 cm beyond the ulcer, indicating that TFF1 plays an important part in protection and restitution of gastric mucosa. The expression of TFF1 is increased in peripheral tissues of gastric carcinoma and gastric mucosa with atypical hyperplasia, but is decreased in cancer tissues, implying that TFF1 may be related to suppression and differentiation of carcinoma. The weaker expression of TFF1 in poorly-differentiated carcinoma may be related to the destruction of glands and cells in cancer tissues and the decrease in secretion of TFF1.



**Key words:** Trefoil factor 1; Gastric mucosa protection; Carcinoma suppression

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## INTRODUCTION

Trefoil factor 1 (TFF1) is a member of the trefoil factor family, which is a group of small molecule polypeptides mainly secreted by gastrointestinal mucous cells. TFF1 is mainly expressed in epithelial cytoplasm of the mucosa in gastric body and antrum[1]. The specificity of expression disappears in case of gastrointestinal mucosa injury[2]. TFF1 can be expressed in the whole injured mucosa, and its expression in injured mucosa is much higher than that in normal mucosa[3]. The purpose of this experiment was to observe the expression and patterns of TFF1 in normal and abnormal gastric tissues (gastric carcinoma, atypical

hyperplasia, and intestinalization) and to investigate the role TFF1 in the carcinogenesis and progression of gastric carcinoma and its molecular biological mechanism underlying gastric mucosa protection.

## MATERIALS AND METHODS

### Materials

Gastric antral specimens were obtained from a cohort undergone upper gastrointestinal endoscopy without abnormal appearance at Zhongshan Hospital affiliated to Xiamen University in June 2003. Two specimens were taken from each person and preserved in liquified nitrogen.

Paraffin specimens were obtained from 160 patients who underwent gastroscopic biopsy or radical gastric carcinomectomy during January 2002-June 2003 in Zhongshan Hospital affiliated to Xiamen University. The age of the patients ranged from 40 to 70 (53.2 ± 6.5) years. Of these 160 patients, 35 had normal gastric antrum mucosa, esophageal, gastric and duodenal tissues in gastroscopy and normal gastric mucosa in pathologic examination; 38 were diagnosed as gastric adenocarcinoma by pathologic examination; 36 as atypical hyperplasia; 35 as intestinalized gastric mucosa; and 20 as esophageal squamous carcinoma.

### Reagents

Sheep anti-human TFF1 multiclonal antibody (first antibody) and second antibody were purchased from Beijing Zhongshan Jinqiao Biotechnology Company, Ltd. EDTA, SDS and β-mercaptoethanol were obtained from Sigma Company. Acrylamide, protein-determination reagent and NC film were bought from Bio-Rad Company. ECL Western blotting reagents were from Amersham Company. Mice anti-human TFF1 monoclonal antibody, S-P super sensitive kit and DAB display kit were from Fuzhou Maixin Biotechnology Development Company.

### Western blot

Normal gastric mucosa specimens were added into buffer solution (containing 0.2 mmol/L sucrose, 10 mmol/L Tris, 1.5 mmol/L EDTA, 20 mmol/L β-mercaptoethanol, 1% PMSF, 1% DTT) preset at 4℃. After the solution was uniformly mixed, protein was extracted and its concentration was measured. One hundred and twenty ug of protein from each specimen was added into buffer solutions of the same volume. The solutions were boiled at 100℃ for 5 min. The proteins were isolated by acrylamide gel electrophoresis (5% aggregation gel of 70 V and 20% isolation gel of 120 V) for about 5 h. NC films were metastasized by 250 mA electricity in icy water for 90 min by wet metastasis method. The proteins were kept in confining liquid at room temperature for 30 min, inoculated in 1:200 first antibody at room temperature for 3 h and cleansed by PBS-T, and then inoculated in 1:4000 second antibody for 3 h, cleansed by PBS-T, and exposed to ECL in dark room.

### Immunohistochemical method

Specimens from normal and abnormal gastric tissues (gastric carcinoma, atypical hyperplasia, and intestinalized gastric mucosa) were fixed in 10% formaldehyde, routinely dehydrated, cleaned, infiltrated with wax, embedded and made into serial 4 μm thick sections. The sections were dewaxed, stained with SP method, displayed by DAB, stained again with hematoxylin and blown dry. The specific operations were carried out following the instructions on the S-P test kit.

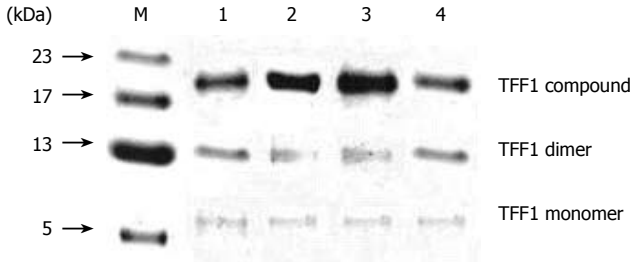


**Figure 1** Molecular forms of TFF1 in normal gastric mucosa by Western blot.

### Statistical analysis

All the data were analyzed by *t*-test using SPSS 10.0 software, and expressed as mean ± SD.

## RESULTS

### Western blot

Three patterns of TFF1 were found in normal gastric mucosa: monomer, dimer and TFF1 compound whose molecule weight is about 21 kDa. Among these three patterns, the concentration of TFF1 compound was the highest, followed by that of dimer, and monomer (Figure 1).

### Immunohistochemical method

Each section was photographed under 100 × high-power microscope. Motic Imaged Advanced 3.0 software was used to estimate the average positive *AO* of 20 glands randomly selected to reflect the intensity of TFF1 expression. The higher the *AO* was, the stronger the expression was.

TFF1 was expressed mainly in epithelial cytoplasm of the mucosa in gastric body and antrum, especially around the nuclei. The cytoplasm of positive cells was buffy after staining. The closer the TFF1 to the lumen, the deeper the color was. The expression of TFF1 in peripheral tissues of gastric carcinoma (0.51 ± 0.07) was higher than that in normal gastric mucosa (0.44 ± 0.06, $P < 0.001$). The expression of TFF1 in gastric adenocarcinoma was positively related to the differentiation of adenocarcinoma. The lower the differentiation of adenocarcinoma was, the weaker the expression of TFF1 was. No TFF1 was expressed in poorly-differentiated adenocarcinoma. The expression of TFF1 in moderately-well differentiated adenocarcinoma (0.45 ± 0.07) was a little lower than that in normal mucosa ($P > 0.05$). The expression of TFF1 in gastric mucosa with atypical hyperplasia (0.57 ± 0.03) was significantly higher than that in normal gastric mucosa ($P < 0.001$). No TFF1 was expressed in intestinalized gastric mucosa. There was no statistically significant difference between the expressions of TFF1 in gastric mucosa

**Table 1 Expression of TFF1 in gastric carcinoma and pathological changes prior to carcinogenesis (mean ± SD)**

| Classification | *n* | Average positive A |
|---|---|---|
| Normal | 35 | 0.44 ± 0.06 |
| Low-differentiated gastric carcinoma | 27 | None |
| Middle and highly differentiated gastric carcinoma | 11 | 0.41 ± 0.07[a] |
| Peripheral tissues of gastric carcinoma | 38 | 0.51 ± 0.07[b] |
| Atypical hyperplasia | 36 | 0.57 ± 0.03[b] |
| Intestinalization | 35 | None |
| Peripheral tissues of intestinalized mucosa | 35 | 0.45 ± 0.07[a] |
| Esophageal carcinoma | 20 | None |
| Peripheral tissues of esophageal carcinoma | 20 | None |

[a]$P > 0.05$ *vs* normal mucosa; [b]$P < 0.001$ *vs* normal mucosa.

around the intestinalized tissues (0.45 ± 0.07) and normal gastric mucosa ($P > 0.05$). No TFF1 was expressed in 20 specimens from patients with esophageal carcinoma (including peripheral tissues of carcinoma) (Table 1).

## DISCUSSION

Trefoil factor family (TFF) is a group of small molecule polypeptides and mainly secreted by gastrointestinal mucous cells. At present there are three kinds of trefoil peptides found in mammals, which are breast cancer-associated peptide (pS2 or TFF1), spasmolytic polypeptide (SP or TFF2) and intestinal trefoil factor (ITF or TFF3). These three patterns of TFF have a special structure, P-structure domain. This structure is composed of 38-39 amino acids sequenced by 6 highly conservative cysteine residues linked by three intramolecular disulfide bonds which make the whole peptide chain twisted and folded into trefoil-shaped structure[1]. Studies at home and abroad have proven that TFF plays an important role in protection and restitution of gastrointestinal mucosa[4-6].

TFF1, a member of TFF, was obtained from MCF-7 cell line of human mammary carcinoma by estrogen induction[7]. Each TFF1 molecule is composed of 60 amino acids and its molecular weight is 6674 kDa, including[8] cysteine residues, of which 6 take part in the constitution of P-structure domain and the 7th lies in the third base to the end of carboxyl side, i.e. $Cys^{58}$. Chadwick *et al*[5] replaced $Cys^{58}$ of recombinant TFF1 protein with $Ser^{58}$ and analyzed TFF1 containing $Ser^{58}$ by equilibrium ultracentrifugation, gel filtration, polyacrylamide gel electrophoresis, and mass spectrum. They found that homologous dimer could present in TFF1, but not in TFF1 analog containing $Ser^{58}$, suggesting that $Cys^{58}$ can form intermolecular disulfide bond and shape the dimer. In the present study, three patterns of TFF1 were found in normal gastric mucosa: monomer (6.5 kDa), dimmer and TFF1 compound whose molecule weight is about 21 kDa. The concentration of TFF1 compound was the highest among these three patterns, followed by that of dimer and monomer, indicating that the biological activity of TFF1 may be related to the formation of homologous dimmer or other oligomers composed of heterogenous proteins.

At present, there are two hypotheses concerning the mechanism underlying trefoil peptide protection in gastric mucosa. Trefoil peptide could bind to glycoprotein in mucus to form stable gel compounds, which could reinforce the mucous gel layer and decrease injury to mucosa by harmful substance in gastric surface and mechanical stress, *etc*. *In vivo* and *vitro* studies have shown that trefoil peptide can increase viscosity of mucous gel layer, decrease the ability of H+ to penetrate mucous gel layer and injuries to gastric mucosa[9]. Trefoil peptide is likely to accomplish its biologic function by binding to its receptor or transport protein. To find the possible receptor or transport protein and explore their mechanism are one of the hot topics in international studies on trefoil peptide[10-12]. TFF1 compound found in this study is likely composed of TFF1 and its receptor, transport protein or some glycoproteins. However, different molecules binding to TFF1 have been reported by different scholars. This diversity might be related to different experimental conditions, the ability of trefoil peptide to bind to different molecules, and the unstable structure of molecules binding to trefoil peptide. All the molecules are parts of the possible binding molecule. No consensus has been reached upon the real causes of the diversity. Moreover, no breakthrough has been made in finding the possible receptor or transport protein.

TFF1 is mainly expressed in epithelial cells of the mucosa in gastric body and antrum, but the specificity of expression disappears in pathological tissues[2]. TFF1 can be expressed in injured parts of gastrointestinal mucosa[3], much higher than that in normal mucosa. In the present study, the expression of TFF1 was higher in mucosa with peptic ulcer or gastritis, mucosa around gastric carcinoma and mucosa with stress ulcer induced by aspirin in rabbits than that in normal mucosa, which is consistent with other studies[13,14]. It has been proved that the expression of TFF1 is up-regulated in malignant carcinoma tissues. A hot topic of studies at home and abroad is whether carcinoma leads to the excessive expression of trefoil peptide. In mouse model of TFF1 gene knockout, all gastric epithelial cells showed severe hyperplasia, high dysplasia and adenoma in gastric antrum, which may develop into gastric infiltrative adenocarcinoma[15]. However, the excessive expression of TFF1 in mammal tissues of transgenic rat model does not lead to hyperplasia and dysplasia[16]. TFF1 could restrain proliferation of gastric adenocarcinoma cell line AGS and its inhibition is dose-dependent. TFF1 dimer has a much higher biologic activity than monomer[17], suggesting that TFF1 is a kind of tumor suppression factor. In our experiment, the expression of TFF1 in peripheral tissues of gastric adenocarcinoma was much higher than that in normal gastric mucosa, illustrating that TFF1 is relevant to tumor suppression. The formation of carcinoma promotes the secretion of TFF1 to restrain proliferation of carcinoma, leading to less or no expression of TFF1 in carcinoma tissues. The reasons why the lower the differentiation, the weaker the expression, might be as follows. The decreased expression of TFF1 may lead to formation of carcinoma, and destruction of glands and cells in carcinoma tissues may lead to decreased secretion of TFF1. The poorer the differentiation is, the

more seriously the glands and cells are destructed, the less the TFF1 is secreted and expressed. In our study, the expression of TFF1 in moderately-well differentiated adenocarcinoma was a bit lower than that in normal mucosa, but there was no statistically significant difference due to the limited specimens.

At present, more and more attention has been paid to the relationship between precancerous change and carcinogenesis. Intestinalized gastric mucosa and atypical hyperplasia are the most common pathological changes. Our study showed that the expression of TFF1 in atypical hyperplasia was much higher than that in normal mucosa. When the glands of gastric mucosa are not destroyed in early stage of gastric carcinogenesis, the secretion of TFF1 might be promoted to protect gastric mucosa against damage. in intestinalized gastric mucosa, TFF1 was expressed in peripheral tissues of intestinalized mucosa but not in intestinalized gastric. The possible reasons might be as follows. Goblet cells could not express TFF1, and lack of TFF1 induces intestinalization of epithelial cells in gastric mucosa, indicating that TFF1 might play a role in gastric carcinogenesis. Some studies showed that TTF1 can protect mucosa against damage and suppress carcinogenesis, while other studies showed that TTF1 can restrict cell adherence, promote cancer cell invasion, and block necrosis of cancer cells. These findings indicate that TFF1 may be an indicator of metastasis, carcinoma invasion, and poor prognosis[18,19]. Further study is needed to explore the relationship between trefoil peptide and carcinoma.

In addition, no TFF1 is expressed in esophageal carcinoma and its peripheral tissues, and TFF1 is irrelevant to esophageal carcinogenesis and suppression.

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*RAPID COMMUNICATION*

# Rapid quantification of hepatitis B virus DNA by real-time PCR using efficient TaqMan probe and extraction of virus DNA

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## Abstract

**AIM:** To rapidly quantify hepatitis B virus (HBV) DNA by real-time PCR using efficient TaqMan probe and extraction methods of virus DNA.

**METHODS:** Three standards were prepared by cloning PCR products which targeted S, C and X region of HBV genome into pGEM-T vector respectively. A pair of primers and matched TaqMan probe were selected by comparing the copy number and the Ct values of HBV serum samples derived from the three different standard curves using certain serum DNA. Then the efficiency of six HBV DNA extraction methods including guanidinium isothiocyanate, proteinase K, NaI, NaOH lysis, alkaline lysis and simple boiling was analyzed in sample A, B and C by real-time PCR. Meanwhile, 8 clinical HBV serum samples were quantified.

**RESULTS:** The copy number of the same HBV serum sample originated from the standard curve of S, C and X regions was $5.7 \times 10^4$/mL, $6.3 \times 10^2$/mL and $1.6 \times 10^3$/mL respectively. The relative Ct value was 26.6, 31.8 and 29.5 respectively. Therefore, primers and matched probe from S region were chosen for further optimization of six extraction methods. The copy number of HBV serum samples A, B and C was $3.49 \times 10^9$/mL, $2.08 \times 10^6$/mL and $4.40 \times 10^7$/mL respectively, the relative Ct value was 19.9, 30 and 26.2 in the method of NaOH lysis, which was the efficientest among six methods. Simple boiling showed a slightly lower efficiency than NaOH lysis. Guanidinium isothiocyanate, proteinase K and NaI displayed that the copy number of HBV serum sample A, B and C was around $10^5$/mL, meanwhile the Ct value was about 30. Alkaline failed to quantify the copy number of three HBV serum samples. Standard deviation (SD) and coefficient variation (CV) were very low in all 8 clinical HBV serum samples, showing that quantification of HBV DNA in triplicate was reliable and accurate.

**CONCLUSION:** Real-time PCR based on optimized primers and TaqMan probe from S region in combination with NaOH lysis is a simple, rapid and accurate method for quantification of HBV serum DNA.



**Key words:** Hepatitis B virus; Serum DNA; Real-time PCR; Extraction method

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## INTRODUCTION

Hepatitis B virus (HBV) is a human hepadnavirus that causes acute and chronic hepatitis and hepatocellular carcinoma[1]. Although an effective vaccine has been available for two decades, an estimated 350 million people worldwide are chronically infected with HBV[2]. The conventional ways available for curing this disease are not very efficient. A significant proportion of chronic infections terminate in hepatocellular carcinoma, leading to more than one million deaths annually[3]. The diagnosis and clinical monitoring of HBV infection are based on the detection of viral antigens, antibodies to viral proteins, and circulating viral genome (HBV DNA)[4-6]. There is increasing evidence that measuring the level of HBV DNA in serum is useful in monitoring the efficacy of antiviral therapy, detecting the occurrence of drug-resistant mutants and relapse after discontinuing antiviral therapy[7-9]. An accurate quantitative assay for serum HBV

DNA may monitor residual viral load during treatment and allow the timely detection of flares of viral replication that accompany the emergence of variants.

In China, enzyme-linked immunosorbent assay (ELISA) is still a main detection method for HBV infection, but ELISA results neither efficiently reflect serum viral load or hepatitis activity nor monitor the efficacy of antiviral treatments. In recent years, real-time PCR detection assays based on SYBR Green I[10,11] and TaqMan probe[12-19] have been proposed for quantification of HBV DNA in serum. In the former assay, SYBR Green I can specifically bind to double-stranded DNA (dsDNA) rather than to single-stranded DNA (ssDNA). Fluorescence is monitored once each cycle after product extension and increases above background fluorescence at a cycle number that is dependent on the initial template concentration. Unfortunately, the dye detects all dsDNAs, including primer dimer and other nonspecific products.

The real-time PCR method based on the TaqMan probe uses a dual-labeled fluorescent probe containing a reporter dye that is quenched by a second fluorescent dye[20]. Hydrolysis of the probe by polymerase exonuclease activity separates the reporter from the quencher as the amplification proceeds, the fluorescence signal from the reporter increases cumulatively. The cycle at which fluorescence exceeds background, known as the threshold cycle, is inversely related to the initial copy number, thus allowing quantitative analysis.

The efficacy and accuracy of real-time PCR largely depend on the primers and probe[14,21], and are also related to the extraction method of HBV DNA[22,23]. A simple, rapid, efficient method for DNA extraction is crucial to the success of real-time PCR and the subsequent analysis. We describe here a rapid, convenient real-time quantitative assay for serum HBV DNA which combines optimized primers and probe with simple HBV DNA extraction method. The assay was designed to enable accurate quantification of clinical serum samples, which makes it a useful clinical test to monitor serially the efficacy of antiviral therapy.

## MATERIALS AND METHODS

### Preparation of standards

Serum HBV DNA was extracted by NaOH lysis as previously described[26]. In brief, 50 μL of HBV positive serum was mixed with an equal volume of 0.4 mol/L NaOH and then the mixture was incubated at 80℃ for 10 min, followed by centrifugation for 30 s at 15000 × *g*. After that the supernatant was carefully transferred to a new microcentrifuge tube and supplemented with 25 μL of 0.4 mol/L Tris-HCl (pH7.5). Two microliters of HBV DNA was used as a template in PCR.

Three pairs of primers were designed by software Beacon Designer 2.1 in the conserved region of S, C and X gene respectively and synthesized by Sangon Co., Ltd, Shanghai. The sequences of each pair of primers were as follows: RSU (5'-AGAATCCTCACAATACCGCAGAGT-3') and RSL (5'-CACACGGTAGTTCCCCCTAGAA-3'), RCU (5'-GTCTTTCGGAGTGTGGATTCG-3') and RCL (5'-CGGCGATTGAGACCTTCGT-3'), RXU (5'-ACTCCCCGTCTGTGCCTTCT-3') and RXL (5'-CATTCGGTGGGCGTTCAC-3').

Three amplified products were cloned into the pGEM-T vector (Promega, USA), and then the recombinant HBV plasmids pGEM-S, pGEM-C and pGEM-X were serially diluted from $10^7$ to $10^3$ copies/μL after identification by PCR. One microliter of each diluted recombinant plasmid was used as a standard PCR template.

### TaqMan probes

Taqman probes targeting S, C and X genes were designed by software Beacon Designer 2.1 and synthesized in Sangon Co., Ltd, Shanghai. The sequences of PS, PC, and PX were 5'FAM-AGACTCGTGGTGGACTTCTCTCAAT-TAMARA3', 5'FAM-TCCCCTAGAAGAAGAACTCCCTCGCCTC-TAMARA3', and 5'FAM-CCGGACCGTGTGCACTTCGCTT-TAMARA3', respectively.

### DNA extraction

Six methods (including guanidinium isothiocyanate, proteinase K, NaI, NaOH lysis, alkaline lysis, as well as boiling) were used to extract serum HBV DNA from three serum samples[24-28]. Two microliters of HBV DNA was used as a template in real-time PCR.

In the methods of GuSCN, proteinase K and NaI lysis, protocol after the lysis process was the same, though components of lysis were different. GuSCN lysate consisted of 1 mol/L Tris-HCl (pH8.0), 0.5 mol/L EDTA (pH8.0), 100 g/L SDS, 200 mg/L proteinase K[24]. Proteinase K lysate contained 10 mmol/L Tris-HCl (pH8.0), 10 mmol/L EDTA (pH8.0), 0.5% SDS, 150 mmol/L NaCl and 200 mg/L proteinase K[27]. NaI extraction buffer included 6 mol/L NaI, 0.5% SDS, 26 mmol/L Tris-HCl (pH8.0) and 13 mmol/L EDTA (pH8.0)[25]. First, 100 μL lysate was added into an equal volume serum, then the mixture was incubated at 37℃ for 1 h, 55℃ for 2 h and 60℃ for 15 min in GuSCN, proteinase K and NaI lysis methods respectively. After that, 200 μL chloroform/isoamyl alcohol (24:1) was added to extract products and the supernatants were removed into new eppendorf tubes after centrifugation at 15000 × *g* for 15 min, followed by alcohol precipitation and further washing[24,25,27]. At last, naturally dried DNA precipitates were dissolved in 30 μL 0.1 × TE.

The easily manipulated alkaline lysis and boiling extraction were similar to the above NaOH lysis, except for the lysate. The lysate used in alkaline lysis was composed of 1 mol/L NaOH, 2 mol/L NaCl and 0.5% SDS[26], while the boiling extraction consisted of an equal volume of PBS[28].

### Optimization of primers and probes

Real-time PCR amplification was performed in 20 μL

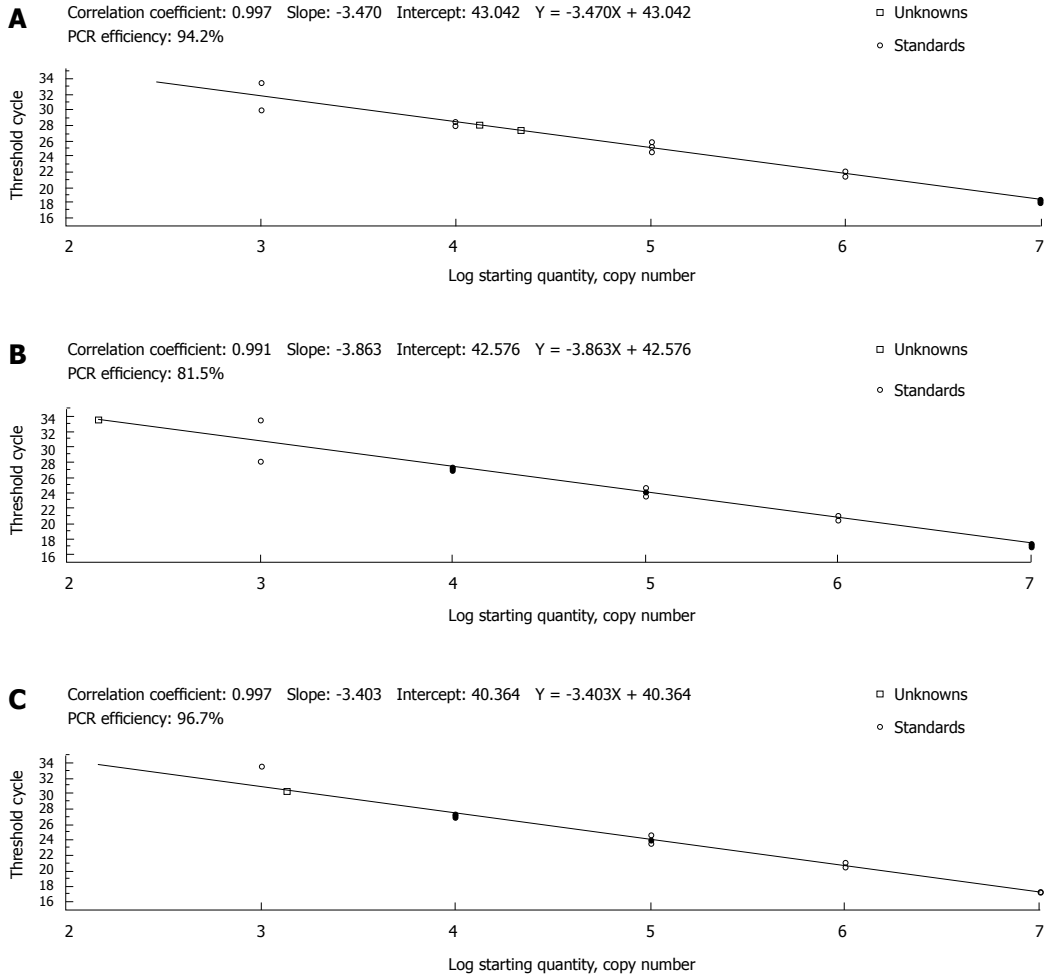


**Figure 1** Ct values and copy number of HBV serum samples originated from three standard curves corresponding to the S (**A**), C (**B**) and X (**C**) regions respectively. Each real-time PCR reaction was performed in triplicate.

reaction mixture containing 1 μL standard DNA or 2 μL isolated serum HBV DNA, 2 × PCR reaction buffer [100 mmol/L Tris-HCl (pH8.3), 100 mmol/L KCl, 7.0 mmol/L $MgCl_2$, 400 μmol/L each of the deoxynucleotide triphosphates (dNTP), 1U hot star DNA polymerase Takara, Japan], 5pmol of each pair of primers and 2.5 pmol of corresponding TaqMan probe. All amplification reactions were performed in triplicate. After preparation of the reaction mixtures in 96-well plates, amplification was performed as follows: initial denaturation at 94℃ for 3 min, followed by 40 cycles at 94℃ for 20 s, 60℃ for 40 s. Fluorescence readings were recorded at 60℃ in each cycle. Results were analyzed by the software iCycler$^{TM}$iQ 3.0a provided with the iCycler system (Bio-Rad, USA).

### *Evaluation of six DNA extraction methods*

To evaluate HBV DNA extraction methods, RSU, RSL primers and PS probe were used in real-time PCR. Amplification and analysis were performed as above.

### *Quantification of HBV viral DNA in sera by real-time PCR*

NaOH lysis was performed to extract serum HBV DNA from 8 clinical serum samples. The selected RSU, RSL and PS were used in real-time PCR. The procedures of real-time PCR and standard curve analysis were described as above.

**Table 1 Copy number and Ct values of HBV serum samples derived from three different standard curves (mean ± SD)**

| Standard curve | Ct | | Copy number ( /mL) | |
|---|---|---|---|---|
| | Mean ± SD | CV(%) | Mean ± SD | CV (%) |
| S | 26.6 ± 0.061 | 0.2 | $5.7 \times 10^4 \pm 2.29 \times 10^3$ | 4.0 |
| C | 31.8 ± 0.073 | 0.2 | $6.3 \times 10^2 \pm 2.73 \times 10^1$ | 4.3 |
| X | 29.5 ± 0.357 | 1.2 | $1.6 \times 10^3 \pm 1.77 \times 10^2$ | 11.1 |

CV: Coefficient variation.

## RESULTS

### *Linear standard curve and optimization of primers and TaqMan probes*

Three recombinant plasmids pGEM-S, pGEM-C and pGEM-X were used as standards to set up the real-time protocol and construct the standard curve. Forty cycles of amplification allowed us to obtain linear quantification between $1 \times 10^3$ and $1 \times 10^7$ copies/μL per reaction (Figure 1). The highest copy number ($5.7 \times 10^4$/mL) and the lowest Ct value (26.6) of the same HBV serum samples were observed from the standard curve originated from S region (Table 1). The lowest copy number ($6.3 \times 10^2$/mL) and the highest Ct value (31.8) were displayed from the

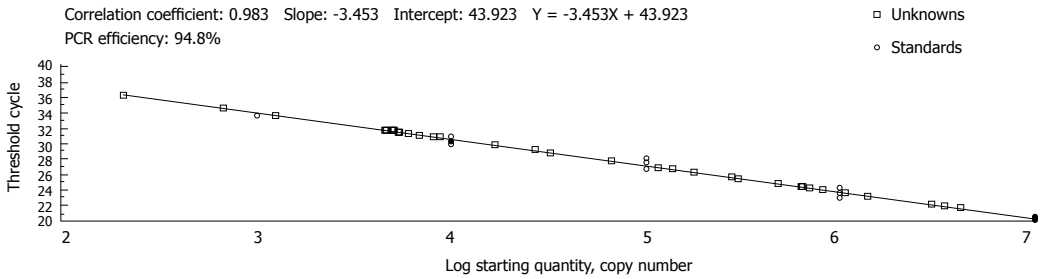


**Figure 2** A standard curve for evaluation of HBV DNA extraction methods.

**Table 2 Copy number and Ct values of HBV serum samples detected by six different extraction methods (mean ± SD)**

| Method | Copy number/mL and Ct value | | |
|---|---|---|---|
| | Sample A | Sample B | Sample C |
| Guanidinium isothiocyanate | $1.41 \times 10^5 \pm 1.23 \times 10^4$ | $1.92 \times 10^5 \pm 1.70 \times 10^4$ | $3.41 \times 10^4 \pm 3.65 \times 10^3$ |
| | 31.3 ± 0.16 | 30.9 ± 0.07 | 33.3 ± 1.59 |
| Proteinase K | $2.99 \times 10^5 \pm 2.88 \times 10^4$ | $2.21 \times 10^5 \pm 2.97 \times 10^4$ | $3.59 \times 10^5 \pm 5.10 \times 10^4$ |
| | 30.5 ± 0.19 | 30.3 ± 0.38 | 29.6 ± 0.38 |
| NaI | $2.51 \times 10^5 \pm 2.91 \times 10^4$ | $2.12 \times 10^5 \pm 3.01 \times 10^4$ | $9.12 \times 10^4 \pm 9.65 \times 10^3$ |
| | 30.7 ± 0.17 | 30.5 ± 0.66 | 31.4 ± 0.14 |
| NaOH lysis | $3.49 \times 10^8 \pm 2.72 \times 10^7$ | $2.08 \times 10^6 \pm 2.80 \times 10^5$ | $4.40 \times 10^7 \pm 2.06 \times 10^6$ |
| | 19.9 ± 0.18 | 30.0 ± 0.52 | 26.2 ± 0.78 |
| Alkaline lysis | N/ N | N/ N | N/ N |
| Boiling | $2.14 \times 10^8 \pm 2.16 \times 10^7$ | $1.83 \times 10^6 \pm 1.05 \times 10^5$ | $1.48 \times 10^7 \pm 1.22 \times 10^6$ |
| | 23.9 ± 0.10 | 30.1 ± 0.69 | 27.4 ± 0.94 |

N: Negative.

**Table 3 Copy number and Ct values of 8 clinical HBV serum samples detected by optimized primers, TaqMan probe from S region and NaOH lysis method (mean ± SD)**

| Sample | Ct | | Copy number /mL | |
|---|---|---|---|---|
| | Mean ± SD | CV (%) | Mean ± SD | CV (%) |
| 1 | 25.4 ± 0.24 | 0.9 | $1.4 \times 10^9 \pm 2.38 \times 10^8$ | 17.0 |
| 2 | 31.9 ± 0.20 | 0.6 | $1.4 \times 10^7 \pm 1.87 \times 10^6$ | 13.4 |
| 3 | 31.5 ± 0.52 | 1.7 | $1.8 \times 10^7 \pm 2.32 \times 10^6$ | 12.9 |
| 4 | 27.3 ± 0.14 | 0.5 | $3.7 \times 10^8 \pm 3.73 \times 10^7$ | 10.0 |
| 5 | 34.0 ± 0.27 | 8.1 | $6.4 \times 10^6 \pm 6.08 \times 10^5$ | 9.5 |
| 6 | 32.3 ± 0.52 | 0.2 | $1.1 \times 10^7 \pm 4.43 \times 10^6$ | 4.0 |
| 7 | 27.0 ± 0.23 | 0.8 | $4.6 \times 10^8 \pm 7.22 \times 10^6$ | 15.7 |
| 8 | 33.9 ± 0.12 | 0.4 | $3.1 \times 10^6 \pm 2.76 \times 10^5$ | 8.9 |

CV: Coefficient variation.

standard curve of C region. The highest copy number from S region was about 90.5 and 35.6 multiple to the C and X regions. Hence, RSU, RSL primers and PS probe were chosen for further analysis because of the efficient amplification.

### *Different DNA extraction methods*

To explore a simple and fast method for serum HBV DNA extraction, six different methods were compared by real-time PCR in combination with selected primers and TaqMan probe from S region. The copy number and Ct values of three HBV serum samples on the standard curve (Figure 2 and Table 2) showed that NaOH lysis and boiling were predominately advantageous over the other methods. Adversely, alkaline lysis failed to detect any HBV DNA in all samples. There was no significant difference among the other three methods.

### *Quantitation of HBV viral DNA in sera by real-time PCR*

Table 3 indicated the copy number and Ct values of 8 positive HBV serum samples based on the optimized primers and TaqMan probe as well as DNA extraction. Their SD and CV unfolded that quantification of hepatitis B virus DNA in triplicate by real-time PCR was reliable and accurate.

## DISCUSSION

Detection and analysis of PCR products should be carried out simultaneously with temperature cycling during amplification. If the fundamental properties of DNA, such as product size, quantity, sequence, or melting profile can specifically identify the products during PCR, no further analysis is required.

Real-time PCR is a powerful diagnostic tool capable of rapidly generating reliable and reproducible results with reduced risks of cross contamination[29]. The amplification efficiency can be detected with fluorescent probes. All steps are accomplished automatically by computer except for sample preparation, which can monitor PCR reaction timely.

To make our assay more sensitive and efficient, we

optimized three pairs of primers and probes targeting the conserved S, C and X regions of HBV genome respectively. The highest copy number and Ct value of HBV serum samples were observed on the standard curve originated from S region. This indicated that the sensitivity of real-time PCR can be improved by adjusting primes and probe[21].

HBV DNA extraction from serum is a key step in real-time PCR, as it directly affects the accuracy of quantification of viral load. NaOH lysis showed that the highest copy number of HBV serum samples A, B, and C was $3.49 \times 10^8$/mL, $2.08 \times 10^6$/mL and $4.40 \times 10^7$/mL respectively. Boiling was as effective as NaOH lysis in DNA extraction from HBV serum samples A, B, and C. The copy number of HBV serum samples A, B, and C detected by boiling was $2.14 \times 10^8$/mL, $1.83 \times 10^6$/mL, and $1.48 \times 10^7$/mL, respectively. However, boiling was unsuitable for DNA extraction from serum with high viscidity[28].

Compared with the methods of NaOH lysis and boiling, guanidinium isothiocyanate, proteinase K and NaI decreased the copy number of HBV serum samples A, B and C to approximately $10^2$-$10^3$/mL. Proteinase K lysis was a bit better than the other two methods. In all three methods, the lysis was mixed with serum and extracted with chloroform/isoamyl alcohol (24:1), followed by precipitation and washing with absolute alcohol. A large quantity of DNA was lost due to the complicated process of these methods. DNA extraction with alkaline failed to detect serum DNA in our experiment, possibly due to the high concentration and pH value. On the contrary, 0.4 mol/L NaOH lysis followed by neutralization with 0.4 mol/L Tris-HCI (pH7.6) showed excellent efficacy because of a moderate alkaline concentration and lower pH value which may significantly improve the efficiency of PCR amplification.

In conclusion, real-time PCR based on the optimized primers, probe and DNA extraction is a simple, accurate, specific and sensitive method for the measurement of HBV viral load in serum. NaOH lysis for HBV serum DNA extraction is rapid, simple and efficient, making the assay suitable for handling a large number of clinical HBV serum samples. Since there are about 200 million HBV carriers in China, doctors need to know the HBV status of patients before starting any medical treatment. This assay may be especially useful for monitoring the therapeutic effects in chronically infected patients on antiviral therapy.

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*RAPID COMMUNICATION*

# Diagnosis and treatment of small intestinal bleeding: Retrospective analysis of 76 cases

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**Key words:** Small intestine; Hemorrhage; Neoplasia; Meckel's diverticulum

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## Abstract

**AIM:** To investigate the causes of small intestinal bleeding as well as its diagnosis and therapeutic approaches.

**METHODS:** A retrospective analysis was conducted according to the clinical records of 76 patients with small intestinal bleeding admitted to our hospital in the past 5 years.

**RESULTS:** In these patients, tumor was the most frequent cause of small intestinal bleeding (37/76), followed by Meckel's diverticulum (21/76), angiopathy (15/76) and ectopic pancreas (3/76). Of the 76 patients, 21 were diagnosed by digital subtraction angiography, 13 by barium and air double contrast X-ray examination of the small intestine, 11 by $^{99m}$Tc-sestamibi scintigraphy of the abdominal cavity, 6 by enteroscopy of the small intestine, 21 by laparoscopic laparotomy, and 4 by exploratory laparotomy. Although all the patients received surgical treatment, most of them (68/76) received part enterectomy covering the diseased segment and enteroanastomosis. The follow-up time ranged from 1 year to 5 years. No case had recurrent alimentary tract bleeding or other complications.

**CONCLUSION:** Tumor is the major cause of small intestinal bleeding followed by Meckel's diverticulum and angiopathy. The main approaches to definite diagnosis of small intestinal bleeding include digital subtraction angiography, $^{99m}$Tc-sestamibi scintigraphy of the abdominal cavity, barium and air double contrast X-ray examination of the small intestine, laparoscopic laparotomy or exploratory laparotomy. Part enterectomy covering the diseased segment and enteroanastomosis are the most effective treatment modalities for small intestinal bleeding.



## INTRODUCTION

Due to the lack of specific clinical symptoms and physical signs of small intestinal bleeding and the limitations in the present diagnostic methods for tumors inside the alimentary tract, early diagnosis of small intestinal bleeding is difficult[1-3]. In this article, we retrospectively reviewed and analyzed the diagnostic and therapeutic experiences with 76 cases of small intestinal bleeding patients admitted to our hospital between April 1999 and April 2004.

## MATERIALS AND METHODS

### *Clinical data*

A total of 76 patients with small intestinal bleeding (44 males and 32 females) were included in the present study. Their age ranged from 15 to 74 years with a mean of 43.5 years. Alimentary tract hematorrhea occurred in all 76 patients. Intermittent dark and bloody stools were found in 57 patients and recurrent dark and bloody stools in 19 with hemorrhagic shocks. Forty patients had accompanying abdominal pain, 4 had accompanying fever and 6 had tangible abdominal lumps.

### *Diagnosis procedures*

All the 76 patients underwent gastroduodenoscopy, colonscopy, barium meal examination or barium enema to exclude the possible lesions in the stomach, duodenum, colon or rectum. Barium and air double contrast X-ray examinations of small intestine were performed in 43 patients, of them 12 were diagnosed having small intestinal tumors including 1 having Meckel's diverticulum. $^{99m}$Tc-sestamibi scintigraphy of the abdominal cavity (ECT) was performed in 36 patients (Figure 1A and 1B). Of them, 16 were definitely diagnosed having small intestinal

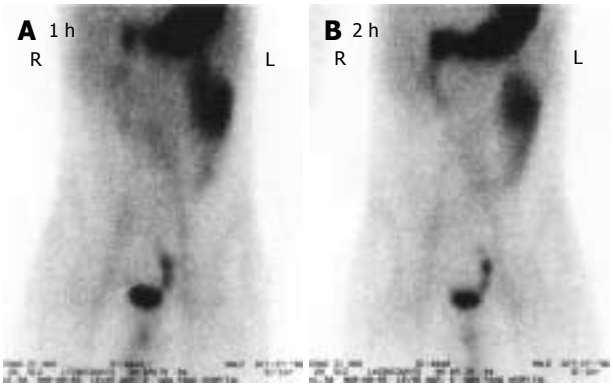


**Figure 1** ECT examination showing small intestinal bleeding at 1 h (**A**) and 2 h (**B**) after intravenous injection of the tracer. Serial planar imaging (1, 5, 15 and 30 min, 1 and 2 h) of the whole abdomen was performed. There was a radioactive accumulation at 5 min on the upper-left corner of the gall bladder, becoming increasingly dense till 2 h. Its emerging time was almost simultaneously as the image of the stomach with the density being slightly lower than that of the stomach image. During the whole period of imaging, its location was comparatively stable. The dynamic imaging of the whole abdomen showed an abnormal radioactive imaging near the upper-left corner of the gall bladder, which was considered the ectopic stomach mucous tissue inside the Meckel's diverticulum.

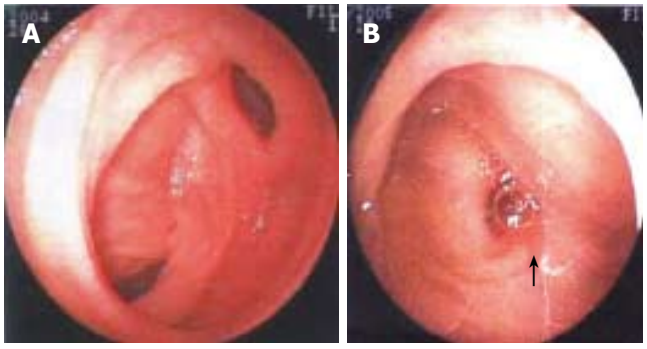


**Figure 2** Double-balloon enteroscopy showing small intestinal bleeding (**A**) and Meckel's diverticulum (**B**). Double-balloon enteroscope was pushed 200 cm into the ileum through the anus. There was a diverticulum in the ileum 90-100 cm away from the ileocecal valve, at the opening of which there was an ulcer (1.2 cm × 1.0 cm). The ulcer was covered by a thin lichenoid substance without active bleeding. No other abnormalities were found. The diagnosis of Meckel's diverticulum was established.

bleeding including 4 having Meckel's diverticulum and 2 having small intestinal tumor. Of the 25 patients who received small intestine enteroscopy, 11 had suspected small intestinal bleeding, and 6 had Meckel's diverticulum (Figure 2A and 2B). Of the 24 patients who received digital subtraction angiography (DSA), 18 were found to have definite small intestinal bleeding, 2 having Meckel's diverticulum and 6 having small intestinal tumor. Of the 21 patients who received laparoscopic laparotomy, 4 had Meckel's diverticulum, 4 had definite small intestinal bleeding (Figure 3A and 3B).

## RESULTS

Tumor was the major cause of small intestinal bleeding, which was found in 37 patients. Of these patients 29 were found to have benign stromal tumor, 3 had malignant stromal tumor and 5 had adenocarcinoma (Figure 4A and

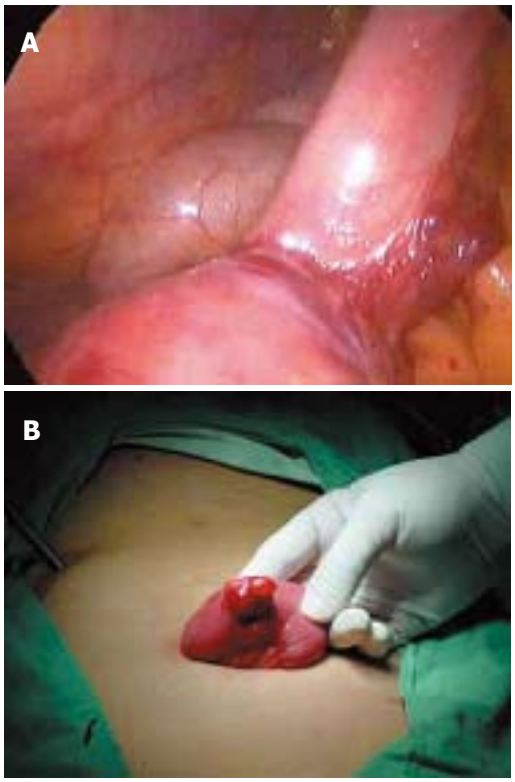


**Figure 3** Laparoscopic laparotomy showing small intestinal bleeding (**A**) and oval stromal tumor (**B**). An oval stromal tumor was found on the upper segment of jejunum with clear borderline and smooth surface but without adhesion to other tissues during laparoscopic laparotomy. Expansive abdominal excision was performed to draw out the tumor.

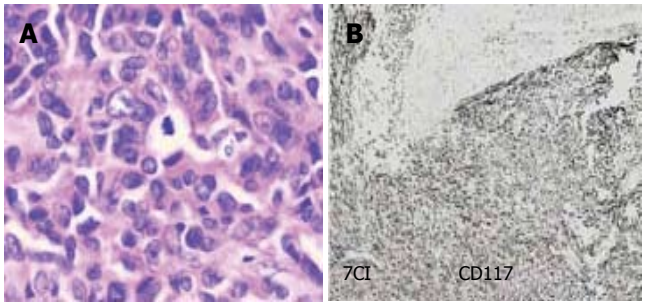


**Figure 4** Pathological and immunohistological examination of small intestinal bleeding caused by small intestinel stromal tumor. Arrangement of tumor cells in interlock weaving form with abundant cytoplasm, light staining, unclear borderline and diffused infiltration of chronic inflammatory cells but without necrosis and hemorrhage, karyokinesis could be seen (in the middle of **A**). Immunohistochemical findings were as follows: CD117 (+) (**B**), CD34 (-), S-100 (-), Vimentin (weak +) (HE × 200).

4B). Other causes of small intestinal bleeding included Meckel's diverticulum, angiopathy and ectopic pancreas, which were observed in 21, 15 and 3 patients, respectively. Jejunum bleeding in the small intestine was found in 38 (50%), ileum bleeding in 31 (40%) and multi-foci bleeding in 7 (10%) patients, respectively.

All the patients received surgical treatment, of them 49 received part enterectomy covering the diseased segment and enteroanastomosis, 12 had laparoscopic part enterectomy covering the diseased segment and enteroanastomosis, 9 had laparoscope-assisted

part enterectomy covering the diseased segment and enteroanastomosis, 3 had partial excision of ileum and half of the right colon for intestinal cancer, and 3 had conservative excision of intestinal cancer.

One patient with small intestine stromal tumor had recurrent bleeding 10 d after the operation. Stomal ulcer was confirmed by laparotomy and the stoma was then removed. Postoperative death occurred in 3 patients, of them 2 died of disseminated intravascular coagulation (DIC) and 1 of severe general infection. The rest 73 patients were cured and discharged from the hospital. Their follow-up time ranged from 1 year to 5 years with a mean of 2.8 years. By the time of this retrospective analysis, all these people were alive except for 2 deaths due to recurrent malignant stromal tumor of the small intestine and 3 deaths due to small intestinal adenocarcinoma. Those who were still alive at the time of this analysis had no alimentary tract bleeding or other complications.

## DISCUSSION

The 3-5 m long small intestine accounts for 75% of the whole gastrointestinal tract. The ansa intestinalis is long-winding and overlapping with active peristalsis and its position in the abdominal cavity varies greatly. Therefore, quick and accurate determination of the cause and foci of small intestinal bleeding is a great challenge for surgeons in clinical practice[1-4]. For unknown causes of alimentary tract bleeding, endoscopy should be first performed to exclude the possible lesions in stomach, duodenum, colon or rectum in order to confirm the bleeding originating from small intestinal segment inferior to duodenum. Subsequently, the bleeding causes can be diagnosed through related examinations.

Small intestine enteroscopy or barium and air double contrast X-ray examination of small intestine can be performed in patients with their bleeding arrested or with little bleeding and stable vital signs to determine the cause of bleeding[2,4]. ECT, DSA, exploratory laparotomy or laparoscopic laparotomy should be performed in patients with unstable vital signs and active bleeding as early as possible to determine the bleeding foci. B-mode ultrasonography or CT scanning should be carried out in patients with abdomen lumps to determine the origin and type of lumps as well as the interrelation with its neighboring organs.

Due to the lack of specific clinical symptoms and physical signs of small intestinal bleeding and the limitations of the present diagnostic approaches to finding lesions inside the alimentary tract, the early diagnosis of small intestinal bleeding is difficult. Barium and air double contrast X-ray examinations of small intestine are the most widely used methods for the diagnosis of small intestinal bleeding. However, since the foci of small intestinal bleeding usually grow outwards, it is difficult to show the tumor, thus resulting in false negatives. Of the 43 patients who received double-contrast radiology of small intestine, 13 were found to be positive for small intestinal bleeding, including 12 cases of small intestinal tumor and 1 case of Meckel's diverticulum.

Diagnosis of small intestinal bleeding by enteroscopy is time-consuming[5-7]. It is unpleasant for patients and may result in complications of bleeding and perforation or has a high rate of false negative, therefore, its clinical application is confined[8,9]. Recently, a capsule endoscope is under clinic experiment, but this examination cannot perform biopsy and make pathological diagnosis[10-12]. Of the 25 patients who received enteroscopy of small intestine more than once, 11 had suspected small intestinal bleeding and 6 had small intestinal bleeding. The diagnosis rate was less than 8%. Small intestine enteroscopy was performed 5 times in one stromal tumor patient with no positive findings. DSA is the most effective approach to diagnosis of alimentary tract bleeding which shows specific signs of tumor and angiopathy, while embolismic therapy can be performed during DSA diagnosis[3,4]. However, embolismic therapy for small intestinal bleeding has unsatisfying therapeutic efficacy. Even if the embolism is hyper-selective, hematorrhea could reoccur in several hours. Of the 24 patients who underwent DSA, 18 were found to be positive for small intestinal bleeding (including angiopathy in 3, small intestinal stromal tumorin 5, and small intestinal diverticulum in 2 patients). Hematorrhea reoccurred 4-6 h after the embolismic therapy in all the patients.

$^{99m}$Tc-marked erythrocytes for alimentary bleeding imaging are more sensitive to minor intestinal bleeding, and can only determine the position of the bleeding foci[4]. Of the 36 patients who received $^{99m}$Tc-sestamibi scintigraphy, 16 had positive findings of Meckel's diverticulum. Abdominal B-mode ultrasonography and CT scanning have limited diagnostic value because stromal tumor, angiocavernoma and Meckel's diverticulum of small intestine are usually small[13]. Of the patients who received CT scanning, only 4 were found to have abdominal lumps before the operation and none of them could be located. However, CT scaning can display the surrounding tissues of tumor and the involved organs, showing the origin and type of tumor as well as interrelation with its neighboring organs. Based on such findings, distant metastasis can be judged, suggesting that this diagnostic way provides valuable reference for surgical treatment.

Tumor is the major cause of small intestinal bleeding followed by diverticulum and angiopathy of small intestine. Most stromal tumors found in patients with intestinal bleeding caused by tumor are usually benign in nature. Stromal tumors are more common than small intestinal adenocarcinoma, which is not entirely consistent with other studies. Meckel's diverticulum and angiopathy are the second and third-frequent causes of small intestinal bleeding in such patients. The data reveal that jejunum bleeding often indicates small intestine stromal tumor, while ileum bleeding often originates from Meckel' s diverticulum. There is no definite location of small intestinal angiopathy, but most cases of angiopathy occur in the middle section of small intestine. Angiocavernoma is commonly restricted yet vascular malformation is usually multi-foci, distributing in segments or even spreading along the entire small intestine and its mesentery.

Because of the difficult location and diagnosis of small intestinal bleeding as well as the cautious attitude of surgeons, surgical treatment is only considered for patients with more-than-once negative test results,

recurrent hematorrhea and therapeutic failure. Exploratory laparotomy should be performed as early as possible for patients with unknown alimentary tract bleeding causes or for patients who have no specific cause discovered in various clinical examinations. However, patients with hematorrhea in small intestine are generally in poor conditions with unstable vital signs and are usually too vulnerable to receive major operation because they have missed the best time for operation. All the 3 deaths occurred due to the delayed operation. Once the diagnosis is made, different surgical procedures should be adopted according to the nature, location and area of the lesion. Part enterectomy covering the diseased segment and enteroanastomosis should be performed for patients with small intestinal stromal tumor, Meckel's diverticulum, ectopic pancreas, individual hemangioma and confined vascular malformation. The complete diseased segment of small intestine should be removed in patients with recurrent hemangioma and extensive vascular malformation. But excessive removal of small intestine lesion has to be prevented because short intestinal syndrome may occur.

Laparoscopic laparotomy can clearly and conveniently observe the small intestinal serosa and mesentery. It can assist in the management of discovered lesions. When the focus is discovered by laparoscopy, either laparoscope-assisted enterectomy covering the diseased segment and enteroanastomosis or laparoscopic enterectomy covering the diseased segment and enteroanastomosis can be performed[14-18]. Since December 2002, we have made efforts in applying laparoscope to examine unknown alimentary tract hematorrhea causes that were formerly imperative to laparotomy in 21 patients. The results show that laparoscope-assisted operation has the advantages of quicker location of the focus with noninvasive trauma, less pain and shorter recovery time. Therefore, laparoscope-assisted operation should be set up as the routine method for the treatment of small intestinal hematorrhea[13,15,17,18].

Patients whose bleeding causes could not be specified by laparoscopic laparotomy have only less bleeding from the small intestine that is mostly caused by ectopic pancreas or small intestinal stromal tumor, which is also difficult to locate by exploratory laparotomy. Alternatively, small intestinal enteroscopy during laparoscopy can be carried out by making use of the light-source atop the enteroscope to take fluoroscopy of the suspected area. This in combination with what is seen under the laparoscope usually leads to positive findings. Of the patients who received small intestinal enteroscopy during laparoscopy in this study, 4 had lesions which were cured.

In conclusion, tumor is the major cause of small intestinal bleeding followed by Meckel's diverticulum, angiopathy, and ectopic pancreas. The main diagnostic approaches are barium and air double contrast X-ray examinations of small intestine, DSA and ECT. Laparoscopic laparotomy or exploratory laparotomy should be performed for patients with long-term alimentary tract bleeding for unknown reasons as soon as possible. Enterectomy covering the diseased segment and enteroanastomosis are the most effective treatment modalities.

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*RAPID COMMUNICATION*

# Splenectomy with endoscopic variceal ligation is superior to splenectomy with pericardial devascularization in treatment of portal hypertension

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## Abstract

**AIM:** To investigate the therapeutic efficacy and complications of splenectomy with endoscopic variceal ligation (EVL) and splenectomy with pericardial devascularization (i.e. Hassab's operation) in patients with portal hypertension.

**METHODS:** A total of 103 patients with liver cirrhosis and portal hypertension were randomly selected to receive either splenectomy with EVL ($n$ = 53, group A) or Hassab's operation ($n$ = 50, group B).

**RESULTS:** The portal blood flow volume, the presence of portal vein thrombosis, gastric emptying time and free portal venous pressure (FPP) before and after the operation were determined. Patients were followed up for up to 64 mo with an average of 45 mo, and the Dagradi classification of variceal veins and the grading of portal hypertension gastropathy (PHG) were evaluated. It was found that all esophageal varices were occluded or decreased to grade Ⅱ or less in both groups. There was little difference in the recurrence rate of esophageal varices (11.9% *vs* 13.2%) and the re-bleeding rate (7.1% *vs* 5.3%) between groups A and B. The incidence of complications and the percentage of patients with severe PHG after the operation were significantly higher in group B (60.0% and 52.0%) than in group A (32.1% and 20.8%, $P < 0.05$). No patients died of operation-related complications. There was no significant difference in gastric emptying time, FPP and portal blood flow volume between the two groups.

**CONCLUSION:** The results suggest that splenectomy with EVL achieves similar therapeutic efficacy to that of Hassab's operation in terms of the recurrence rate of esophageal varices and the re-bleeding rate, but the former results in fewer and milder complications.



**Key words:** Portal hypertension; Splenectomy; Endoscopic varices ligation; Hassab's operation

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## INTRODUCTION

Recurrent bleeding occurs in over 70% of portal hypertension patients with a variceal bleeding history[1]. It is a general consensus that all patients with a variceal bleeding history should accept further treatment to prevent re-bleeding. Surgical operations still play a role in this process[2]. Since hepatitis-induced cirrhosis is the main cause of portal hypertension in China, which is associated with increased hepatocellular damage and worsened liver function, splenectomy with pericardial devascularization (i.e. Hassab's operation) has been widely used because of its minor influence on liver function[3,4]. However, this operation has its limitations, including a high incidence of portal vein thrombosis, serious gastric mucosa damage and gastric emptying delay after operation[5-8].

The benefits of endoscopic management in preventing re-bleeding have been explicitly illustrated in a series of well designed randomized controlled trials[9,10]. Sclerotherapy significantly decreases the recurrence of bleeding and mortality, but it is associated with serious complications, the most common of which are esophageal strictures and bleeding from treatment-induced ulcers[11]. More recently, endoscopic variceal ligation (EVL) has been proven as an endoscopic therapy at least as effective as sclerotherapy, but with fewer complications. Meta-analyses have revealed that variceal ligation can more significantly reduce re-bleeding than sclerotherapy (POR = 0.53, 95% CI: 0.42 to 0.67), without significant heterogeneity amongst trials[12,13]. However, EVL still results in the re-bleeding rate as high as 30%, and does not help to relieve

portal hypertension and hypersplenism[14].

Theoretically, combining splenectomy with EVL should relieve not only portal hypertension but also hypersplenism, and thus prevent the bleeding of esophageal varices[15]. Meanwhile, avoiding the procedure of devascularization may overcome its associated limitations as mentioned above. In this study, we compared the therapeutic efficacy of splenectomy combined with EVL with that of Hassab's operation and their respective complications in patients with portal hypertension.

## MATERIALS AND METHODS

### Patients

From January 1999 to June 2002, 103 consecutive patients (61 males and 42 females) with portal hypertension caused by hepatitis-induced cirrhosis were admitted to the Third Affiliated Hospital of Sun Yat-Sen University Hospital. All these patients were recruited in this prospective study.

The inclusion criteria included clinically diagnosed portal hypertension caused by hepatitis-induced cirrhosis in patients with endoscopically confirmed esophageal varices and a history of upper gastrointestinal hemorrhage, and functional hepatic reserve of grade A or B according to the Pugh-modified Child's scales.

The exclusion criteria included portal hypertension caused by reasons other than hepatitis-induced cirrhosis in patients with functional hepatic reserve of grade C by the Child-Pugh classification, peptic ulcer or other gastrointestinal complications, severe diseases in major organs other than in liver and/or age over 70 years.

The recruited patients were randomly assigned to group A or B with the use of opaque, sealed envelopes that contained a treatment assignment derived from computer-generated random numbers. Randomization was stratified according to the severity of liver failure (assessed by the Child-Pugh classification system). All the patients were cared for at the Department of General Surgery, Third Affiliated Hospital of Sun Yat-Sen University, Guangzhou, China. A written informed consent was obtained from all the patients, and the trial was approved by the Ethics Committee of Sun Yat-Sen University. The study protocol is shown in Figure 1.

### Pre-operative endoscopic examination

All the patients received a pre-operative endoscopic examination. The esophageal varix was evaluated by Dagradi classification[16,17], while the portal hypertensive gastropathy (PHG) was graded as mild, moderate or severe as previously described[18,19].

### Gastric emptying assessment

Gastric emptying was assessed with a standardized scintigraphy method[20]. In brief, a test meal was prepared by mixing sixty grams of fat-parched flour (16 g protein, 3.24 g fat and 80 g carbohydrate, 924.7 KJ per 100 g) with hot water containing 37-74 mBq of 99 mTc-DTPA to form a portion of semi-solid general meal. Each patient consumed a portion of the test meal within 5 min at a sitting position. Time to half-emptying of gastric contents, lag-phase calculation and residual gastric contents at 60, 120 and 240 min were calculated by a GENESYS SPECT system (ADAC, Laboratories, Milpitas, CA, USA).

### Splenectomy combined with endoscopic varices ligation

Patients assigned to group A ($n$ = 53) initially underwent routine splenectomy, and EVL was performed right after the splenectomy. After local application of lidocaine, an endoscope (GIF 240 or 260, Olympus Optical, Tokyo) was introduced, and ligation was carried out 6-12 times by placing a single rubber band (Bard Interventional Products, Tewksbury, Mass USA) over a varix each time. The ligation was repeated every 2 wk till the varices completely disappeared under the endoscope.

### Hassab's operation

Patients assigned to group B ($n$ = 50) underwent Hassab's operation as previously described in detail by Yang and Qiu[3]. In brief, extended left subcostal incision or L incision of the left upper abdomen was used for extreme splenomegaly. After routine splenectomy, the gastric branch and 5-8 small branches of the gastric coronary veins were disconnected. The esophageal branch was disconnected and suture-ligated. The gastric posterior vein was ligated by suturing, and then the left subphrenic vein was ligated as well[21]. In addition, the arteries accompanied by the veins including the left gastric artery, left gastroepiploic artery, gastric posterior artery and left subphrenic artery, were disconnected.

### Determination of portal vein hemodynamics

Free portal venous pressure (FPP) was measured before and after splenectomy (after the first EVL in group A) by inserting the measuring tube in the main portal vein through the right gastroepiploic vein during the operation, as described previously[22].

### Color Doppler ultrasound

Color Doppler ultrasound detection was performed 2 wk before and 3 wk after the operation by a color Doppler ultrasound system (Biosound AU 4, Esaote, Italy). For each measurement, at least 3 reproducible patterns were created to calculate the mean maximum portal blood velocity ($V_{max}$) to ensure the measurement accuracy. Mean portal blood velocity ($V_{mean}$) was calculated (cm/s) by the equation "$V_{mean} = 0.57 \times V_{max}$" as described by Moriyasu *et al*[23]. The cross-sectional area ($cm^2$) was also recorded at the site of the main portal vein where the portal blood velocity was measured. Portal blood flow volume (PBF) was obtained (mL/min) by the equation "PBF = Area × $V_{mean}$ × 60". The presence of portal vein thrombosis was examined as well[24,25].

### Follow-up of patients

All patients were followed up by endoscopy at wk 2, 4 and 12 after operation, and every 12 wk thereafter (Figure 1). Gastrointestinal symptoms such as refractory hiccup, bilious vomiting, hematemesis and dark stools were recorded. The Dagradi classification and PHG grading were evaluated at the follow-up endoscopy as described above.

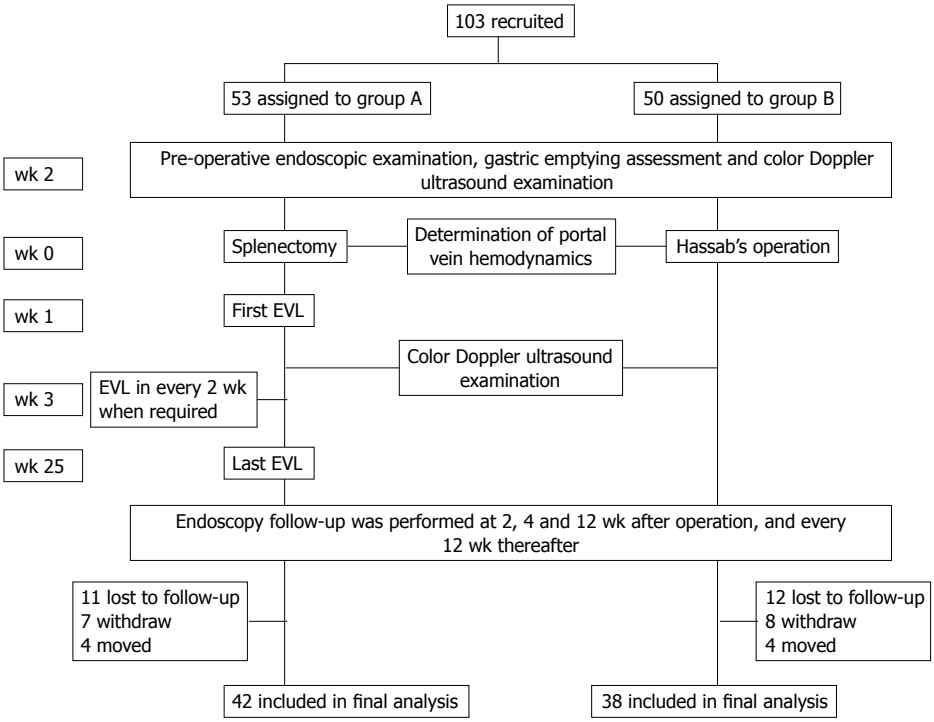


**Figure 1** The study protocol and the number of patients studied.

### *Statistical analysis*

Values were expressed as mean ± SD. Mean differences between the two groups were calculated by unpaired *t* test. Mann-Whitney test was used for the analysis of ranked data. The comparison of categorical data was performed by the $\chi^2$ test. $P < 0.05$ (2-tailed test) was considered statistically significant.

## RESULTS

### *Efficacy on esophageal varices*

A total of 154 EVLs were performed in group A, with a mean of 3.2 (2-5) EVL sessions per patient. All esophageal varices were occluded or decreased from grade Ⅲ to grade Ⅱ (Dagrade classification) in both groups.

A total of 80 patients were followed up for 24.5-64 mo with an average of 45 mo. Eleven patients in group A and 12 patients in group B were not followed up due to death or other reasons (3 died of liver cancer,5 died of hepatic failure, 15 lost contact). The recurrence rate of esophageal varices was 11.9% (5/42) in group A and 13.2% (5/38) in group B, while the re-bleeding rate was 7.1% (3/42) in group A and 5.3% (2/38) in group B ($P > 0.05$).

### *Complications*

No patients in both groups died of operation-related complications. The common complications included infection, worsened liver function (i.e. increasing jaundice), refractory ascites and pleural effusion. The incidence of complications in group B was higher than that in group A (60% *vs* 32.1%, $P < 0.01$). Mild pharyngodynia and/or retrosternal pain were found in most patients in group A, but they were relieved 3-5 d later without medical treatment. Esophageal ulcer-induced re-bleeding was found in 2 patients at 2 wk after the operation, but the bleeding was stopped by repeated EVL and medical treatment.

**Table 1 Incidence of complications after operation**

| Complications | Group A ($n$ = 53) | | Group B ($n$ = 50) | | Value | |
|---|---|---|---|---|---|---|
| | $n$ | % | $n$ | % | $\chi^2$ | $P$ |
| Total cases with complications | 17 | 32.1 | 30 | 60 | 4.85 | 0.01 |
| Refractory ascites | 3 | 5.7 | 9 | 18 | 4.65 | 0.03 |
| Pleural effusion | 5 | 9.4 | 16 | 32 | 5.63 | 0.02 |
| Esophageal ulcer | 2 | 3.8 | 0 | 0 | 6.56 | 0.03 |
| Portal vein thrombosis | 5 | 9.4 | 11 | 22 | 3.25 | 0.03 |
| Refractory hiccup | 2 | 3.8 | 15 | 30 | 5.46 | 0.02 |
| Bilious vomiting | 3 | 5.7 | 16 | 32 | 3.65 | 0.01 |

Incomplete portal vein thrombosis was found in 7 patients (13%) in group A as detected by color Doppler ultrasound, and disappeared after anticoagulation treatment. In group B, 14 patients (28%) suffered from portal vein thrombosis ($P < 0.01$, compared with group A), while one patient presented with widespread thrombi in the main portal vein and the portal vein branch, and died of sponge-like lesions in the portal vein and liver function failure 6 mo after the operation, despite thrombolysis treatment. Significant differences were found in the incidence of refractory hiccup, bilious vomiting and the degree of gastric motility between the two groups ($P < 0.05$) (Table 1).

### *Endoscopic follow-up of PHG*

The PHG grading increased significantly after the operation in patients of group B, but not in patients of group A (Table 2). The percentage of patients with severe PHG after the operation was 20.8% in group A and 52.0% in group B ($P < 0.05$) (Table 2). Three patients in group B suffered from hematemesis, which was evidenced by dark stools and/or a positive fecal occult blood test more than

**Table 2 PHG grading before and after operation *n* (%)**

| PHG grading | Group A (*n* = 53) | | Group B (*n* = 50)[a] | |
|---|---|---|---|---|
| | Pre-operation | Post-operation | Pre-operation | Post-operation |
| Normal | 3 (5.66) | 2 (37.74) | 5 (10.00) | 2 (4.00) |
| Mild | 25 (47.17) | 23 (43.40) | 27 (54.00) | 8 (16.00) |
| Moderate | 17 (32.08) | 17 (32.08) | 13 (26.00) | 14 (28.00) |
| Severe | 8 (15.09) | 11 (20.75) | 5 (10.00) | 26 (52.00)[c] |

[a]$P < 0.05$ *vs* post-operation; [c]$P < 0.05$ *vs* post-operation in group A.

**Table 3 Alteration of gastric emptying time after operation (mean ± SD)**

| Time points | Gastric emptying time (min) | |
|---|---|---|
| | Group A (*n* = 53) | Group B (*n* = 50) |
| Before operation | 55 ± 10 | 54 ± 8 |
| 3 wk after operation | 53 ± 8 | 64 ± 7 |
| 6 wk after operation | 51 ± 9 | 62 ± 6 |
| 12 wk after operation | 54 ± 8 | 58 ± 8 |

There was no significant difference in the gastric emptying time between the two groups at each time point, or among the four time points in each group.

**Table 4 Alterations of portal vein pressures and hemodynamic variables (mean ± SD)**

| | Group A (*n* = 53) | | | Group B (*n* = 50)) | | |
|---|---|---|---|---|---|---|
| | Pre-operation | Post-operation | *P* | Pre-operation | Post-operation | *P* |
| Free portal venous pressure ($cmH_2O$) | 37.1 ± 4.67 | 29.02 ± 4.34 | 0.02 | 38.1 ± 4.47 | 31.02 ± 4.3 | 0.02 |
| $V_{max}$ (cm/s) | 19.51 ± 4.30 | 15.90 ± 3.12 | 0.01 | 18.54 ± 3.30 | 14.90 ± 2.12 | 0.01 |
| Blood flow (mL/min) | 1100.84 ± 351.8 | 852.25 ± 292 | 0.02 | 1120.84 ± 341.84 | 852.25 ± 292.35 | 0.02 |

1 wk, and further confirmed as PHG-related hemorrhage by endoscopic examination.

### *Alteration of gastric empty time*

No significant difference was found in gastric emptying time between the two groups at each time point before and after the operation. Moreover, the gastric emptying time did not change after the operation in both groups (Table 3).

### *Alterations of portal vein pressure and hemodynamic variables*

In both groups after the operation, the directions of the portal venous flow were maintained towards the liver, but the FPP, $V_{max}$ and the blood flow were decreased significantly compared with those before the operation ($P < 0.05$). However, there was no significant difference in the changes of these variables between the two groups ($P > 0.05$) (Table 4).

## DISCUSSION

Portosystemic shunt and Hassab's operation are commonly used in the treatment of portal hypertension. Although both operations are effective in control of bleeding, portosystemic shunt has increasingly been replaced by devascularization in recent years in China because of its technical difficulty and poor long-term outcome[3,4]. A prospective study of 435 cases[3] showed that the 5- and 10- year survival rates of patients after devascularization are 94.1% and 70.0%, respectively, while the recurrent bleeding rate is 6.2% and 13.3%, respectively. The same study [3] also showed that the bleeding control rate of emergency operation is 96.9%, and the overall operative mortality rate is only 4.5%. However, the portal blood flow may decrease to some extent and re-bleeding is likely to occur because of the portal hyperdynamic state, and gastric congestion may exacerbate portal hypertensive gastropathy[4].

With the introduction of multishot band devices which allow the placement of 5-10 bands at a time, EVL has become a more effective method with fewer side effects compared with previous operations. After several repeated sessions at 2 wk intervals, obliteration of varices can be achieved in about 90% of patients[9]. However, since portal hypertension and hypersplenism cannot be eliminated by EVL resulting in the recurrence of varices after EVL, 20%-75% of patients require repeated EVL sessions[10]. As the hyperkinetic circulatory state of the splenic vein is a potential factor for the development of bleeding in esophageal varices, we believe that splenectomy in combination with EVL may eliminate not only hypersplenism but also the hyperkinetic circulatory state of the portal vein system, which may prevent the recurrence of esophageal varices after EVL.

In the present study, splenectomy in combination with EVL showed a similar efficacy to Hassab's operation in the treatment of esophageal varices and in the prevention of re-bleeding. However, the former demonstrated several advantages over the latter, including a lower incidence of overall and individual complications such as refractory ascites, pleural effusion, esophageal ulcer, portal vein thrombosis, refractory hiccup and bilious vomiting and lower percentage of patients with severe PHG.

EVL may cause fibrosis in the mucous layer where the esophageal varices are located, but has no effect on the formation of collateral circulation in the muscular layer. Moreover, EVL may not injure the collaterals of the portal vein near the esophagus, thus decreasing the portal vein pressure by keeping the natural portal azygous shunt. In addition, EVL is a safe operation with few mild complications that can be tolerated by patients even with poor liver functions, thus allowing the extension of the indications for such a surgical intervention.

Thrombosis in the portal vein is a major complication of

devascularization, which may be caused by the decreased blood flow in the portal vein system, porto-azygos disconnection, damage to the endangium and increased platelets after splenectomy. Acute thrombosis in the portal vein may lead to intestinal infarction, which is characterized by abdominal pain, hematochezia and diffuse peritonitis[3]. In our study, portal vein thrombosis was present in 28% (14/50) of patients receiving Hassab's operation and 13% (7/53) of patients receiving splenectomy in combination with EVL, indicating that the latter can offer a significant benefit for patients with portal vein thrombosis.

In addition to portal vein thrombosis, vagal nerve injury is another commonly reported complication of devascularization[3], which may greatly increase the incidence of gastric emptying delay and bilious vomiting by decreasing the gastric motivation. It is presumably a major cause for PHG. However, this kind of injury is likely to be related to the operational skills. Theoretically, devascularization may accompany a higher incidence of gastric emptying delay. However, no difference was found in the gastric emptying time between the two groups in the present study, suggesting that this phenomenon cannot be ascribed to any influence of EVL on gastric emptying.

Based on the observations of the present study, splenectomy in combination with EVL is as effective as Hassab's operation in preventing the recurrence of esophageal varices and the re-bleeding rate, but has a lower incidence of PHG than Hassab's operation. However, repeated EVL sessions (an average of 3.2 times) are necessary to maintain the occlusion of varices, which requires long-term follow-up and thus increasing the cost of therapy.

In conclusion, splenectomy in combination with EVL achieves a similar therapeutic efficacy to Hassab's operation in terms of the recurrence rate of esophageal varices and the re-bleeding rate, but the former results in fewer and milder complications.

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*RAPID COMMUNICATION*

# A series of 64 cases of pancreatic cystic neoplasia from an institutional study of China

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## Abstract

**AIM:** To recognize cystic neoplasia of the pancreas and thus to identify a panel of curable diseases.

**METHODS:** Sixty-four cases of cystic neoplasia of the pancreas, including 28 cases of intraductal papillary mucinous neoplasia (IPMN), 12 cases of serous cystic neoplasia (SCN), 11 cases of mucinous cystic neoplasia (MCN), 11 cases of solid pseudo-papillary neoplasia (SPN), and 2 cases of solid tumor with cystic degeneration were examined immunohistochemically for their expression of MUC1, MUC2, MUC4, MUC5AC, and MUC6, as well as other related antigens.

**RESULTS:** Adenoma type of IPMN and borderline lesions exhibited high expressions of MUC2, and MUC5AC. In contrast, IPMN with invasive carcinoma component showed MUC1 immunoreactivity. SCN was mainly positive for MUC1 and MUC6, while negative for MUC2, MUC4 and MUC5AC. Noninvasive MCN, regardless of its cellular atypia degree, was positive for MUC5AC and negative for MUC1. MUC1 expression was only observed in patients with an invasive component. No mucin expression was found in SPN.

**CONCLUSION:** Mucin profile may, in conjunction with histologic study, provide important information on tumor types and patient treatment of cystic neoplasia of the pancreas.



**Key words:** Pancreas; Cystic neoplasm; Mucin; Differential diagnosis; Diagnosis

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## INTRODUCTION

Cystic neoplasia of the pancreas accounts for about 10%-15% of all cystic pancreatic lesions[1]. The majority of cystic lesions of the pancreas are pseudocysts. Although cystic neoplasia of the pancreas is rare, it encompasses a spectrum of benign, borderline and malignant neoplasiae. In contrast to solid tumors of the exocrine pancreas, which are exclusively malignant (85%-90%), the clinical challenge is the differential diagnosis and management of cystic neoplasia.

The broad spectrum of pancreatic neoplasia and tumor-like lesions with cystic features has been recently reviewed[1-3]. The incidence or frequency of pancreatic cystic neoplasia varies among different institutes. Because there has been no comprehensive study in a large series of cases from China comparing the incidence and biology to Western series, we reviewed all cystic neoplasiae collected in pathological files of Zhongshan Hospital over five years. Most of the neolpasms in our series were well documented and have been included in the recent World Health Organization (WHO) classification[4]. The four most common cystic neoplasiae of the pancreas are intraductal papillary mucinous neoplasia (IPMN), serous cystic neoplasia (SCN), mucinous cystic neoplasia (MCN), and solid pseudo-papillary neoplasia (SPN).

Mucins are a group of high molecular weight glycoproteins that are widely expressed in epithelial cells. Fourteen mucin genes have been identified thus far[5-8]. They can be further grouped into two subfamilies: secreted and transmembrane mucin genes. Among these, MUC2, MUC5AC and MUC6, known as the gel-forming mucins, are expressed in the pancreas either under normal physiologic or tumoral conditions. Among membrane-bound mucins, MUC1 and MUC4 are the two main mucins

associated with pancreas[7,8].

In the present study, we examined the expression of the well-characterized MUCs (MUC1, 2, 4, 5AC and 6) in pancreatic cystic neoplasia and compared the expression profiles with those observed in adenocarcinoma and endocrine tumor of pancreas with cystic feature. The overall goal was to identify the mucin profile that is potentially a specific marker for the differential diagnosis of cystic neoplasia of the pancreas.

## MATERIALS AND METHODS

Pancreatic cystic tumors diagnosed between January 1999 and June 2005 in the Department of Pathology of Zhongshan Hospital, Fudan University were identified, reviewed, and classified according to the recent World Health Organization classification of pancreas neoplasia[6]. During this period, 248 patients were operated on in our hospital for pancreatic lesions. Clinical information was obtained from the patient records or the clinicians in charge. The tumors were removed surgically from all the patients. All specimens were fixed in buffered-formalin and embedded in paraffin. Deparaffinized sections were stained with haematoxylin and eosin, Alcian blue and periodic acid-Schiff. One or two representative blocks from each case were selected. Immunohistochemical analysis was carried out on serial sections cut from the cystic epithelial neoplasia using the Envision (DAKO, Carpiteria, CA) method with several antisera (Table 1).

All cases were analyzed by 2 pathologists in accordance with the WHO criteria. In the current study, SCN, MCN, IPMN were classified into three classes according to the degree of cytologic and structural atypia including increased nuclear-to-cytoplasmic ratio, loss of polarity, pleomorphism, hyperchromatism, prominent nucleoli, abnormal mitosis, cribriform pattern and multilayer, and presence of invasion. Immunohistochemical evaluation was independently performed by two authors (Ji Y, and Zhu XZ) without knowledge of the clinical data. For each marker, the positive cell distribution in tumor tissue and staining pattern (cytoplasmic, membranous, or nuclear) were recorded. For mucins, we evaluated the staining of cytoplasm and luminal surface. The cells were considered positive when at least one of the components was positive. Percentages of the positively stained neoplastic cells were as follows: -: < 5% of neoplastic cells stained; +: 5%-20% of neoplastic cells stained; ++: 20%-50% of neoplastic cells stained; +++: > 50% of neoplastic cells stained.

## RESULTS

This series was comprised of 64 cases, including 28 cases of IPMN, 12 cases of SCN, 11 cases of MCN, 11 cases of SPN, 1 case of ductal adenocarcinoma with cystic feature (cDAC), and 1 case of endocrine tumor with cystic feature (cPET). All cystic neoplasiae of the pancreas in order of their relative frequency are listed in Table 2.

The details of the cases included in the study are shown in Table 3. The immunostaining findings are listed in Tables 4 and 5.

**Table 1 Antibodies used for immunostaining of cystic neoplasia of the pancreas**

| Antibody | Clone | Type | Producer | Dilution | Commence |
|---|---|---|---|---|---|
| PanCK | AE1/AE3 | MoAb | DAKO | 1:100 | Membrane |
| CK 7 | OV-TL | MoAb | DAKO | 1:200 | Membrane |
| CK 8/18 | RCK108 | MoAb | DAKO | 1:200 | Membrane |
| CA19-9 | 116-NS-19-9 | MoAb | DAKO | 1:50 | Cytoplasmic |
| CEA | A0115 | PoAb | DAKO | 1:200 | Cytoplasmic |
| EMA | E29 | MoAb | DAKO | 1:100 | Membrane |
| MUC1 | Ma695 | MoAb | Novacastra | 1:100 | Apical membrane |
| MUC2 | Ccp58 | MoAb | Novacastra | 1:1000 | Cytoplasmic |
| MUC4 | | MoAb | Zymed | 1:100 | Apical membrane |
| MUC5AC | CLH2 | MoAb | Novacastra | 1:1000 | Cytoplasmic |
| MUC6 | CLH5 | MoAb | Novacastra | 1:100 | Cell membrane |
| Synaptophysin | | MoAb | DAKO | 1:100 | Cytoplasmic |
| Chromogranin-A | DAK-A3 | MoAb | DAKO | 1:20 | Cytoplasmic |
| NSE | | PoAb | DAKO | 1:200 | Cytoplasmic |
| α1-AT | | PoAb | DAKO | 1:500 | Cytoplasmic |
| α1-ACT | | PoAb | DAKO | 1:250 | Cytoplasmic |
| Vimentin | V9 | MoAb | DAKO | 1:100 | Cytoplasmic |
| α-inhibin | | MoAb | DAKO | 1:50 | Nuclear |
| β-catenin | | MoAb | DAKO | 1:100 | Membrane/ nuclear |

CK: cytokeratin; CEA: carcinoma-embryonal antigen; EMA: epithelial membrane antigen; NSE: neural specific enolase; α1-AT: alpha-1-antitrypsin; α1-ACT: alpha-1-antichymotrypsin.

**Table 2 Distribution of 64 cases of cystic neoplasia of the pancreas**

| Type | | *n* | Percentage |
|---|---|---|---|
| Intraductal papillary mucinous neoplasia | | 28 | 43.8 |
| | Adenoma | 5 | |
| | Borderline | 4 | |
| | Carcinoma | 19 | |
| Serous cystic neoplasia | | 12 | 18.8 |
| | Adenoma | 11 | |
| | Borderline | 0 | |
| | Carcinoma | 1 | |
| Mucinous cystic neoplasia | | 11 | 17.3 |
| | Adenoma | 8 | |
| | Borderline | 1 | |
| | Carcinoma | 2 | |
| Solid pseudo-papillary neoplasia | Borderline (Uncertain malignant potential) | 11 | 17.3 |
| Cystic ductal adenocarcinoma | High-grade malignant | 1 | 1.6 |
| Cystic pancreas endocrine tumor | Low-grade malignant | 1 | 1.6 |
| Total | | 64 | 100 |

### *IPMN*

Macroscopically, neoplasia could be classified as main pancreatic duct type in 15 (50.4%) patients (Figure 1A), and branch type in 5 patients, both main and branch duct types in 8 patients, respectively. On histologic analysis of the entirely resected specimens, IPMN showed simple hyperplasia (adenoma) in 5 patients, atypical hyperplasia

**Table 3 Clinicopathologic data of 40 cases of cystic neoplasia of the pancreas**

| Clinical feature | Type | IPMN *n* = 28 | SCN *n* = 12 | MCN *n* = 11 | SPN *n* = 11 | cDAC *n* = 1 | cPET *n* = 1 |
|---|---|---|---|---|---|---|---|
| Age (yr) | Mean (range) | 65 (45-80) | 52.7 (36-69) | 51.7 (29-72) | 39 (22-52) | 58 | 45 |
| Sex | Male:Female | 4:3 | 3:10 | 1:10 | 1:10 | 1:0 | 1:0 |
| Location | Head | 16 | 5 | 2 | 3 | 1 | 1 |
| | Body/tail | 12 | 7 | 9 | 8 | | |
| Tumor size | Range (mean) | 2-7.5 (4.5) | 3-12 (6) | 2-13 (7.8) | 2-21 (6.5) | 5 | 4.5 |

IPMN: intraductal papillary mucinous neoplasia; SCN: serous cystic neoplasia; MCN: mucinous cystic neoplasia; SPN: solid pseudo-papillary neoplasia; cDAC: cystic ductal adenocarcinoma; cPET: cystic pancreatic endocrine tumor.

**Table 4 Immunoreactivity of cystic neoplasia of the pancreas**

| Type | IPMN *n* = 28 | SCN *n* = 12 | MCN *n* = 11 | SPN *n* = 11 | cDAC *n* = 1 | cPET *n* = 1 |
|---|---|---|---|---|---|---|
| CK8/18 | + | + | + | - | + | + |
| CK19 | ++ | + | + | - | + | +/- |
| CA19-9 | + | - | +/- | - | + | - |
| CEA | + | - | -/+ | - | + | - |
| SYN | +/- | - | +/- | +/- | - | + |
| CHG-A | +/- | - | +/- | - | - | + |
| NSE | + | +/- | +/- | + | | |
| α-AT | - | - | - | + | - | - |
| α-ACT | - | - | - | + | - | - |
| Vim | - | - | | + | - | - |
| α-inhibin | - | + | +[1] | +/- | | |
| β-catenin | - | + | - | +/- | - | - |

[1]Positive α-inhibin in MCN is limited in ovarian-like stromal cells. IPMN: intraductal papillary mucinous neoplasia; SCN: serous cystic neoplasia; MCN: mucinous cystic neoplasia; SPN: solid pseudo-papillary neoplasia; cDAC: cystic ductal adenocarcinoma; cPET: cystic pancreatic endocrine tumor.

(borderline lesion) in 4 patients, *in situ* carcinoma in 6 patients, and invasive carcinoma in 13 patients. The epithelial proliferative lesions in all patients involving the main or secondary pancreatic duct showed a papillary architecture (Figure 1B). Moderate to severe fibroatrophic changes of obstructive pancreatitis were observed in 21 patients.

Immunochemically, neoplasia could be classified as intestinal type (MUC1-, MUC2+) in 11 patients (Figure 1C), pancreaticobiliary type (MUC1+, MUC2-) in 9 patients, gastric type (MUC1-, MUC2-, MUC5AC+) in 6 patients (Figure 1D), and oncocytic type in 2 patients. Of the 13 IPMN patients with invasive carcinoma, 5 showed the features of mucinous carcinoma in their invasive component and 8 showed ductal carcinoma in their invasive component with MUC1+. The mucin profiles of the mucinous or ductal component corresponded to the intestinal or pancreaticobiliary type, respectively. All IPMN samples stained for CEA, CK8/18, and CK19 were negative for CK20, α1-AT. However, single endocrine cells were detected by SYN and CHG-A in 10 intestinal types and 4 pancreaticobiliary types of IPMN. They expressed both gastric hormones (serotonin and gastrin) and/or pancreatic hormones (glucagon, PP) focally.

**Table 5 Mucin profiles of cystic neoplasia of the pancreas**

| Type | IPMN *n* = 28 | SCN *n* = 12 | MCN *n* = 11 | SPN *n* = 11 | cDAC *n* = 1 | cPET *n* = 1 |
|---|---|---|---|---|---|---|
| MUC1 | 7/28 + | 1/12 +/- | 2/11 + | - | ++ | - |
| MUC2 | 15/28 ++ | 1/13 +/- | - | - | - | - |
| MUC4 | - | - | -- | - | ++ | |
| MUC5AC | 28/28 + | 2/12 + | 10/11 ++ | - | + | - |
| MUC6 | - | 11/12 ++ | - | - | + | - |

IPMN: intraductal papillary mucinous neoplasia; SCN: serous cystic neoplasia; MCN: mucinous cystic neoplasia; SPN: solid pseudo-papillary neoplasia; cDAC: cystic ductal adenocarcinoma; cPET: cystic pancreas endocrine tumor.

### SCN

The most common subtype of SCN was serous microcystadenoma (SMA, 8 cases), characterized by a central stellate scar, sharp demarcation from surrounding pancreatic tissue, and its predominant occurrence in women. SMA was comprised of numerous small cysts with a diameter ranging from 0.01 to 0.5 cm and a few larger cysts up to 1.5 cm in diameter. The cysts contained eosinophilic fluid and were lined by a single layer of cuboidal or flattened epithelial cells with pale to clear cytoplasm. The tumor cells were centrally located with round to oval nuclei. The cytoplasm was positive for granular periodic acid-Schiff. The usually thin fibrous septae were acellular. The central scar was composed of hyalinized tissue. Three cases of serous oligocystic ill-defined adenoma (SOIA) showed only a few, usually rather large cysts (0.2-2.5 cm in diameter) filled with clear or brown fluid (Figure 2A). The cyst epithelium was cytologically identical to that of SMA (Figure 2B). However, unlike SMA, SOIA was not well-defined due to the extension of small cysts into the adjoining pancreatic tissue. The tumors also lacked a central fibrous scar. One serous cystadenocarcinoma (SCC) was found to have a liver metastatic lesion during operation. The primary tumor of pancreas, which was similar to SMA, showed no distinct atypia.

Besides stained for CK8/18, CK19, the cells were also positive for neuron specific enolase (NSE) and negative for alpha-1-antitrypsin (α1-AT) and alpha-1-antichymotrypsin (α1-ACT). Of the mucins tested, MUC1 (75%) (Figure 2D) and MUC6 (66%) (Figure 2C) had the highest positive rates. MUC2 and MUC5AC staining was completely lacking. In

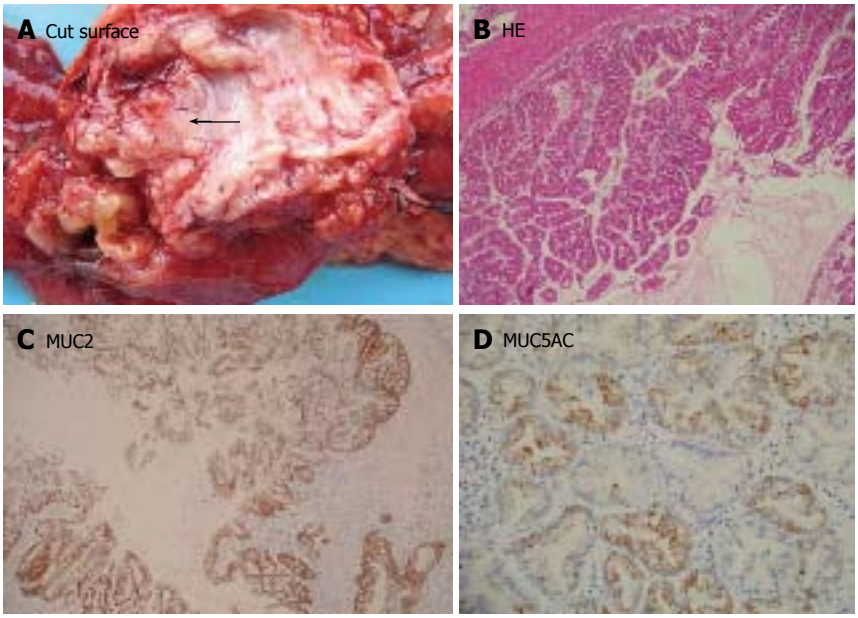


**Figure 1** Intraductal papillary mucinous neoplasia. **A**: Sectioning of the pancreas revealing papillary tumors (arrow) with sticky mucin in dilated main panceatic ducts; **B**: Epithelial proliferative lesions that involve the main or secondary pancreatic duct showing a papillary architecture (HE × 40); **C**: MUC2 diffuse expression in papillary epithelial cells of intestinal type (Envison × 40); **D**: Focally stained MUC5AC in cytoplasm of gastric subtype tumor cells (Envison × 100).

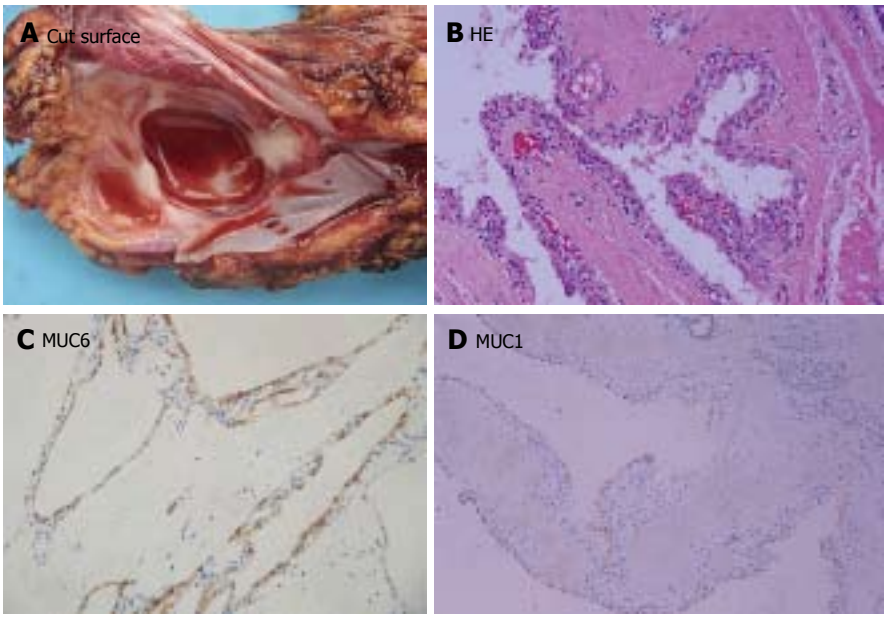


**Figure 2** Serous cystic neoplasm. **A**: Smooth and thin cyst wall on cut surface containing clear red-brown fulid; **B**: Cysts wall lined by a single layer of cuboidal or flattened epithelial cells with pale to clear cytoplasm, and centrally located tumor cells with round to oval nuclei (HE × 100); **C**: Tumor cells strongly positive for MUC6 (Envison × 100); **D**: MUC1 expression in some tumor cells (Envison × 40).

the normal pancreatic tissue entrapped into the tumors, small duct cells and centroacinar cells were apically positive for MUC6 and MUC1. In addition, the epithelial cells were stained for α-inhibin.

### *MCN*

MCN was studied in 11 patients. The tumors presented as a large round cystic mass with a unilocular or multilocular cut surface (Figure 3A). Micorscopically, the cysts were lined by mucin-producing epithelial cells with variant degrees of dysplasia supported by an ovarian-like stroma (Figure 3B). MCN was classified as adenomas (MCA) in 8 patients, borderline tumor (MCB) in 1 patient, and carcinomas (MCC) in 2 patients with invasive component. The epithelial component of MCN was stained for CK8/18, CK19, and CEA. MUC1 positivity was, however, found in the invasive components of MCC. All of our MCN patients were negative for MUC2, except for goblet cells, which were quite numerous in MCB and rare in MCA. MUC5AC expression was found in all the 11 MCN patients. The cytoplasmic staining intensity increased with progressive nuclear atypia and was the strongest in invasive carcinomas (Figure 3C). A few cells were stained faintly for MUC6 (Figure 3D). In addition, endocrine cells detected by CHG-A, were found in 3 MCN patients, of them 2 also had expression of gastrin and serotonin, 1 had expression of only serotonin.

### *SPN*

The extent of cystic changes in 11 SPN patients varied from tumor to tumor, affecting either part of the tumor tissue, or almost the entire tumor tissue. The cystic structures contained hemorrhagic debris and were usually surrounded by hemorrhagic-necrotic tissues (Figure 4A). Microscopically, SPN showing solid areas was composed of monotonous polygonal epithelioid cells, often

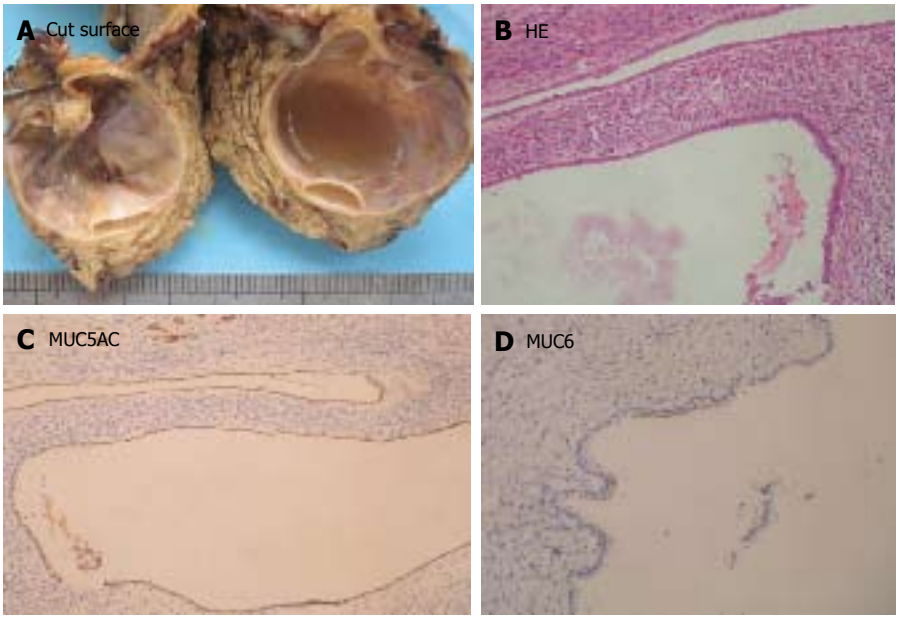


**Figure 3** Mucinous cystic neoplasm. **A**: Tumors presented as large round cystic masses with a unilocular cut surface; **B**: Cysts lined by mucin-producing epithelial cells with variant degrees of dysplasia supported by an ovarian-like stroma; **C**: MUC5AC expression in cytoplasm; **D**: Faint MUC6 expression in apical tumor cells (Envison × 100).

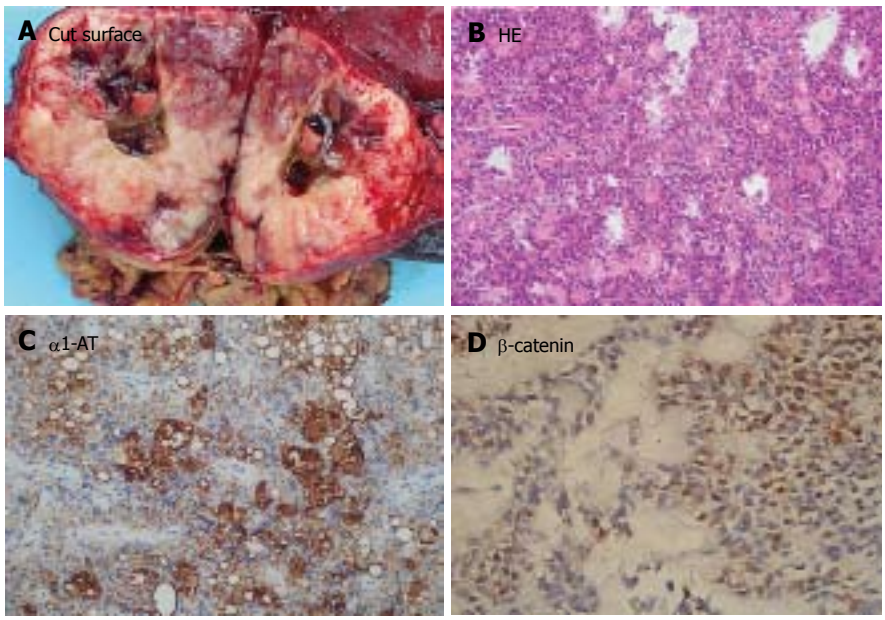


**Figure 4** Solid pseudo-papillary neoplasia. **A**: Cystic structures containing hemorrhagic debris surrounded by hemorrhagic-necrotic tissue; **B**: SPN showing solid areas consisting of monotonous polygonal epithelioid cells, often with minimal intervening stroma accompanied with innumerable capillary-sized vessels. In the pseudopapillary regions, the cells away from the small vessels appeared to have dropped away, leaving an irregular cuff of cells surrounding each vascular core (HE × 100); **C**: SPN showing a consistent pattern of reactivity for α-1-AT (Envison × 100); **D**: Positive β-catenin and progesteron receptor limited to the nuclei of tumor cells (Envison × 200).

with minimal intervening stroma accompanied with innumerable capillary-sized vessels (Figure 4B). Some areas showed more extensive stromal fibrosis, with round aggregates of perivascular hyalinized stroma imparting a cylindromatous appearance. In the pseudopapillary regions, the cells located away from the small vessels appeared to have dropped away, leaving an irregular cuff of cells surrounding each vascular core.

Immunohistochemical studies revealed that SPN showed a consistent pattern of reactivity for vimentin, α-1-antitrypsin (α-1-AT) (Figure 4C) and α-1-antichymotrypsin (α-1-ACT), but an inconsistent positive pattern for CD10 and S-100 protein. None of these proteins was reactive for mucins and pancreatic hormones. Staining of the pancreatic enzyme trypsin was consistently negative, as the specific endocrine marker chromogranin. Seven cases were focally positive for synaptophysin and the less specific marker NSE. Positive β-catenin (Figure 4D) and progesteron receptors were limited to the nuclei of tumor cells.

### *cDAC*

MUC1 and MUC4 were observed in invasive component. CEA, CK7, 19 and CA19-9 with focal staining of MUC5AC did not exceed 30% of the tumor, in which MUC2 was absent (Figure 5A-5D).

### *cNET*

The NET which was not encapsulated in the pancreatic head, infiltrated the duodenal wall and was considered malignant. As the typical NET had no cystic change, NET was diffusely stained for SYN, CHG-A and NSE, as well as pancreatic hormones and glucagons (Figure 6A-6D).

## DISCUSSION

Cystic tumor of the pancreas accounts for 10%-15%

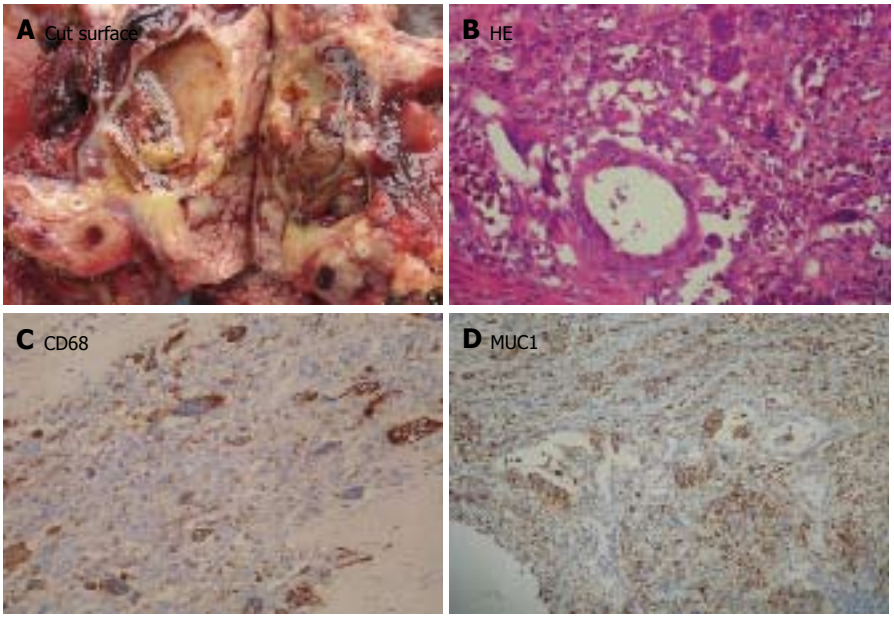


**Figure 5** Cystic ductal adenocarcinoma. **A**: Part of the tumor showing cystic wall with brown color; **B**: Cystic wall consisting of multinuclear giant cells (HE × 100); **C**: Giant tumor cells positive for CD68 (Envison × 100); **D**: Diffusely stained MUC1 in neoplastic cells, including duct and giant tumor cells (Envison × 100).

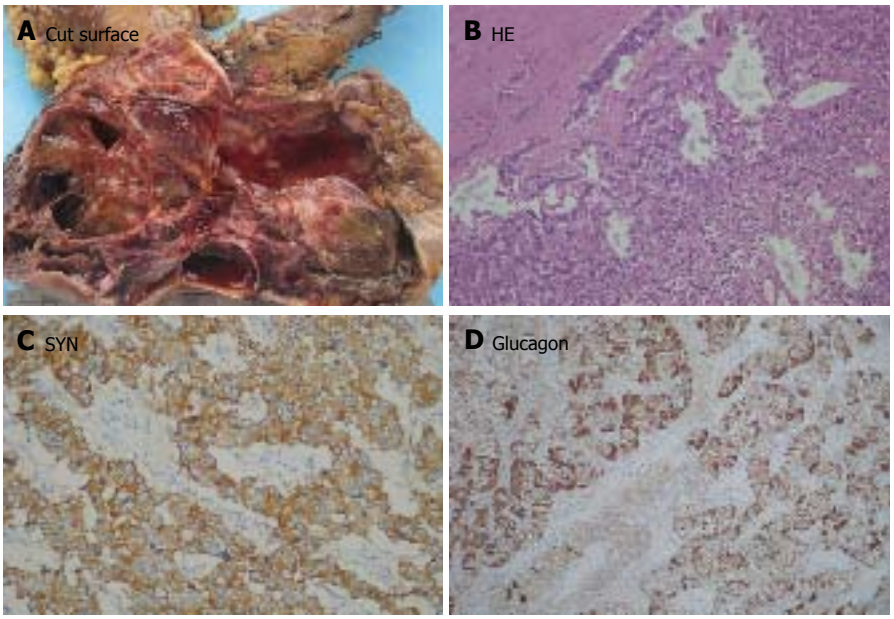


**Figure 6** Cystic pancreatic endocrine tumor. **A**: Cut surface of a large PET showing degeneration and cystic change; **B**: Tumor cells arranged as trabecular nests in the remnant area (HE × 100); **C**: SYN presented in all tumor cells (Envison × 200); **D**: Most tumor cells expressing glucagon in cytoplasm (Envison × 100).

of pancreatic cystadenomas and 1% of pancreatic carcinomas[1-3]. With the increased availability of imaging techniques and improved feasibility of surgical resection, an increasing number of cystic pancreatic neoplasiae have been identified in several institutions, thus their diagnosis can be established more easily.

The clinical challenge is the differential diagnosis and management of cystic neoplasm, which represents a wide range of biologic behavior. A variety of biochemical, genetic, and imaging techniques have been developed for the diagnosis and monitoring of the outcome of cystic neoplasia of the pancreas. In consideration of the potentials and limitations inherent in each of these methods, the need for efficient differential diagnostic markers that specifically discriminate pancreatic cystadenocarcinoma from pancreatic carcinoma cannot be overemphasized. In this regard, mucins may represent potential candidates for such a purpose, with respect to their biochemical properties relating to malignant conditions[9,10]. Analysis of mucin expression profiles in PCN patients has brought some light on their prognosis[5,6]. MUC1 is thought to have an inhibitory role in cell-cell and cell-stroma interactions as well as in immunoresistance[5]. MUC1also acts as a signal transducer, interacting with and promoting the activities of EGFR, MAP kinase and Wnt signaling pathway[11]. In pancreatic neoplasia, MUC1 has been found to be a marker of an aggressive phenotype expressed in high-grade PanINs, and more importantly, presents uniformly in infiltrating conventional ductal adenocarcinoma[5,6,9]. On the other hand, MUC2 functioning as a protective barrier in the intestinal epithelium[12,13], appears to be a marker of an indolent phenotype in the pancreas[5]. MUC2 is not expressed in the normal pancreas, PanINs or ductal adenocarcinoma, but is often detectable in IPMNs and uniformly presents in colloid carcinomas.

IPMN is a mucin-producing epithelial neoplasia in-

volving the main and/or branch pancreatic ducts. Recent data suggest that a more heterogeneous profile of IPMN has different mucin patterns and clinical prognosis[11,12]. In consistent with previous reports, the intestinal type of our IPMN patients expressed MUC2, MUC5AC and MUC4, but not MUC1. It had a lower invasive and metastatic potential in comparison to DACs and therefore had a better clinical outcome. The pancreatobiliary type of IPMN is MUC1 positive and MUC2 negative with an increased tendency to invade, and seems to be related to DAC[13,14]. The third group with a gastric foveolar differentiation which is MUC5AC positive but usually does not express either MUC1 or MUC2, was not identified in this study. The lack of this type in our series may be due to the misdiagnosis of MCN for their similar mucin profile.

Pancreatic SCN is usually considered benign. Whether SCN is malignant is still controversial. However, the malignancy of pancreatic SCN has been recognized since 3% of reported SCNs are cancerous in nature[15]. In many reported cases of malignant SCN, there are multifocal lesions in the liver or additional lesions, but pancreatic tumor itself shows no signs of malignancy. Amorphous nuclei, irregular nuclear margins, and coarse nuclear chromatin in SCN suggest that it has a malignant potential. In the present study, SCN exhibited a similar if not identical immunoprofile-positivity for MUC1 and MUC6, whereas no SCN expressed MUC5AC, although mucin production is not a histologic feature of SCN. The mucin profile of SCN that was positive for MUC6 supports the hypothesis of a direction of differentiation toward centroacinar cells, since the ultrastructural features of tumor cells are similar to those of centroacinar cells[15]. This hypothesis is further supported by the centroacinar localization of incipient serous cystic tumors observed incidentally[16]. In immunostaining, helpful markers for SCN are α-inhibin and MUC6.

MCN was first considered as a premalignant or overtly malignant cystic neoplasia. Because of the reputed difficulty in classifying MCN as a "benign" cystadenoma, some pathologists classify MCN of the pancreas with only small areas of epithelial hyperplasia or dysplasia as grade 1 cystadenocarcinoma, thereby implying a more aggressive natural history and predetermining their future management and follow-up as a malignancy despite successful resection. When these neoplasiae become malignant, only portions of the tumor dedifferentiate (i.e., become proliferative, develop nuclear dysplasia, and eventually invade the stroma). These areas may not be sampled unless the entire neoplasm is examined. In addition, separation of cystic mucinous neoplasia from non-invasive IPMN and high-grade ductal adenocarcinoma that incidentally have cystic areas (related to necrosis or ductal obstruction) requires both adequate sampling and clinicoradiologic and pathologic correlation. Mucin gene expression may discriminate between MCN and DAC[7]. In this study, noninvasive MCN, regardless of the degree of cellular atypia, was positive for MUC5AC and negative for MUC1. MUC1 expression was observed only in patients with an invasive component. MUC2 perinuclear expression was restricted to goblet cells scattered within the epithelium of MCN, often accompanied with endocrine cells, a further indication of intestinal differentiation. As MUC5AC is a gastric foveolar mucin, and expressed during intestinal ontogenesis, further investigation is required before we understand the role of MUC5AC in MCN. In addition, the expression of α-inhibin in stromal cells of MCN may be useful in the diagnosis of cystic pancreatic lesions.

SPN of the pancreas is a rare neoplasia with distinctive pathologic features and low-grade malignant potential that preferentially affects young women. SPN is composed of uniform and polygonal epithelioid cells, which are arranged in a discohesive pattern. SPN can be readily diagnosed by routine histologic examination, but immunohistochemical studies are frequently performed to confirm the diagnosis. Tumor cells of SPN are characteristically positive for vimentin and α-1-antitrypsin, and may also show focal immunoreactivity for cytokeratin and synaptophysin and abnormal nuclear localization of β-catenin. Staining of chromogranin and the acinar markers trypsin and chymotrypsin is consistently negative. SPN is composed of monomorphous cells with uncertain lineage or histogenesis[17]. The phenotype of SPN surprisingly does not resemble that of any normal epithelial cell lines of the pancreas.

Cystic NET is relatively easy to be distinguished from other epithelial cystic neoplasiae by the lack of mucin expression and presence of neuroendocrine markers[18].

In general, mucins follow a defined spatial and temporal pattern of expression throughout the development of an organ. However, recent studies demonstrate that deregulated expression of mucins is associated with various types of neoplasia. In the normal adult pancreas, MUC1 expression is confined to the apical membrane of intralobular ductules, possibly a manifestation of the "lumen maintenance" role of MUC1. MUC1 expression is not detectable in larger ducts or other pancreatic elements (islets or acini)[19]. MUC6 mRNA is also detectable in acini, in which the signal is largely restricted to centroacinar cells (Table 5). MUC2 or MUC5AC is not expressed in normal adult pancreas[20,21]. In pancreatic adenocarcinoma, tumors and tumor cell lines can overexpress MUC1. Additionally, an aberrant expression of MUC4 in pancreatic tumor and cell lines has been reported, a MUC that is usually undetectable in normal pancreas.

It is important to distinguish between a pseudocyst tumor and a cystic tumor because they are frequently confused with one another and the management of these entities is entirely different.

In summary, mucin profile may lead not only to the early diagnosis of pancreatic tumors but also to the accurate diagnosis of neoplasia. Mucin alteration may, in conjunction with histologic study, provide important information on tumor type and patient treatment.

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*RAPID COMMUNICATION*

# Expression of semaphorin 6D in gastric carcinoma and its significance

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## Abstract

**AIM:** To investigate the protein and mRNA expression of semaphorin 6D in gastric carcinoma and its significance.

**METHODS:** The protein and mRNA expression of semaphorin 6D was detected by semi-quantitative reverse transcription PCR and Western blotting respectively in 30 cases of gastric carcinoma and normal gastric mucosa.

**RESULTS:** The protein and mRNA expression of semaphorin 6D in gastric carcinoma was significantly higher than that in normal gastric mucosa (0.24 ± 0.06 *vs* 0.19 ± 0.07, 0.26 ± 0.09 *vs* 0.20 ± 0.10, $P < 0.05$).

**CONCLUSION:** Semaphorin 6D may play an important role in the occurrence and development of gastric carcinoma, and is related to tumor angiogenesis.



**Key words:** Semaphorin 6D; Gastric carcinoma; Normal gastric mucosa; Angiogenesis

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## INTRODUCTION

The semaphorin family is characterized by a phylogenetically conserved sema domain in the extracellular region[1]. This family has been subdivided into eight groups, which play a crucial role in formation of nervous system, embryogenesis, angiogenesis, immunoreaction, and carcinogenesis[1-5]. Semaphorin 6D is a novel member of class 6 semaphorin gene, mapped on the chromosome 2, and it is grouped into five isoforms through different splicing[6]. At present, little is known about the function of semaphorin 6D in carcinogenesis. Therefore, we detected the protein and mRNA expression of semaphorin 6D in gastric carcinoma and normal gastric mucosa by semi-quantitative RT-PCR and Western blotting in order to explore the expression of semaphorin 6D in gastric carcinoma and its significance.

## MATERIALS AND METHODS

### *Patients*

Thirty fresh gastric carcinoma specimens and thirty normal gastric mucosa specimens were obtained from patients who underwent surgery for gastric carcinoma between April 2006 and August 2006 in General Hospital of Chinese PLA and analyzed. The diagnosis of gastric carcinoma was confirmed by pathological examination. The patients with gastric carcinoma consisted of 11 women and 19 men. Their mean age was 54 years and ranged from 32 to 74 years. Of the 30 patients, 13 showed moderate differentiation, 17 poor differentiation.

### *Reverse transcription PCR (RT-PCR)*

Total mRNA was isolated by Trizol reagent according to the procedure of supplier (BioDev-tech, Beijing, China). The concentration was determined by measuring the absorbance at 260 nm and using the following equation: one optical density unit = 40 mg of RNA/mL. A 1.5 μg aliquot of total RNA from each specimen was reverse-transcribed into single-strand cDNA using oligo (dT)16 primer for 2 h at 37℃. Each single-strand cDNA was used for subsequent PCR amplification of semaphorin 6D and β-actin with the latter used as a quantitative control. PCR was carried out in a reaction volume of 50 μL: an initial denaturation for 5 min at 95℃, followed by 37 cycles at 94℃ for 50 s, at 55℃ for 50 s, and at 72℃ for 1 min, then a final extension at 72℃ for 10 min on the authorized thermal cycler for PCR. The primer sequences used for amplification were 5'-CTCAGTCGCTGTGAGCGTTAT-3' and 5'-CAGATGTTGGACCGCCAAATA-3' for samephorin 6D, 5'-CGCACCACTGGCATTGTCAT -3' and 5'-TTCTCCTTGATGTCACGCAC -3' for β-actin. The primer sequences were synthesized by Beijing Genomics Institute (China). The PCR products were resolved in 2% agarose gels and visualized by staining with

ethidium bromide. To quantify the PCR products, the bands representing amplified products were analyzed by Quantity One Analysis Software (BIO-RAD Co., USA).

### *Western blotting*

Expression of the semaphorin 6D protein was detected using the Western blot method. After washed in ice-cold PBS, the samples were finely minced and suspended in ice-cold homogenization buffer (2 mL/g of tissue) containing protease inhibitors to minimize protein degradation. The suspension was homogenized, then centrifuged at 12000 × *g* for 30 min at 4℃ to remove nuclei and cell debris. The supernatant (total protein extract) was collected. An equal amount (50 μg) of proteins was run on a 10% SDS-PAGE gel and electrotransferred onto hybond-polyvinylidene difluoride membranes (Amersham, Arlington Heights, USA). The membranes were blocked for 2 h at room temperature, followed by incubation with 3 μg/mL primary anti-semaphorin antibody (R&D system, Minneapolis, USA) at 4℃ overnight. The primary antibody was diluted in TBST containing fat-free milk. After three washes (10 min each time) in TBST, membrane was incubated in peroxidate-conjugated second antibody (Sigma, St. Louis, USA) diluted 1:800 at room temperature for 1 h. Immunoreactive proteins were visualized on autoradiogram using ECL Western blotting detection reagents (Amershan Pharmacia, Uppsala, Sweden) and exposed to X-Omat BT film (Kodak, New York, USA). Bands were analyzed by Quantity One Analysis Software normalized with respect to β-actin as an internal control.

### *Statistical analysis*

Results were expressed as mean ± SD. The statistical differences between different groups were analyzed by paired *t*-test. $P < 0.05$ was considered statistically significant. All analyses were performed by SPSS12.0 statistical software.

## RESULTS

### *Expression of semaphorin 6D mRNA*

Two percent of agarose gel electrophoresis showed a 232bp semaphorin 6D fragment by RT-PCR amplification from gastric caner and normal gastric mucosa specimens (Figure 1). The semaphorin 6D mRNA amplification was successful in all tissues. The expression level was 0.24 ± 0.06 in tumor, much higher than that in normal gastric mucosa (0.19 ± 0.07, $P < 0.05$).

### *Expression of semaphorin 6D protein*

The affinity-purified anti-semaphorin 6D antibody detected a major band at 100 kDa in protein extracts from all samples tested (Figure 2). The expression was 0.26 ± 0.09 in tumor, much higher than that in normal gastric mucosa (0.20 ± 0.10, $P < 0.05$), which was consistent with that of RT-PCR.

## DISCUSSION

The semaphorin family is characterized by a phylogenetically conserved sema domain in the extracellular region.

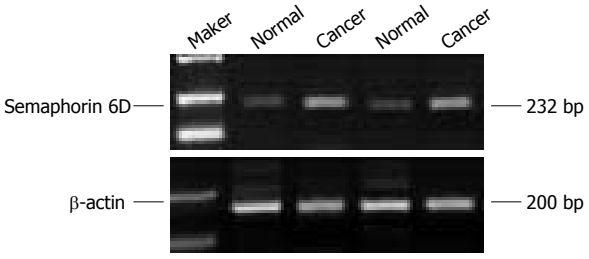


**Figure 1** Expression of semaphoring 6D mRNA detected by RT-PCR. The expression of semaphoring 6D mRNA was significantly higher in gastric cancer than in normal gastric mucosa ($P < 0.05$).

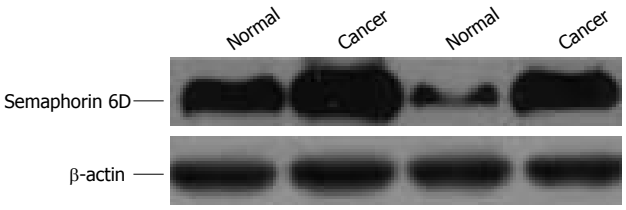


**Figure 2** Protein expression of semaphoring 6D detected by Western blot. The protein expression of semaphoring 6D was significantly higher in gastric cancer than in normal gastric mucosa ($P < 0.05$).

On the basis of additional structural features, such as the presence or absence of transmembrane domains, Ig-like domains, thrombospondin repeats, and glycophosphatidy linositol linkage sites, the family has been subdivided into eight groups, including virally derived proteins[1]. Despite the fact that a number of semaphorins have been known to play a crucial role in the hard wiring of the nervous system, including fasciculation, axon branching, and target selection as axonal guidance cues[2,3]. There is increasing evidence that semaphorins play a significant role in angiogenesis. For example, secreted class Ⅲ semaphorins, regulating axonal growth during the development of central nervous system[7,8], can initiate signaling events in a variety of tissues that influence vascular morphogenesis and endothelial cell motility[9-11]. It was reported that semaphorin 4D is highly expressed in endothelial cells and promotes endothelial cell migration and tubulogenesis[12].

Gastric carcinoma is one of the most common malignant tumors. As other malignant tumors, its growth and metastasis require induction of angiogenesis (the growth and remodeling of new blood vessels from a preexisting vascular network) to ensure the delivery of oxygen, nutrients, and growth factors to rapidly divide transformed cells and to provide tumor cell access to systemic circulation[13-15]. Without the ability to induce angiogenesis, most neoplasms would fail to grow (> 2 mm in diameter) or metastasize[15]. Semaphorin 6D is a novel class 6 semaphorin gene, mapped on the chromosome 2, and can be grouped into five isoforms through different splicing, including one short isoform (only consisting of extrocellular domain) and four long isoforms[6]. It was reported that semaphorin 6D can inhibit axonal extension of NGF-differentiated PC12 cells and to collapse growth cones of chick DRG and rat hippocampal neurons[6]. It was also reported that semaphorin 6D also can attract or

inhibit epithelial cell migration depending on different special regions through different receptor complex in cardiac morphogenesis of chick embryos[16], suggesting that semaphorin 6D may regulate angiogenesis *in vivo*, and increase the intriguing possibility, thus playing a role in tumor-induced angiogenesis, including gastric carcinoma. Therefore, we detected the protein and mRNA expression of semaphorin 6D in gastric carcinoma and normal gastric mucosa by semi-quantity RT-PCR and Western blotting. The results showed that the expression of semaphorin 6D was significantly higher in gastric carcinoma than in normal gastric mucosa, suggesting that semaphorin 6D plays an important role in the development of gastric carcinoma. Semaphorin 6D may induce tumor angiogenesis, thereby enhancing tumor growth, survival, and metastasis.

In conclusion, this is the first time to investigate the expression of semaphorin 6D in gastric carcinoma and its clinical significance. The results of our study shed some light on the pathogenesis of gastric carcinoma, and may represent a new therapeutic target for gastric carcinoma treatment.

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# Cytomegalovirus enterocolitis in a patient with diffuse large B-cell lymphoma after chemotherapy with rituximab

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## Abstract

Rituximab has been associated with the development of cytomegalovirus enterocolitis in immunosuppressed patients. A 51-year-old patient with diffuse large B-cell lymphoma who received a conditioning chemotherapy regimen (RCVP and RICE) consisting of rituximab before bone marrow transplantation went on to develop cytomegalovirus enterocolitis. This supports evidence from previously described cases that rituximab may be associated with cytomegalovirus enterocolitis.



**Key words:** Rituximab; Cytomegalovirus; Enterocolitis; Diffuse large B-cell lymphoma; Immunosuppression

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## TO THE EDITOR

In response to Unluturk *et al*'s letter to the editor entitled 'Cytomegalovirus gastritis after rituximab treatment in a non-Hodgkin's lymphoma patient[1], a similar case of cytomegalovirus (CMV) enterocolitis after treatment with rituximab has been described in a similar patient.

Rituximab, a monoclonal antibody targeting CD20 molecules on B-lymphocytes, can be given in combination with the chemotherapy regimen CVP (cyclophosphamide, vincristine and prednisolone), as the first treatment for people who have advanced (stage three or four) follicular lymphoma when first diagnosed or for people with advanced (stage three and four) follicular lymphoma, whose lymphoma has come back after initial chemotherapy[2].

A 51-year old patient diagnosed with follicular lymphoma in August 2004 was treated with a splenectomy and eight courses of RCVP (rituximab, cyclophosphamide, vincristine and prednisolone) combined chemotherapy, which led to a partial remission. In March 2006 the follicular lymphoma transformed to diffuse large B-cell lymphoma (DLBCL) and he was then treated with three courses of RICE (rituximab, ifosfamide, cytosine-arabinoside and etoposide) combined chemotherapy, which again led to only a partial remission.

It was decided to proceed with an autologous peripheral blood stem cell transplant in August 2006. In the week preceding transplant he was treated with a conditioning chemotherapy regimen consisting of carmustine, etoposide, cytarabine, and melphalan. His pre-tansplant CMV PCR status was negative. In the immediate days post-transplant he developed conditioning chemotherapy-related mucositis affecting his whole gastrointestinal tract, which was treated symptomatically. Faecal microscopy and stool cultures at the time were negative. The mucositis resolved two weeks post transplant but then in the third week he developed profound diarrhoea passing two litres of liquid faeces per day. CMV was detected by PCR, but bacteriological analysis of the faeces was otherwise negative. With a provisional diagnosis of CMV enterocolitis, a colonoscopy was performed and biopsies were taken from the terminal ileum and colon. Histological analysis of the biopsy material revealed evidence of CMV inclusions. He was started on ganciclovir with marked symptomatic improvement and resolution of his diarrhoea.

This case supports Unluturk *et al*'s case that CMV enterocolitis may occur after treatment with rituximab in patients with non-Hodgkin's lymphoma. Whether this is an opportunistic reactivation infection related to rituximab-induced immunosuppression needs further research.

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# Meetings

## MAJOR MEETINGS COMING UP

First Biennial Congress of the Asian-Pacific Hepato-Pancreato-Biliary Association
March, 2007
Fukuoka, Japan
http://www.congre.co.jp/1st-aphpba

American College of Gastroenterology Annual Scientific
20-25 October 2006
Las Vegas, NV

14th United European Gastroenterology Week, UEGW
21-25 October 2006
Berlin, Germany

APDW 2006: Asian Pacific Digestive Week 2006
26-29 November 2006
Lahug Cebu City, Philippines

## EVENTS AND MEETINGS IN THE UPCOMING 6 MONTHS

Falk Symposium 151: Emerging Issues in Inflammatory Bowel Diseases
24-25 March 2006
Sydney - NSW
Falk Foundation e.V.
symposia@falkfoundation.de

10th International Congress of Obesity
3-8 September 2006
Sydney
Event Planners Australia
enquiries@ico2006.com
www.ico2006.com

Easl 2006 - the 41st annual
26-30 April 2006
Vienna, Austria
Kenes International

Prague hepatology 2006
14-16 September 2006
Prague
Foundation of the Czech Society of Hepatology
veronika.revicka@congressprague.cz
www.czech-hepatology.cz/phm2006

12th International Symposium on Viral Hepatitis and Liver Disease
1-5 July 2006
Paris
MCI France
isvhld2006@mci-group.com
www.isvhld2006.com

Falk Symposium 152: Intestinal Disease Part I, Endoscopy 2006 - Update and Live Demonstration
4-5 May 2006
Berlin
Falk Foundation e.V.
symposia@falkfoundation.de

Falk Symposium 153: Intestinal Disease Part II, Immunoregulation in Inflammatory Bowel Disease - Current Understanding and Innovation
6-7 May 2006
Berlin
Falk Foundation e.V.
symposia@falkfoundation.de

ILTS 12th Annual International Congress
3-6 May 2006
Milan
ILTS
www.ilts.org

Internal Medicine: Gastroenterology
22 July 2006-1 August 2006
Amsterdam
Continuing Education Inc
jbarnhart@continuingeducation.net

6th Annual Gastroenterology And Hepatology
15-18 March 2006
Rio Grande
Office of Continuing Medical Education
cmenet@jhmi.edu
www.hopkinscme.net

World Congress on Gastrointestinal Cancer
28 June 2006-1 July 2006
Barcelona, Spain
c.chase@imedex.com

International Conference on Surgical Infections, ICSI2006
6-8 September 2006
Stockholm
European Society of Clinical Microbiology and Infectious Diseases
icsi2006@stocon.se
www.icsi2006.se/9/23312.asp

7th World Congress of the International Hepato-Pancreato-Biliary Association
3-7 September 2006
Edinburgh
Edinburgh Convention Bureau
convention@edinburgh.org
www.edinburgh.org/conference

Society of American Gastrointestinal Endoscopic Surgeons
26-29 April 2006
Dallas - TX
www.sages.org

Digestive Disease Week 2006
20-25 May 2006
Los Angeles
www.ddw.org

Annual Postgraduate Course
25-26 May 2006
Los Angeles, CA
American Society of Gastrointestinal Endoscopy
www.asge.org/education

American Society of Colon and Rectal Surgeons
3-7 June 2006
Seatlle - Washington
www.fascrs.org

## EVENTS AND MEETINGS IN 2006

10th World Congress of the International Society for Diseases of the Esophagus
22-25 February 2006
Adelaide
isde@sapmea.asn.au
www.isde.net

Falk Symposium 151: Emerging Issues in Inflammatory Bowel Diseases
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www.cag-acg.org

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Falk Seminar: XI Gastroenterology Seminar Week
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Falk Symposium 152: Intestinal Disease Part I, Endoscopy 2006 - Update and Live Demonstration
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Falk Symposium 153: Intestinal Disease Part II, Immunoregulation in Inflammatory Bowel Disease - Current Understanding and Innovation
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www.uegw2006.de

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mangosee.com/mangosteen/hepatitis2006/hepatitis2006.htm

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27-31 October 2006
Boston, MA
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13-16 December 2006
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*EDITORIAL*

# Acute fatty liver of pregnancy: An update on pathogenesis and clinical implications

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## Abstract

Acute fatty liver of pregnancy (AFLP) is a serious maternal illness occurring in the third trimester of pregnancy with significant perinatal and maternal mortality. Till recently, it has been considered a mysterious illness. In this editorial, we review the recent advances in understanding the pathogenesis of AFLP and discuss the studies documenting a fetal-maternal interaction with a causative association between carrying a fetus with a defect in mitochondrial fatty acid oxidation and development of AFLP. Further, we discuss the impact of these recent advances on the offspring born to women who develop AFLP, such that screening for a genetic defect can be life saving to the newborn and would allow genetic counseling in subsequent pregnancies. The molecular basis and underlying mechanism for this unique fetal-maternal interaction causing maternal liver disease is discussed.



**Key words:** Acute fatty liver of pregnancy; HELLP syndrome; Mitochondrial Trifunctional Protein; Mitochondrial fatty acid oxidation

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## INTRODUCTION

Acute fatty liver of pregnancy (AFLP) is a maternal liver disease unique to pregnancy. It was first described in 1934 as "yellow acute atrophy of the liver[1]" and was described as a specific clinical entity in 1940[2]. AFLP is a rare, but serious condition that occurs in the third trimester. It carries a significant perinatal and maternal mortality[3,4]. AFLP can lead to hepatic failure and encephalopathy and, if diagnosis is delayed, death for the fetus and mother. Clinical findings in AFLP vary and its diagnosis is complicated by a significant overlap in clinical and biochemical features with the HELLP (hemolysis, elevated liver enzymes, and low platelets) syndrome. The cause for AFLP is unknown. Recent molecular advances suggest that AFLP may result from mitochondrial dysfunction. Some of the clinical and pathological features of AFLP are similar to those found in certain autosomal, recessively inherited disorders of fatty acid oxidation, and hence, it was suggested that AFLP may result from defects in the β-oxidation of fatty acids[5-7]. Below, we review recent advances that link AFLP to disorders of fatty acid oxidation and the clinical impact for both the mother and the newborn.

## CLINICAL FEATURES OF ACUTE FATTY LIVER OF PREGNANCY

AFLP has an incidence of 1 per 13 000 deliveries[4]. It affects women of all ages and races and there is no distinctive epidemiologic feature that has been related to geographic areas or ethnic groups. The onset of AFLP is between the 30$^{th}$ and 38$^{th}$ wk of gestation although an early occurrence at 26 wk has been reported[8]. There are rare reports of AFLP diagnosis in the second trimester[9-11]. It is more frequent in primaparous than multiparous women but can occur after multiple uneventful pregnancies[12]. Several reports have documented recurrence of AFLP in subsequent pregnancies[13,14].

The initial manifestations of AFLP are nonspecific and include headache, fatigue, nausea and vomiting[15,16]. Seventy percent of patients present with nausea and vomiting, whereas 50%-80% complain of right upper quadrant or epigastric pain. AFLP can be complicated at an early stage by an upper gastrointestinal hemorrhage due to coagulation abnormalities, acute renal failure, infection, pancreatitis, or hypoglycemia[15,16]. Hepatic encephalopathy occurs later in the disease and should immediately alert the physician to the possibility of AFLP. Most patients improve 1-4 wk postpartum[17].

## DIAGNOSIS OF ACUTE FATTY LIVER OF PREGNANCY

The diagnosis of AFLP is suggested by the clinical setting,

which can be confirmed by a liver biopsy[17]. Since AFLP is a medical and obstetric emergency, early recognition and prompt treatment improves both maternal and fetal survival. In most cases, reliance on clinical and laboratory findings and physician experience to make a differential diagnosis and therapeutic decisions are critical, even without a liver biopsy. Although the gold standard for a definitive diagnosis of AFLP is through a liver biopsy, it is not routinely done for the obvious reasons of urgent delivery and coagulopathy. Thus, in most cases, reliance on laboratory data without waiting for a histologically proven diagnosis is made.

Patients with AFLP have elevated serum aminotransferase levels. White-cell count can be elevated and peripheral blood smears may demonstrate thrombocytopenia and normoblasts[18]. Disseminated intravascular coagulopathy (DIC) is relatively common[19,20]. Prothrombin time (PT), partial thromboplastin time (PTT) and fibrinogen may be abnormal. Blood urea nitrogen and creatinine may be elevated with uric acid increased. Alkaline phosphatase is 3-4 times normal. Ammonia level may be elevated and hypoglycemia may occur. Ultrasound, computerized tomography scan, and magnetic resonance imaging have been considered as noninvasive tools for diagnosis of AFLP but their value remain limited[21]. Histologically, AFLP is characterized by microvesicular hepatic steatosis. On Oil Red O staining, one observes the cytoplasmic vesiculation as a result of microvesicular fat[22,23].

Although there are many causes of hepatic disease in pregnancy, a few disease states pose difficulty in the differential diagnosis. AFLP is often difficult to distinguish clinically from fulminant viral hepatitis since both may present suddenly and may progress to hepatic failure[24]. Severe HELLP syndrome may be impossible to distinguish from AFLP in some cases. HELLP syndrome is a severe form of preeclampsia that threatens the patient and her fetus occurring in 4%-20% of women with severe preeclampsia[25]. Typically, hypoglycemia, and prolongation of prothrombin time may distinguish AFLP from HELLP syndrome. Histological examination of liver biopsies from HELLP syndrome cases reveal periportal hemorrhage and fibrin deposition while those from AFLP cases are characterized by microvesicular fatty infiltration of the liver[27].

## PATHOGENESIS OF ACUTE FATTY LIVER OF PREGNANCY

Until recently the pathogenesis of AFLP was unknown and still has not been fully elucidated. Recent molecular advances suggest that AFLP may result from mitochondrial dysfunction. As we discuss below, several reports have documented a strong association between AFLP and a deficiency of the enzyme long-chain 3-hydroxyacyl-CoA dehydrogenase (LCHAD) in the fetus, a disorder of mitochondrial fatty acid beta-oxidation.

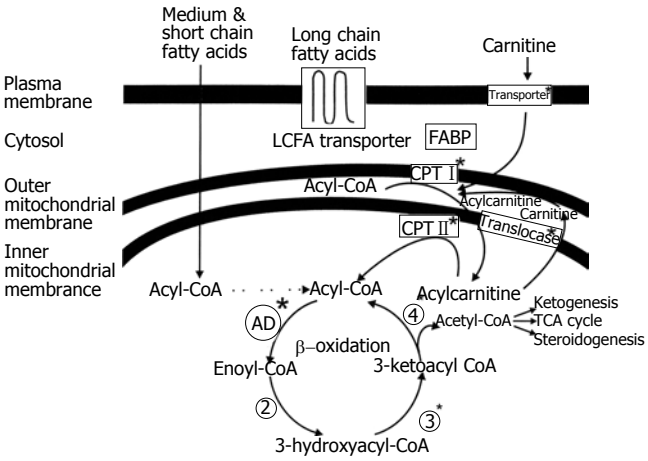


**Figure 1** Fatty Acid Import into the Mitochondria and Fatty Acid β-oxidation. Long chain fatty acids are actively transported across the plasma membrane, esterified to coenzyme A, carried by fatty acid binding proteins through the cytoplasm to the mitochondria and translocated across the mitochondrial membranes by the carnitine shuttle into the mitochondrial matrix. The fatty acid is subsequently cleaved into two shorter carbon entities by the β-oxidation spiral.

## MITOCHONDRIAL FATTY ACID BETA-OXIDATION

The β-oxidation of fatty acids is the major source of energy for skeletal muscle and the heart, while the liver oxidizes fatty acids primarily under the conditions of prolonged fasting, during illness, and at periods of increased muscular activity. Fatty acid oxidation (FAO) also plays an essential role in the intermediary metabolism in the liver. The oxidation of fatty acids in the liver fuels the synthesis of ketone bodies, 3-hydroxy butyrate and acetoacetate, which are utilized as alternative sources of energy by extrahepatic organs, like the brain, when blood glucose levels are low[18].

Mitochondrial β-oxidation of fatty acids is a complex process that consists of multiple transport steps and four enzymatic reactions resulting in the sequential removal of two-carbon, acetyl-coenzyme A units. Plasma long chain fatty acids are transported actively across the plasma membrane, esterified to coenzyme A, carried by fatty acid binding proteins through the cytoplasm to the mitochondria, and translocated across the mitochondrial inner membrane by the carnitine shuttle to the mitochondrial matrix (Figure 1). Once in the mitochondrial matrix, the fatty acid is sequentially cleaved, two carbons shorter, by the four reactions of the β-oxidation spiral. Each step in the spiral is catalyzed by 2-4 distinct enzymes, encoded by separate nuclear genes that exhibit different overlapping substrate specificities. The first step in the spiral, as shown in Figure 2, is an acyl-CoA dehydrogenase reaction, catalyzed by very long-chain acyl-CoA dehydrogenase (VLCAD) and its homologous enzymes, long-chain acyl-CoA dehydrogenase (LCAD), medium-chain acyl-CoA dehydrogenase (MCAD) or short-chain acyl-CoA dehydrogenase (SCAD). The second step in the pathway adds a water across the double bond and is catalyzed by either a long-chain 2,3-enoyl-CoA hydratase

(LCEH) or a short-chain 2,3-enoyl-CoA hydratase (SCEH) which hydrates the 2,3 enoyl-CoA across the double bond. The third step is catalyzed by a long-chain 3-hydroxyacyl-CoA dehydrogenase (LCHAD) or a short-chain 3-hydroxyacyl-CoA dehydrogenase (SCHAD) which oxidizes the 3-hydroxy position producing a 3-ketoacyl-CoA. The fourth and last step in the spiral is mediated by a long-chain 3-ketoacyl-CoA thiolase (LKAT), medium-chain 3-ketoacyl-CoA thiolase (MKAT), or short-chain 3-ketoacyl-CoA thiolase (SKAT), that shortens the fatty acyl-CoA substrate by two carbons by cleaving off acetyl-CoA. The shortened acyl-CoA can then reenter the fatty acid β-oxidation spiral until the fatty acid is completely broken down into a 2-carbon or 3-carbon specie. The acetyl-CoA produced can be used for ketogenesis, steroid genesis and as a substrate for the tricarboxylic acid cycle. For long chain length fatty acids, the last three steps are mediated by an enzyme complex called trifunctional protein[29,30].

In 1992, two groups of investigators, independent of each other, reported that LCHAD is part of an enzyme complex, the mitochondrial trifunctional protein (MTP) which is associated with the inner mitochondrial membrane[31,32]. MTP, also known as trifunctional protein (TFP), is a heterooctamer of 4α- and 4β- subunits. The α-subunit amino-terminal domain contains the long-chain 3-enoyl-CoA hydratase enzymatic activity while the LCHAD enzymatic activity resides in the carboxy-terminal domain. The β-subunit has the long-chain 3-ketoacyl-CoA thiolase enzymatic activity. The association of the α and β subunits to form the enzyme complex is necessary for membrane translocation and for the catalytic stability of the 3 enzymes[33,34]. The human cDNAs encoding both α and β subunits have been isolated and characterized[29,35]. Both subunit genes, HADHA and HADHB, have been localized to chromosome 2p23 using fluorescence *in situ* hybridization (FISH)[36]. Figure 3 shows a schematic representation of the exons of the α- and β-subunits composing the MTP subunit cDNAs and the corresponding functions of the encoded protein. Both genes have been found to be arranged in a head-to-head manner and share a promoter that is bidirectional[37].

## FATTY ACID OXIDATION DISORDERS

Fatty acid oxidation disorders have become an important group of inherited metabolic disorders characterized by a wide array of clinical presentations and as important causes of pediatric morbidity and mortality. The first documented inherited disorder of fatty acid oxidation was described in 1973 of a muscle carnitine palmitoyl transferase (CPT) deficiency[38]. Since then, more than 20 different disorders that affect β-oxidation have been identified. LCHAD deficiency was first described in 1989[39]. MTP deficiency was first reported in 1992[40], which cleared the confusion of interpreting the combined defects of LCEH, LCHAD, and LKAT.

Fatty acid oxidation disorders, if unrecognized and untreated, can cause sudden unexpected death. Such deaths can be certified as sudden infant death syndrome (SIDS), if fatty acid oxidation defects are not suspected.

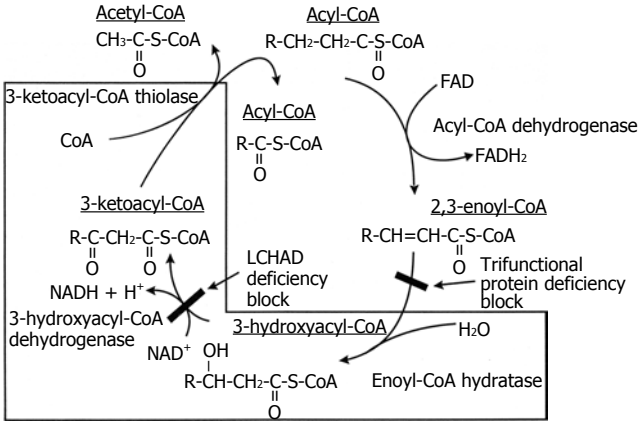


**Figure 2** The Biochemistry of TFP and LCHAD Deficiencies. The last three reactions of the mitochondrial fatty acid β-oxidation spiral where the trifunctional protein catalyzes long chain fatty acids substrates. In isolated LCHAD deficiency, the pathway is blocked after the enoyl Co-A hydratase reaction and before the 3-hydroxyacyl Co-A dehydrogenase reaction, causing the accumulation of medium- and long-chain 3-hydroxy fatty acids and their metabolites. In complete TFP deficiency, the pathway is blocked after the acyl Co-A dehydrogenase reaction and before the enoyl Co-A dehydrogenase reaction causing the accumulation of straight chain fatty acids and their metabolites.

Postmortem studies on SIDS victims attributed a small, but significant, number of SIDS to undiagnosed fatty acid oxidation disorders at the time of the sudden infant death[41].

## MITOCHONDRIAL TRIFUNCTIONAL PROTEIN DEFECTS

MTP defects have recently emerged as an important group of errors of metabolism because of their clinical implications. Human defects in the MTP complex are recessively inherited and cause either isolated LCHAD deficiency, with normal or partially reduced thiolase and hydratase activity, or complete MTP deficiency with markedly reduced activity of all 3 enzymes. Patients have been described with either an isolated LCHAD deficiency or MTP deficiency. In the literature, the majority of patients reported have been described as having isolated LCHAD deficiency[42].

A few hours or several months after birth, children with these recessively inherited disorders present with nonketotic hypoglycemia and hepatic encephalopathy, which may progress to coma and death if untreated[43]. They can also present with cardiomyopathy, slowly progressing peripheral neuropathy, skeletal myopathy, or sudden, unexpected death[44,45].

## MOLECULAR BASIS OF MTP DEFECTS

With the explosion in human molecular genetics over the last decade, we and others began to characterize normal and mutated MTP genes using molecular approaches to delineate disease-causing MTP mutations. Two teams, independent of each other, delineated the G1528C mutation in exon 15 of the α-subunit which alters amino acid 474 from glutamic acid to glutamine (E474Q) and

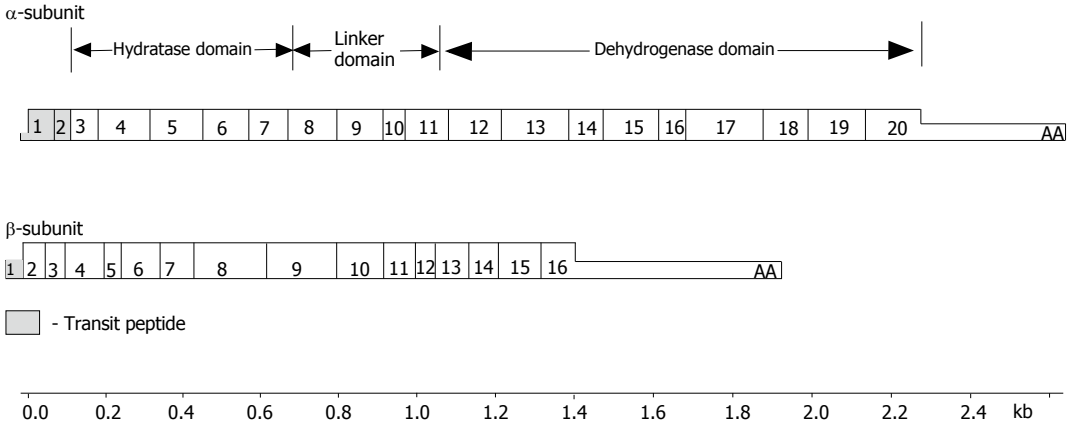


**Figure 3** The structure of MTP subunit cDNA's. The MTP α-subunit contains 20 exons. Exons 1 and 2 encode the transit peptide that directs uptake into the mitochondria which is proteolytically cleaved during translocation into the mitochondria. Exons 3-8 encode the N-terminus of the mature α-subunit containing the long-chain 2,3-enoyl-CoA hydratase activity. Exon 9 encodes the region known as a linker domain. Exons 11-20 encode the C-terminus of the mature α-subunit containing the LCHAD activity. The MTP β-subunit has 16 exons that encode the long-chain 3-keto-acyl CoA thiolase activity.

**Table 1 Genotypes and phenotypes in 24 families with MTP defects**

| Number of families | Biochemical phenotype | Pediatric phenotype | Protein expression | Pediatric phenotype | Maternal phenotype |
|---|---|---|---|---|---|
| 19 | LCHAD | Homozygous G1528C (8) | Normal | Hepatic (7)<br>Mixed (1) | AFLP (6)<br>Normal (1)<br>HELLP (1) |
| | | Compound heterozygous (11)<br>G1528C/splice site (3)<br>G1528C/stop codon (7)<br>G1528C/?[1] | Reduced | Hepatic (9)<br>Mixed (1)<br>Unknown (1) | AFLP (7)<br>HELLP (1)<br>Normal (3) |
| 5 | MTP | Homozygous (1)<br>(A→7G missplice), exon 7 | Absent | Cardiac | Normal |
| | | Compound Heterozygous (2)<br>(G + 1A missplice/A<br>+ 3G missplice), exon 3<br>( C2026T/C2027A), exon 19 | Absent<br>Absent | Cardiac<br>Cardiac | Normal<br>Normal |
| | | Homozygous (T845A), exon 9<br>Compound heterozygous<br>(T914A,/ C871T), exon 9 | Reduced<br>Reduced | Neuromuscular<br>Neuromuscular | Normal<br>Normal |

[1]Unknown mutation; AFLP–acute fatty liver of pregnancy; HELLP–hemolysis, elevated liver enzymes and low platelets syndrome.

replaces the acidic and negatively charged side-chain with a neutral, amide-containing residue[35,46]. In a subsequent study, we have reported the α-subunit molecular defects and phenotypes in 24 patients with documented isolated LCHAD deficiency or complete MTP deficiency[42]. Of the 24 patients, 19 were diagnosed with isolated LCHAD deficiency and presented with the hepatic phenotype, 5 were diagnosed with complete trifunctional protein deficiency where three displayed the cardiac phenotype and the other two presented with the neuromuscular phenotype. Patients with isolated LCHAD deficiency presented predominantly with a Reye-like syndrome of liver dysfunction and carried the prevalent G1528C missense mutation on one or both alleles. Of the 19 subjects, 8 were homozygous for the common mutation G1528C while 11 were compound heterozygotes, where one allele carried the common mutation and the other allele had a mutation other than the common G1528C mutation[42]. The genotypes and phenotypes of these 19 patients are presented in Table 1.

The likely mechanism by which the E474Q mutation causes isolated LCHAD deficiency is that the mutation inactivates LCHAD directly within the catalytic domain, and hence, preserves the other MTP enzyme activities. The likely mechanism has been elucidated by Barycki and colleagues[42] based on the crystal and structural analysis of x-ray diffraction data from the human SCHAD which is highly homologous to the LCHAD domain. E170 in SCHAD, a residue that is analogous to LCHAD E474, is located in the NAD-binding domain within the active catalytic site. E170 is also in a position to interact with another residue, H158 which serves as a base abstracting a proton from the 3-hydroxy group of the substrate. Substitution of E170 in SCHAD with glutamine disrupts the electrostatic interaction between E170 and H158, which is essential for catalysis. Similar to what happens to

E170Q mutation in SCHAD, the E474Q mutation causes isolated LCHAD deficiency and blocks the β-oxidation resulting in the accumulation of 3-hydroxy fatty acid metabolites.

All the five patients with the complete MTP deficiency presented with cardiomyopathy or neuromyopathy and carried mutations other than the E474Q mutation (Table 1). Three of the five patients with MTP deficiency presented with cardiomyopathy. All three patients had splice site and missense mutations that caused the absence of the MTP protein complex[42]. This condition blocks the second step of the β-oxidation spiral that results in the accumulation of long-chain 3-enoyl fatty acid metabolites. The other two patients with complete MTP deficiency presented with neuromyopathy. These two patients carried mutations in exon 9 that allowed stable MTP expression[43]. Exon 9 in the MTP α-subunit encodes a region described as a linker domain that may be important in subunit interaction and octamer complex formation as suggested by crystallographic data from short-chain enoyl-CoA hydratase[43]. The exon 9 mutations in the α-subunit seem to result in defective subunit interactions and octamer formation.

## FETAL MITOCHONDRIAL TRIFUNCTIONAL PROTEIN DEFECTS AND MATERNAL LIVER DISEASE

Several studies document strong and somewhat unique causative association between fetal MTP defects and AFLP.

Shoeman and colleagues were first to report an association between recurrent maternal acute fatty liver of pregnancy with a fetal fatty acid oxidation disorder in two siblings who both died at 6 mo of age[43]. The authors speculated that due to the finding of similar hepatic pathology of microvesicular steatosis, AFLP and fatty acid oxidation defects might share a common pathogenesis.

Other case reports in the early 90's have also associated affected infants with LCHAD deficiency to the occurrence of severe preeclampsia, HELLP syndrome or AFLP in the infant's mother during pregnancy. Wilcken *et al*[14] reported 11 pregnancies in 5 mothers where 6 babies had confirmed LCHAD deficiency by enzymatic analysis of cultured skin fibroblasts and that the mothers had either AFLP or HELLP syndrome in all six pregnancies with the LCHAD-deficient fetuses. Treem *et al*[49] reported an LCHAD deficient child born to a pregnancy complicated by AFLP.

In a subsequent report published in 1995, Sims *et al* delineated the molecular basis of pediatric LCHAD deficiency and its association with AFLP in 3 families with LCHAD-deficient infants[35]. The mothers suffered maternal HELLP syndrome or AFLP during pregnancies with the affected children. Molecular analysis in 2 affected children revealed the G1528C mutation on both alleles and the third child was a compound heterozygote with one allele carrying the G1528C.

Another report documented an association between pediatric LCHAD deficiency and occurrence of AFLP in the mother, Isaacs *et al*[35] reported a compound heterozygosity for MTP mutations in an affected child born to a pregnancy complicated by AFLP, with a novel mutation in exon 16 on one allele and the common G1528C mutation on the other allele.

In a study where we examined the association between MTP defects in children and liver disease in their mothers during pregnancy[42], 15 of 24 women (62%) were diagnosed to have had maternal liver disease, while 9 of the 24 women had normal pregnancies, as summarized in Table 1. In 5 of the normal pregnancies, the affected infant did not have the G1528C mutation, but rather other MTP mutations. The remaining four normal pregnancies were associated with fetal LCHAD deficiency. Thus 15/19 pregnancies associated with fetal LCHAD deficiency were complicated by maternal liver disease and none of the pregnancies associated with complete MTP deficiency were complicated by AFLP or HELLP syndrome. The results in this study suggest that when carrying an LCHAD-deficient fetus, there is a 79% chance that the pregnancy will be complicated by AFLP or HELLP syndrome.

In a subsequent study, we evaluated fetal genotypes and pregnancy outcomes in 83 pregnancies in 35 families with documented pediatric MTP defects, 24 pregnancies were complicated by AFLP, HELLP syndrome or severe preeclampsia[51]. Of the 24 pregnancies, 20 were complicated by AFLP, 2 with HELLP syndrome and 2 with preeclampsia and all the LCHAD-deficient fetuses carried the G1528C mutation on one or both alleles. Five pregnancies had fetuses with complete MTP deficiency (none of the mutations were G1528C) but there were no associated maternal complications in those pregnancies.

The above studies provide strong evidence that carrying a fetus with LCHAD deficiency is associated with a high risk for developing AFLP during pregnancy. With the growing evidence suggesting that carrying an LCHAD-deficient fetus is associated with AFLP, we have recommended that neonates born to pregnancies complicated by AFLP be tested for the common G1528C mutation and that this testing when done early after birth can be life saving as it may identify LCHAD-deficient children before they manifest the disease allowing early dietary intervention by institution of a diet low in fat, high in carbohydrate, and by substitution of the long chain fatty acids with medium chain fatty acids.

To further assess the significance of the association between maternal AFLP and fetal LCHAD deficiency, we prospectively screened for MTP mutations in mothers who developed AFLP or HELLP syndrome and their newborn infants[52]. The molecular screening was based solely on the maternal history. We prospectively screened 27 pregnancies complicated by AFLP and 81 pregnancies complicated by HELLP syndrome. Out of the 27 women that developed AFLP, 5 carried fetuses with MTP mutations. Three were homozygous for the G1528C mutation and two were compound heterozygotes with one mutant allele carrying the common G1528C and the other mutant allele carrying a novel mutation. Only one woman diagnosed with HELLP syndrome was heterozygous for the G1528C mutation which was not detected in her infant. None of the children born to the 81 women diagnosed with HELLP syndrome carried MTP mutations. This study

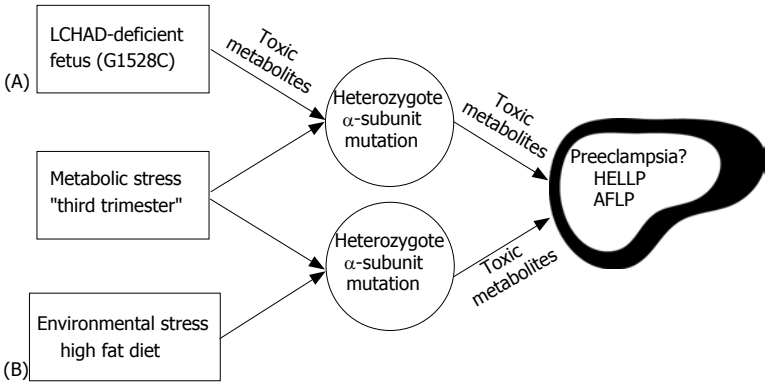


**Figure 4** Hypothesis illustrating the possible role of fetal and maternal MTP mutations in developing AFLP. Carrying an LCHAD deficient fetus (**A**) is the major determining factor in the development of maternal illness. Hepatotoxic metabolites produced by the fetus and/or placenta may cause liver disease in the obligate heterozygous mother when combined with the metabolic stress of the third trimester. Environmental stress (**B**) may lead to the further accumulation of toxic metabolites in the genetically susceptible mother causing maternal liver disease.

documents that in approximately one of five pregnancies complicated by AFLP, the fetus is LCHAD-deficient. This strong association between AFLP and the common G1528C mutation in the fetus is significant and hence screening the offspring of women who develop AFLP at birth for this mutation can be life saving.

In addition, identification of MTP mutations in the offspring of pregnancies complicated by AFLP allows genetic counseling for the mothers. Prenatal diagnosis can be performed in subsequent pregnancies to identify pregnancies at risk for development of AFLP. We performed molecular prenatal diagnosis in 11 pregnancies using chorionic villous samples and successfully identified the fetal genotype in these pregnancies confirmed by biochemical and molecular testing of the newborn or aborted fetuses[53].

## POSSIBLE HYPOTHESIS FOR THE ASSOCIATION BETWEEN FETAL LCHAD DEFICIENCY AND ACUTE FATTY LIVER OF PREGNANCY

The precise mechanism by which an LCHAD-deficient fetus causes AFLP in a heterozygote mother is still unclear. However, several factors appear to contribute to this fetal-maternal interaction as illustrated in Figure 4. First, the heterozygosity of the mother for an MTP defect reduces her capacity to oxidize long-chain fatty acids. Second, the stressful nature of pregnancy with its accompanying changes in metabolism, the increased lipolysis, and the decreased β-oxidation. We speculate that in the presence of the G1528C mutation, potentially hepatotoxic long-chain 3-hydroxyacyl fatty acid metabolites, produced by the fetus or placenta, accumulate in the maternal circulation. There is evidence for fatty acid oxidation in a normal human placenta including LCHAD and SCHAD activity[54]. Another study reported significant expression of fatty acid β-oxidation enzymes in human placenta as assessed by immunohistochemical and immunoblot analyses[55]. A recent study also showed high activity of fatty acid oxidation enzymes in human term placenta and chorionic villus samples[56].

The role of other FAO defects in the development of AFLP is not clear and remains controversial. Few case reports describe possible association between maternal liver disease and fetal fatty acid oxidation defects other than those in MTP. Maternal liver disease consistent with AFLP was associated with carnitine palmitoyl transferase Ⅰ (CPT-1) deficiency. In Innes *et al*[57], the patient developed liver disease consistent with AFLP and in a successive pregnancy, hyperemesis gravidarum. Both children were subsequently shown to have CPT-1 deficiency. In Ylitalo *et al*[58], the patient experienced complications that included a HELLP-like syndrome. She was later diagnosed to be CPT-1 deficient but her child was unaffected. Another report linked AFLP to fetal SCAD deficiency. In Matern *et al*[59], an infant born to a mother who developed AFLP was diagnosed with SCAD deficiency. A report of a pregnancy complicated with HELLP syndrome and associated with fetal MCAD deficiency has also been published[60].

## CONCLUSION

Acute fatty liver of pregnancy is a serious maternal disorder that has been for long considered of mysterious pathogenesis. Recent evidence suggests a fetal-maternal interaction causing acute fatty liver of pregnancy. Approximately one in five women who develop AFLP may carry an LCHAD-deficient fetus. Screening the newborn at birth in pregnancies complicated by AFLP for this fatty acid oxidation disorder can be lifesaving and may allow for genetic counseling in subsequent pregnancies.

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*EDITORIAL*

# Brain edema and intracranial hypertension in fulminant hepatic failure: Pathophysiology and management

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## Abstract

Intracranial hypertension is a major cause of morbidity and mortality of patients suffering from fulminant hepatic failure. The etiology of this intracranial hypertension is not fully determined, and is probably multifactorial, combining a cytotoxic brain edema due to the astrocytic accumulation of glutamine, and an increase in cerebral blood volume and cerebral blood flow, in part due to inflammation, to glutamine and to toxic products of the diseased liver. Validated methods to control intracranial hypertension in fulminant hepatic failure patients mainly include mannitol, hypertonic saline, indomethacin, thiopental, and hyperventilation. However all these measures are often not sufficient in absence of liver transplantation, the only curative treatment of intracranial hypertension in fulminant hepatic failure to date. Induced moderate hypothermia seems very promising in this setting, but has to be validated by a controlled, randomized study. Artificial liver support systems have been under investigation for many decades. The bioartificial liver, based on both detoxification and swine liver cells, has shown some efficacy on reduction of intracranial pressure but did not show survival benefit in a controlled, randomized study. The Molecular Adsorbents Recirculating System has shown some efficacy in decreasing intracranial pressure in an animal model of liver failure, but has still to be evaluated in a phase Ⅲ trial.



**Key words:** Intracranial hypertension; Fulminant hepatic failure; Brain edema

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## INTRODUCTION

Fulminant hepatic failure (FHF) is an infrequent but dreadful disease, defined by the appearance of hepatic encephalopathy within 8 wk after the onset of jaundice in patients with no known chronic liver disease[1]. Most FHF patients rapidly develop electrolyte, metabolic, and coagulation abnormalities[2]. They frequently suffer from acute renal insufficiency and/or adult respiratory distress syndrome (ARDS), leading to multiple organ failure (MOF)[3]. They are very sensitive to infection, and frequently develop a sepsis-like syndrome, with systemic hypotension, low peripheral resistance and increased cardiac output. Modern intensive care units (ICU) have learned to treat all these conditions, and have prolonged FHF patient survival. However, in absence of liver transplantation (LT), the mortality rate of FHF patients remains high (60%-80%). Causes of death of these patients are mainly MOF, sepsis, and/or brain edema leading to intracranial hypertension and secondary brain death.

Brain edema in FHF patients is a relatively recent concept. In a 1944 report of 125 autopsies of military patients dying from what was called fatal hepatitis (previously named idiopathic acute yellow atrophy of the liver), Lucké noted little alteration in the brain, except edema, but he did not describe cerebral herniation[4]. He hypothesized that the cerebral changes of fatal hepatitis might be attributed to loss of detoxifying function of the liver. The first reports of brain edema and cerebral herniation as complications of FHF were published in the 1970's[5,6] and were criticized at that time. Widespread recognition that brain edema and intracranial hypertension are complications of FHF only occurred in the 1980's[7,8]. It is also very interesting to note that brain edema and intracranial hypertension are not recognized common features of terminal chronic liver failure, despite some case reports or small series[9,10]. The recent recognition of brain edema in FHF patients could be due to the advances in FHF patient care. Previously, FHF patients were dying from early hepatocellular insufficiency complications, mainly hemorrhage or sepsis[4]. Improvements in ICU techniques have lengthened the survival of FHF patients. The longer course of the disease may have allowed the development of brain edema, possibly a later complication of FHF (Figure 1). Significant advances in the understanding of FHF brain edema have been made this last decade, but the exact pathophysiological mechanisms underlying development of brain edema and intracranial hypertension in FHF are still not entirely clear and are

likely to be multifactorial[11]. The aim of this paper is to review the pathophysiology of intracranial hypertension in FHF in order to improve understanding and management of this complication.

## PATHOPHYSIOLOGY OF INTRACRANIAL HYPERTENSION IN FHF

Normal intracranial pressure (ICP) is 5 to 10 mmHg and intracranial hypertension becomes clinically relevant when ICP exceeds 20 mmHg. The main complication of severe intracranial hypertension in FHF patients is transtentorial herniation[12]. This herniation may induce (1) compression of the posterior cerebral artery, leading to medial temporal, thalamic, and occipital lobe infarction; (2) cerebral aqueduct and subarachnoid space compression, causing obstructive hydrocephalus; and (3) brain stem compression, resulting in brain stem ischemia, haemorrhage, and death[12]. Additionally, severe intracranial hypertension compromises cerebral perfusion pressure (CPP). By definition, CPP is the difference between mean arterial pressure (MAP) and cerebral venous pressure. As cerebral venous pressure can be approximated by ICP, CPP equals MAP minus ICP[12,13]. An increase in ICP reduces CPP, and thus a decrease in cerebral blood flow (CBF). This reduction in CBF may cause cerebral ischemia or infarction, resulting in neurological deficits in FHF survivors.

A rise in ICP is the mechanical consequence of an increase in the intracranial volume. The central nervous system (CNS) is protected by the skull, which is rigid and incompressible. Inside the skull, 3 different compartments can be defined: the brain, the cerebrospinal fluid (CSF) and the blood. If the volume of one of these elements increases, the volume of another compartment might decrease, resulting in some intracranial compensation capacity or compliance. If the increase in volume exceeds this compliance, any further addition of volume leads to a rise in ICP.

It is generally accepted that CSF volume is not expanded in FHF. During episodes of intracranial hypertension in FHF, ventricular spaces measured by computed tomography (CT) were either unchanged or compressed, suggesting an increase in the brain tissue or blood volume[14,15]. In animal models and in FHF patients there is increased brain volume, secondary to edema[5,16,17]. In this environment even a small increase in cerebral blood volume could significantly increase ICP. In fact, these two phenomena have been proposed to account for intracranial hypertension in FHF: (1) brain edema due to osmotic astrocyte swelling secondary to ammonia-induced accumulation of glutamine (ammonia-glutamine hypothesis); (2) alteration of CBF regulation with increase of the intracranial blood volume.

### *Brain edema in FHF (Figure 2)*

Both vasogenic and cytotoxic mechanisms are implicated in the development of cerebral edema. Vasogenic brain edema occurs as a result of the disruption of the blood-brain barrier (BBB), allowing uncontrolled access of plasma components and water to the extracellular cerebral compartment. Cytotoxic edema is the consequence of impaired cellular osmoregulation in the brain, resulting in an increase of cellular water. In FHF, evidence from experimental animals and postmortem human brain supports aspects of each of these mechanisms, but it is now well established that brain edema in FHF is mainly cytotoxic[18]. In several models of FHF, compounds to which the BBB is normally impermeable (Evans Blue or a-aminoisobutyric acid) were detected in the brain in increased concentrations[19-21]. These observations suggest that BBB permeability is altered in FHF, which is consistent with a vasogenic mechanism. However, the ability of mannitol to reduce ICP in patients with FHF indicates that the BBB remains largely intact[8]. Furthermore, electron microscopic studies of human brain tissue in FHF revealed no alteration in the integrity of tight junctions[22]. Moreover, consistent with cytotoxic edema, marked intracellular swelling of perivascular astrocytes was observed. Recent magnetic resonance imaging of brain of FHF patients confirmed the predominant cytotoxic character of FHF brain edema[23]. This suggests that FHF brain edema primarily develops within the cellular component of the brain as a cytotoxic edema, and changes in the permeability of the BBB may represent secondary events that could exacerbate edema or intracranial hypertension.

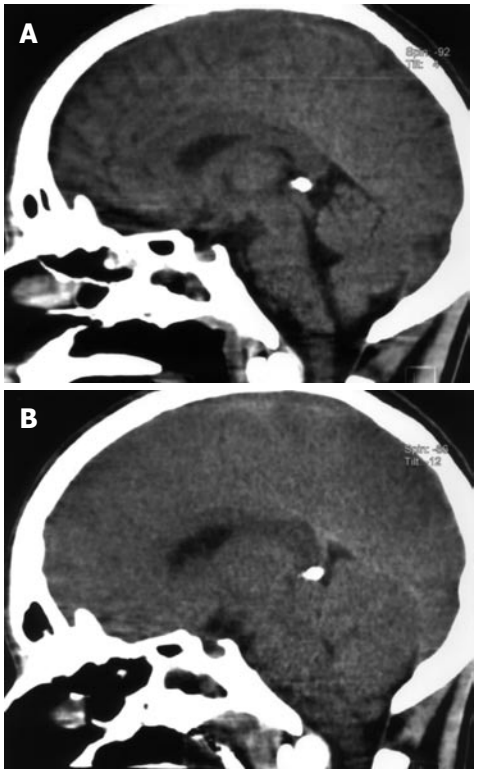


**Figure 1** Computed tomography (CT) (sagittal sections) of the brain of a 17-year old man who developed fulminant hepatic failure. **A**: First CT performed in encephalopathy stage Ⅳ; **B**: Control CT performed when the patient developed unilateral fixed midriasis, showing diffuse edema, compression of the fourth ventricle and of the brain stem. The patient developed brain stem death while waiting liver transplantation, despite medical therapy and MARS treatment.

**The ammonia-glutamine hypothesis:** Cortical astrocyte swelling is the most common observation

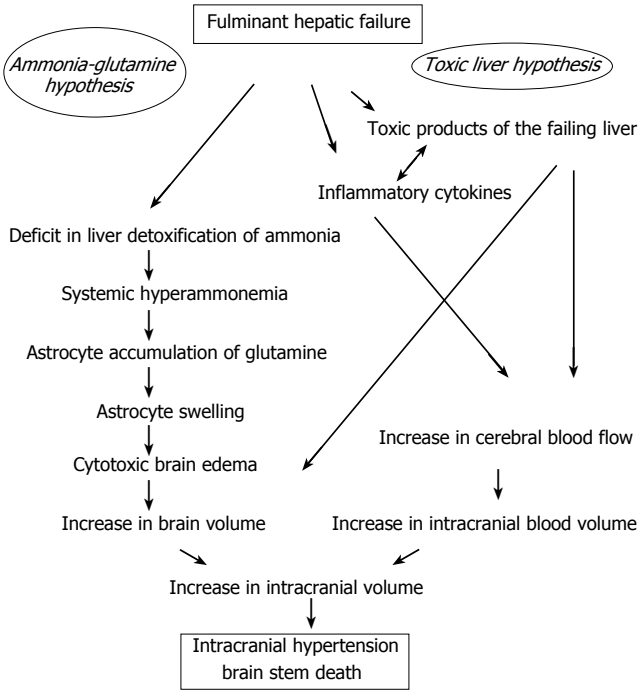


**Figure 2** Schematic representation of the hypotheses explaining intracranial hypertension and brain stem death in fulminant hepatic failure.

in neuropathological studies of brain edema in FHF. Astrocytes are the most numerous cell type in the brain and occupy about one-third of the cortical volume[24]. Astrocytes have several critical metabolic functions involved in the maintenance and regulation of the extracellular microenvironment. They participate in water regulation in the brain, detoxify ammonia and maintain normal levels of extracellular glutamate. Hyperammonemia is prevalent in acute liver failure. In the brain, ammonia is detoxified to glutamine via the amidation of glutamate by an astrocytic enzyme, glutamine synthetase. Glutamine leaves the astrocyte by passive diffusion into the extracellular space where it is taken up by neurons and converted to glutamate. There is clear evidence of increased brain glutamine concentrations in animal FHF models[25] and in postmortem samples of FHF[26]. Brusilow first proposed that this glutamine accumulation is the link between hyperammonemia and edema via altered osmoregulation[27]. In astrocyte cultures, ammonia induces cell swelling[17,28]. Brain swelling and intracranial hypertension have been documented in humans with hyperammonemic conditions[29]. In rats, a continuous infusion of ammonia is associated with brain edema or intracranial hypertension[30] which is reduced by inhibition of glutamine synthesis with methionine-sulfoximine[31-33]. The relationship between hyperammonemia, glutamine and ICP was recently demonstrated in humans[34]. Although there is much clinical and experimental evidence in support of the glutamine hypothesis, prevention of brain edema in FHF by inhibition of glutamine synthesis has not been successful in humans.

### *Increase in cerebral blood flow and intracranial blood volume*

Another phenomenon that has also been involved in intracranial hypertension in FHF is the increase of intracranial blood volume and CBF. Some reports describe decreased CBF in patients suffering from acute liver failure[35,36], but most have found high CBF associated with intracranial hypertension in FHF[37,38]. Paulson suggests that an impairment of vascular autoregulation in the brain could be responsible for this increase in CBF and blood volume[39]. Impaired autoregulation of CBF has been reported in animal models of FHF[40] and in patients with FHF[41]. The exact cause of this increase of CBF in FHF is not known. Nitric oxide (NO) has been implicated but it is possible that the increased NO in the brain of FHF patients is secondary to an increase in CBF, rather than a primary event[18,42]. Inflammation markers (IL-1 $\beta$, TNF $\alpha$, IL-6) and systemic inflammatory response have been associated with increased CBF and ICP in FHF[37,43], with poor outcome[44]. The association of systemic inflammation and impaired regulation of CBF might be related to the role of the necrotic liver in intracranial hypertension in FHF. The observation that brain edema and intracranial hypertension are complications of FHF and not of chronic liver disease lead to the hypothesis that these phenomena may in part result from products of the acutely necrotic liver. There is experimental and clinical evidence to support this theory, the "toxic liver hypothesis". In a rat model, cerebral edema was significantly lower in anhepatic than FHF animals[45]. In pigs, no elevation of ICP was observed after total hepatectomy, whereas a rise in ICP was observed in pigs with FHF secondary to ischemia[46]. Several human observations reinforced this hypothesis. During LT for FHF, it was established that ICP normalizes during the anhepatic phase and may increase during the dissection of the diseased liver and during graft reperfusion[47,48]. The removal of the diseased liver has been linked to ICP normalization and to marked and sustained reduction of several pro-inflammatory cytokines in a case report[49]. Moreover some patients underwent a prolonged period (up to 72 h) of an anhepatic state without neurological sequelae[50,51]. Although these findings are suggestive, the role of products from the necrotic liver in cerebral edema and intracranial hypertension is still unknown.

The respective roles of all of these phenomena in the development of intracranial hypertension in FHF remains to be determined. It can be hypothesized that brain edema (increase in brain volume) secondary to osmotic effect of glutamine in astrocytes, and cerebral hyperemia (increase in blood volume) secondary to vasodilation (cytokines, products of the necrotic liver, glutamine, others…) may contribute to intracranial hypertension leading to brain stem herniation and brain stem death in FHF. During all these FHF phenomena, the brain may respond by altering the expression of genes coding for various proteins whose role may be critical to some CNS functions, including the maintenance of cell volume and neurotransmission. Cerebral gene expression during FHF is modified as demonstrated by differential display in rat models[52,53]. Some genes have been more specifically studied, as GLUT-1[54], aquaporin IV[55], GLT-1[52,56] and others[57]. The exact role of this gene expression observed during FHF is still to be determined.

## DIAGNOSIS OF INTRACRANIAL HYPERTENSION IN FHF PATIENTS

Intracranial hypertension should be clinically suspected in FHF patients with systemic hypertension (sustained ⩾ 160 mmHg or paroxysms ⩾ 200 mmHg), aggravation of hepatic encephalopathy, abnormal pupillary signs, or signs of decerebration. However, most of these clinical signs are not specific, and may be developed by patients in hepatic grade IV encephalopathy without intracranial hypertension. It was reported that brain CT is unreliable in the diagnosis of intracranial hypertension in FHF patients[14], and there have been no reports of the value of brain magnetic resonance imaging in FHF patients for confirming the diagnosis of intracranial hypertension. The most accurate method of diagnosing intracranial hypertension is ICP monitoring. Although the advantages of this monitoring in FHF patients have not yet been demonstrated by a randomized study, ICP monitoring may be very helpful in establishing the presence of intracranial hypertension and in guiding specific therapy. Intracranial hypertension in FHF patients may suddenly rise from normal to life threatening levels within minutes. In this situation, continuous ICP monitoring may allow rapid and specific management. Several groups have included ICP monitoring in the protocol of FHF patient management[14,47,58]. The main argument against ICP monitoring is the enhanced risk of complications in FHF patients, mainly infection and hemorrhage. In a national survey of 262 FHF patients, the complication rate of ICP monitoring was 10%. In this series, intracranial hemorrhages were the cause of death in 7 patients and the epidural transducers had the lowest complication rate (3.7%)[59]. The complication rate of ICP monitoring in FHF patients was reported to be lower in a recent multicenter report, but is still significant[60].

## MANAGEMENT OF INTRACRANIAL HYPERTENSION IN FHF PATIENTS

General management of FHF patients is beyond the scope of this review and was presented elsewhere[2]. The goal in the medical management of FHF patients with intracranial hypertension is to maintain ICP below 20 mmHg and CPP above 70 mmHg. Cerebral ischemia occurs if CPP is less than 40 to 50 mmHg, and LT should be contraindicated if CPP remains below 40 mmHg for two hours[61]. This goal requires intense medical management and nursing. FHF patients should be admitted to the ICU in an institution with an active liver transplant program. FHF patients should be monitored with peripheral arterial catheters. Vital signs, urinary output, arterial blood gases and central hemodynamic parameters should be continuously monitored. FHF patients are treated with standard supportive measures to correct electrolyte, metabolic, respiratory and hemodynamic abnormalities. Hypovolemia may exacerbate hypotension, and may reduce CPP, inducing brain ischemia. Systemic hypertension may also be deleterious by increasing ICP[62]. In this case, β-blockers may be more useful than nitroprusside or calcium-channel inhibitors, because of their potential risk of brain blood vessel dilatation. Patient positioning and nursing is also important in the care of FHF patients with intracranial hypertension. The head should be in the midline because neck rotation or flexion may compromise jugular venous drainage and increase ICP. Head and chest elevation may lower ICP by enhancing CSF drainage and maximizing cerebral venous output[12]. However, the efficacy of this positioning in FHF patients is yet to be proven and further elevations to 40° and 60° may paradoxically increase ICP[63]. Environmental stimulation should be maintained at a minimal level. Most of the FHF patients with encephalopathy grade Ⅲ/Ⅳ are endotracheally intubated to provide airway protection and/or ventilation support. This ventilation may promote some ICP fluctuation. Moreover, positive end-expiratory pressure (PEEP) may increase ICP when mean airway pressures are increased and should be used carefully[12]. Straining against the mechanical ventilator may increase intrathoracic pressure and reduce venous outflow from the head. Coughing, which is a frequent reflex to tracheal tube aspiration, should be avoided for the same reason. Therefore, if necessary, the patient is sedated and/or paralyzed with nondepolarizing neuromuscular blockers.

Specific treatment of intracranial hypertension in FHF patients is aimed at the culprit underlying pathophysiology, for example therapy to reduce brain volume or lower ICP by reducing intracranial blood volume and CBF.

### *Treatments to decrease brain volume*

As hyperammonemia is considered responsible for the cytotoxic brain edema, it seems logical to try to reduce ammonia in FHF patients. There is no randomized study on the effects of lactulose in FHF. In a retrospective study, lactulose administration did not change the outcome of treated patients, and the routine use of lactulose is not recommended[64]. The use of intravenous *mannitol* improved the survival and decreased the ICP level in a controlled trial[8]. Mannitol (0.5 to 1 g/kg iv every 6 h, blood osmolarity < 310 mosmol/L) increases blood osmolarity, thereby inducing fluid movement from brain to blood. Therefore, the efficacy of mannitol depends on an intact BBB. The efficacy of mannitol to reduce ICP may be affected by acute renal failure and oliguria. In order to be able to use mannitol repeatedly, fluid can be taken off with *hemofiltration* (up to 500 mL), which by itself reduces ICP[64]. *Hypertonic saline* has also been evaluated in a small controlled trial to prevent the occurrence of intracranial hypertension. Intravenous hypertonic saline (30%) to maintain serum sodium between 145-155 mmol/L was compared with an untreated group. The treated group suffered smaller increases of ICP[65]. By extension, hyponatremia should be avoided in FHF patients.

### *Treatments to decrease cerebral blood flow and intracranial blood volume*

*Hyperventilation:* In non-FHF patients, hyperventilation induces ICP reduction through vasoconstriction of the brain blood vessels[12]. The duration of this ICP reduction varies, and ICP usually returns to baseline within hours of hyperventilation. In FHF patients, this effect of hyperventilation on intracranial hypertension is not clear[7] but may help to restore cerebral blood flow

**Table 1 Liver transplantation criteria in patients with fulminant hepatic failure**

| |
|---|
| Clichy criteria *(Hepatology* 1991; 14: 49A*)* |
| Grade Ⅲ or Ⅳ encephalopathy and |
| - Factor Ⅴ level < 20% (patients younger than 30) |
| - Factor Ⅴ level < 30% (patients older than 30) |
| King's College criteria *(*Modified from *Gastroenterology* 1989; 97: 439-445*)* |
| Paracetamol intoxication: |
| - pH < 7.3 or |
| - INR > 4 and serum creatinin > 300 μmol/L (> 34 mg/L) and grade Ⅲ or Ⅳ encephalopathy |
| Other causes: |
| - INR > 4 or |
| - 3 of the following criteria: |
| - Age < 10 or > 40 yr |
| - Etiology: NonA nonB hepatitis, halothane hepatitis, idiosyncratic drug reactions |
| - Delay between jaundice and encephalopathy > 7 d |
| - INR > 3.5 |
| - Bilirubin > 300 μmol/L (> 175 mg/L) |

autoregulation[66]. From these observations, it can be stated that hyperventilation may reduce ICP acutely but should not be used over a prolonged period[64]. *Indomethacin* induces cerebral vasoconstriction through inhibition of the endothelial cyclooxygenase pathway, alterations in extracellular pH and reduction in cerebral temperature[64]. Indomethacin has been shown to reduce ICP in an animal model[67] and a small cohort of 12 patients[68], and should be evaluated in a randomized controlled study before wider use. *Thiopental sodium* has also been studied in FHF patients. Its administration results in cerebral vasoconstriction possibly by inhibition of nitric synthase. There is only one small study in the literature involving 13 patients, that showed beneficial effects of thiopental on ICP but its efficacy and side effects have to be evaluated in further randomized studies[69]. *Propofol* in a dose of 6 mg/kg per hour reduces CBF through metabolic suppression[64] and was used in a small report[70]. It could be the sedative of choice in FHF patients.

### *Liver transplantation*

LT has emerged as the most important advance in the treatment of FHF[58,71-74]. To date, transplantation of a functioning graft is the best treatment to achieve control of brain edema and intracranial hypertension. For this reason, every FHF patient should be referred to a transplant center and listed for LT if transplant criteria are met (Table 1)[75,76]. However, some FHF patients in Grade Ⅳ encephalopathy may develop severe cerebral injury or brain death during the perioperative period, and these complications are believed to be secondary to perioperative ICP elevation or CPP reduction. For example, in a study from Paul Brousse's group, 13 patients among 116 (11.2%) who underwent LT for FHF developed brain death during or after the procedure, and 2 others suffered from neurological sequelae[74]. It was demonstrated that during LT, the dissection phase and the graft reperfusion are particularly at risk of ICP elevation, and that the anhepatic phase seems to be more favorable with ICP normalization[47,48].

## FUTURE PROSPECTS IN THE TREATMENT OF INTRACRANIAL HYPERTENSION OF FHF

### *Hypothermia*

The effects of moderate hypothermia (32-34℃) on ICP in FHF patients are currently being investigated. In rat models of FHF, hypothermia lowered brain edema measured by a gravimetric technique, and prolonged survival[77,78]. Several reports have demonstrated that hypothermia causes a significant decrease of ICP in FHF patients[79,80]. Hypothermia could be a very efficient therapy for patients with uncontrolled intracranial hypertension[81] and a multicenter, randomized, controlled trial should be conducted to definitively assess the role of hypothermia in FHF patient management.

### *Liver assist systems*

For more than 50 years, many research groups have attempted to support patients in acute liver failure as a bridge to LT or to recovery of adequate native liver function. Development of such a system presents a unique challenge as it has to reproduce an array of complex liver functions. Moreover, the results of this system have to be evaluated in very sick and unstable patients, in whom large, randomized, controlled trials are very difficult. Plasma exchange, plasmapheresis, blood exchange, hemodialysis, hemofiltration, cross-circulation, and cross-hemodialysis have all been tried without any benefit to patient survival[82]. *Ex vivo* whole (animal and/or human) liver perfusion might be promising in small series, but is limited by several drawbacks that are beyond the scope of this review[82]. Recent advances in semipermeable membranes and hollow-fiber technologies, as well as improved techniques of hepatocyte isolation, have allowed the development of new liver support systems, that may be classified as non-biological blood detoxification systems and liver assist systems with biological components[82]. Two systems have achieved relatively large use in humans. The Molecular Adsorbents Recirculating System (MARS), which is based on the selective removal of albumin-bound toxins from the blood, is commercially available. In a small, randomized trial MARS was shown to improve survival of patients suffering from acute-on-chronic liver failure[83], but controlled clinical data for the use of MARS in FHF patients is lacking, especially its effect on ICP. Recently, it was demonstrated that MARS may attenuate (but not normalize) ICP in a pig model with ischemic liver failure[84]. MARS has still to prove its value in FHF patients in controlled trials.

Multiple liver assist systems with biological components have been tried, in order to construct a liver support that may provide not only detoxification, but also biotransformation and missing liver synthetic function[82]. A lot of systems were based on isolated or cultured hepatocytes and liver tissue slices placed in a variety of perfusion bioreactors[82]. Only one system has completed a controlled trial, the bioartificial liver (BAL). The BAL design is based both on a detoxification part and on a cartridge containing porcine hepatocyte and is explained elsewhere[82]. The BAL has shown some

efficacy to decrease ICP in FHF patients[85] and in patients suffering from acetaminophen-induced liver failure[86]. However a controlled, randomized trial did not show any improvement in survival in the BAL treated group[87] and the Circe company that produced the BAL, has stopped its activity. In conclusion, despite years of scientific efforts, there is no (bio)artificial system that has proved its efficacy on ICP control. The MARS seems promising but has still to prove its role on ICP in FHF patients.

### *Hepatocyte transplantation*

Transplantation of isolated hepatocytes has been shown to provide metabolic support and improve survival in various experimental models of acute liver failure including 90% hepatectomy[88,89], D-galactosamine[90], acetaminophen[91] and ischemic models[92]. Hepatocyte transplantation has also been shown to improve chronic encephalopathy, induced by an end-to-side portocaval shunt in rats[93]. In a pig model of ischemic liver failure, the intrasplenic transplantation of hepatocytes allowed the transplanted animal to maintain normal ICP, compared to the treated group[46].

In two clinical reports, 12 patients were transplanted with a very small number of hepatocytes (0.01%-0.4% of the liver mass) which were infused either intraperitoneally or intrasplenically[94,95]. Although both studies reported improvement in neurologic status and survival of the transplanted patients, the limited number of patients and the lack of appropriate controls do not allow reliable conclusions to be reached. More experiments in large animal models are needed in order to investigate the "neuro-protective" potential of transplanted hepatocytes. In addition, three major problems need to be solved before clinical application of hepatocyte transplantation can be established: (1) how to harvest and store the maximum number of functional hepatocytes from human liver (e.g. hepatic resection specimens, organs rejected for transplantation, *etc*); (2) how to safely perform transplantation of a significant amount of hepatocytes (at least 5% of the liver mass) considering the anatomic limitations and the severe metabolic disturbances of FHF patients (e.g. coagulopathy); (3) how to determine the optimal timing of hepatocellular transplantation in the course of FHF.

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*EDITORIAL*

# Role of Kupffer cells in the pathogenesis of liver disease

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## Abstract

Kupffer cells, the resident liver macrophages have long been considered as mostly scavenger cells responsible for removing particulate material from the portal circulation. However, evidence derived mostly from animal models, indicates that Kupffer cells may be implicated in the pathogenesis of various liver diseases including viral hepatitis, steatohepatitis, alcoholic liver disease, intrahepatic cholestasis, activation or rejection of the liver during liver transplantation and liver fibrosis. There is accumulating evidence, reviewed in this paper, suggesting that Kupffer cells may act both as effector cells in the destruction of hepatocytes by producing harmful soluble mediators as well as antigen presenting cells during viral infections of the liver. Moreover they may represent a significant source of chemoattractant molecules for cytotoxic CD8 and regulatory T cells. Their role in fibrosis is well established as they are one of the main sources of TGFβ1 production, which leads to the transformation of stellate cells into myofibroblasts. Whether all these variable functions in the liver are mediated by different Kupffer cell subpopulations remains to be evaluated. In this review we propose a model that demonstrates the role of Kupffer cells in the pathogenesis of liver disease.



**Key words:** Kupffer cells; Liver disease; Hepatic injury; Liver fibrosis; Hepatocellular carcinoma; Hepatitis; Steatohepatitis

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## INTRODUCTION

The sinusoidal lining of the liver contains the nonparenchymal cell populations which consist of Kupffer cells (KCs), sinusoidal endothelial cells (SEC) and stellate cells (SC). All three cell-types seem to play a crucial role in liver homeostasis and in the pathogenesis of liver disease[1]. KCs constitute 80%-90% of the tissue macrophages in the reticuloendothelial system and account for approximately 15% of the total liver cell population[2]. They are mainly found in the periportal area of the lobule (43%), but KCs also exist in the midzonal (28%) and in the central area (29%)[2]. Despite the view that KCs are fixed tissue macrophages of the liver, there is evidence that they have the ability to migrate along sinusoidal walls with a mean speed of 4.6 ± 2.6 (SD) microns/min[3]. Since the description of these resident liver macrophages in 1876 by von Kupffer various theories have been proposed with regard to their origin and involvement in liver homeostasis and injury. It should be noted that almost all available evidence for the role of Kupffer cells comes from animal models.

KCs are the first cells to be exposed to materials absorbed from the gastrointestinal tract. Their ability to eliminate and detoxify microorganisms, endotoxins, degenerated cells, immune complexes, and toxic agents (e.g. ethanol) is an important physiological function. Due to their key location, KCs might function as antigen-presenting cells[4] and participate in tumour surveillance[5] and the regeneration processes of the liver[6]. They also seem to play a key role in innate immune responses and host defence through the expression and secretion of soluble inflammatory mediators[7]. There is accumulating evidence that the interaction between KC and lipopolysaccharide (LPS) may be the initiating event leading to hepatotoxicity in various types of liver injury including endotoxinaemia, alcoholic liver injury and ischemia/reperfusion injury[8,9] and systemic viral infections[10].

## THE ROLE OF KUPFFER CELLS IN HEPATIC INJURY

Kupffer cells are involved in the pathogenesis of liver injury mediated by chemical substances, toxins and pharmacological agents[7,9] such as carbontetrachloride ($CCl_4$)[11], endotoxin[12], galactosamine[13] and acetaminophen[14] through the release of biologically active substances that promote the pathogenic process[9]. In liver injury and hepatocellular necrosis activated Kupffer cells are a major source of inflammatory mediators including cytokines, superoxide, nitric oxide, eicosanoids, chemokines, lysosomal and proteolytic enzymes and demonstrate increased cytotoxicity and chemotaxis[7,14-16].

Reactive oxygen radicals are released by hepatic mac-

rophages after activation with cytokines, LPS and prostaglandins as a defence against bacterial invasion. These molecules have been implicated in the pathogenesis of liver injury induced in a rat model by sequential administration of endotoxin and *Corynebacterium parvum*[17]. In this model, the products of oxidation of hepatocellular membrane lipids were detected in the systematic circulation and were related with the degree of liver necrosis. Administration of superoxide dismutase, a reactive oxygen radical scavenger, significantly reduced the liver injury and animal mortality[12]. Isolated Kupffer cells from *Corynebacterium parvum*-treated rats demonstrated significantly increased release of superoxide that was further enhanced following administration of endotoxin[17]. The toxicity of reactive oxygen intermediates on hepatocytes has also been demonstrated *in vitro* using cultured rat hepatocytes[18]. However, LPS-treated Kupffer cells are cytotoxic to hepatocytes in co-culture experiments only in the presence of L-arginine, probably in response to simultaneous secretion of nitric oxide by Kupffer cells or induction of production by hepatocytes[19].

Nitric oxide is produced in the liver by Kupffer cells and hepatocytes. Its role in the pathogenesis of hepatic injury is controversial. A protective role has been detected in various conditions such as endotoxemia or $CCl_4$-induced damage where it protects hepatocytes *via* the inhibition of caspases and apoptosis. In other conditions like ischemia/reperfusion injury, shock, and galactosamine induced liver injury, nitric oxide increases oxidative stress *via* its interaction with reactive oxygen species leading to the formation of peroxynitrite or it induces the expression of inflammatory mediators such as TNF-$\alpha$ and IL-1[20]. Adiponectin suppresses TNF-$\alpha$ production and induces IL-10 production by Kupffer cells and administration of galactosamine in adiponectin knock-out mice significantly increases mortality rate compared with wild type animals[21]. It has been suggested that the hepato-protective activity of adiponectin is due, at least in part, to a direct anti-inflammatory effect of adiponectin on Kupffer cells[22].

Cytokine and chemokine production by activated Kupffer cells is involved in the pathogenesis of liver damage. It has been reported that alcohol-induced liver injury is accompanied by increases in the portal concentration of endotoxin, leading to activation of Kupffer cells and subsequent TNF-$\alpha$ production[23]. Other studies have shown a role for the increased production of the chemokine MCP-1 by Kupffer cells in the pathogenesis of acute liver injury due to $CCl_4$[24] or acetaminophen[25] administration. Proteolytic enzymes released by recruited and activated liver macrophages were also found to promote hepatic injury in a rat model of hepatic damage[26].

The pivotal role of Kupffer cells in the initiation of hepatocellular damage is supported by experimental models that have demonstrated a correlation between the degree of activation of Kupffer cells and the degree of hepatocellular destruction[14]. Administration of endotoxin to rats with activated Kupffer cells due to liver resection induced damage of endothelium, sinusoidal fibrin deposition, and lethal massive hepatic necrosis[27]. In another rat model, activation with endotoxin enhanced $CCl_4$-induced liver damage, while pretreatment with polymyxin B or administration of endotoxin in low doses induced immune tolerance which protected the liver from $CCl_4$-induced damage[27]. Other studies demonstrated that activated Kupffer cells express CD95L and could induce apoptosis in $CD95^+$ T lymphocytes and hepatocytes[28].

However, Kupffer cells also participate in protective mechanisms *via* the production of mediators that induce synthesis of the antioxidant agent glutathione[29], or the production of nitric oxide[30,31]. The production of ELR-CXC chemokines such as MIP-2, which induce hepatocyte proliferation also has a protective role in models of hepatotoxicity such as acetaminophen-induced injury[32-34]. This protection is also possibly mediated by the production of IL-10 and IL-18 by Kupffer cells, since depletion of Kupffer cells increases susceptibility of the murine liver to acetaminophen in parallel with a reduction in IL-10 and IL-18[35]. On the other hand, hard evidence for the protective role of Kupffer cells is missing since depletion of Kupffer cells by the traditional method of administration of gadolinium chloride ($GdCl_3$) intraperitoneally might not deplete the liver from Kupffer cells. Instead $GdCl_3$ might change the acinar distribution and phenotype of Kupffer cells promoting the production of TNF-$\alpha$ and IL-6[36-38]. Therefore interpretation of experiments using $GdCl_3$ is difficult. In conclusion, Kupffer cell-induced hepatotoxicity is not only a result of the reaction to hepatotoxins[39], but it might also be a response to an excessive activation or a suppression of hepatoprotective mechanisms[40].

## THE ROLE OF KUPFFER CELLS IN LIVER FIBROSIS

Liver fibrosis is a complex process that involves many cells of the hepatic sinusoid and is characterized by disturbance of the architecture and composition of extracellular matrix in the liver[41,42]. The extracellular matrix in the subendothelial space of Disse mainly consists of collagen type Ⅳ, laminin, and proteoglycans that are progressively replaced during fibrosis by collagen type Ⅰ and Ⅲ. This excess deposition disrupts the normal architecture of the hepatic lobule[43,44].

Ito or stellate cells are the main cellular source of extracellular matrix proteins in the liver[45,46]. The initiation and maintenance of fibrogenesis in the liver is characterized by two processes. The former is characterized by the activation and transformation of Ito cells to myofibroblasts resulting in increased production of collagen types Ⅰ and Ⅲ[47]. In parallel, there seems to be a disturbance of the homeostatic mechanisms involved in extracellular matrix deposition due to reduced expression of the proteolytic enzymes that degrade the extracellular matrix and increased expression of their inhibitors. Thus, maintaining fibrosis involves decreased production of matrix metalloproteinases (MMPs) and increased production of specific (tissue inhibitors of matrix metalloproteinases, TIMPs) or non specific metalloproteinase inhibitors (alpha1-antitrypsin)[48].

Kupffer cells are involved both in processes *via* the production of cytokines and growth factors that induce Ito cell myofibroblastic transformation and also *via* regulation of the production of metalloproteinases and their inhibitors[49]. Kupffer cell-derived TGF-$\beta_1$ has been suggested

to drive Ito cell transformation and to induce production of collagen and proteoglycans by these cells[50]. TGF-$\beta_1$ is considered as the main cytokine that drives fibrosis in various animal models of hepatic damage, including alcoholic liver fibrogenesis[51], schistosomiasis and $CCl_4$-induced fibrosis[52], and one of the major factors involved in fibrosis in patients with chronic liver disease[53].

*In vitro* studies have also shown that Kupffer cells can induce expression of platelet-derived growth factor (PDGF) receptors on Ito cells, thus enhancing Ito cell proliferation in response to PDGF[54]. TNF-α, IL-1 and MCP-1, that are produced by activated Kupffer cells, are also mitogenic and chemoattractant for Ito cells[55,56]. In addition, TGF-β1 and IL-6 were found to induce mRNA expression of metalloproteinases (MMPs) and also their specific inhibitors TIMPs (mostly TIMP-1, in hepatocytes, Kupffer cells and Ito cells in rat liver[57].

Finally another mechanism that could lead to the phenotypic change of Ito cells is the production of gelatinases by Kupffer cells. It has been demonstrated that extracellular matrix proteins play a crucial role in the maintenance of normal function of hepatocytes and Ito cells. Culture of Ito cells on type I collagen or plastic resulted in activation of cells and transformation to myofibroblasts. In contrast, culture of Ito cells in collagen type Ⅳ did not result in phenotypic change[58]. It has been suggested that activation of Kupffer cells and secretion of gelatinase degrades collagen type Ⅳ and therefore triggers the phenotypic change of Ito cells[7,59].

## THE ROLE OF KUPFFER CELLS IN LIVER DISEASES

### *The role of Kupffer cells in liver infections*

Kupffer cells are involved in the defence against infections of the liver. Their major role in the host defence and the prognosis of liver infection is indicated by studies in experimental models of sepsis. LPS pre-treatment has been shown to increase Kupffer cell numbers leading to a reduction of bacterial load and improvement of prognosis in a Salmonella septicemia model[60]. Impairment of the phagocytic function and the production of superoxide by Kupffer cells in models of obstructive jaundice leads to increased susceptibility to infection[61].

Infection of mice with Listeria monocytogenes is a well studied liver infection model. In this model, the accumulation of bacillus in the liver depends on recognition of bacillus surface sugars and lectins by cognate receptors on Kupffer cells. On the other hand, production of inflammatory mediators such as IL-6, IL-12, IL-1β, TNF-α, and nitric oxide by infected Kupffer cells inhibits proliferation of the microorganism[62,63]. At the same time Kupffer cell derived chemokines such as MIP-1α, MIP-1β, MCP-1, and MIP-2, drive monocyte and neutrophil recruitment into the liver in order to control infection[64-66]. Thus as expected, Kupffer cell inactivation results in impaired infection clearance[67]. Being the first line of defence, Kupffer cells also represent the portal of entry for viruses such as cytomegalovirus[68] and parasites such as *Plasmodium berget*[69] and *Leishmania*[70], which enter and proliferate in Kupffer cells and then infect the rest of the liver cells.

In humans, phenomena like the increased frequency of septicaemia and septic shock from Gram negative bacteria that are observed in patients with acute hepatic failure, have been attributed to the inability of Kupffer cells to clear the portal circulation of micro-organisms and endotoxin[71]. Various studies have shown that a large percentage of patients with chronic hepatic disease present with a systematic endotoxinaemia and high titres of antibodies against intestinal bacteria.In contrast, in normal individuals endotoxin is detected only in the portal circulation[72].

Very recently a direct contribution of Kupffer cells to the pathogenesis of hepatitis has been reported[73]. Influenza hepatitis was associated with absence of virus from the liver and foci of CD8+ virus specific T cells in close contact with Kupffer cells. Moreover, elimination of Kupffer cells abrogated the hepatocellular necrosis, despite persistence of CD8+ reactive cells. It seems that activated T cells are trapped and retained in the liver through an antigen-independent mechanism as a possible interaction between activated integrins like LFA-1 on the T cells and constitutively expressed integrin ligands like VCAM and ICAM-1 on sinusoidal endothelium[74,75]. In this model, Kupffer cells are possibly the effector cells killing hepatocytes in an as yet unidentified manner. Kupffer cells can kill hepatocytes either directly *via* activation of fas-dependent or CD95-dependent apoptotic pathways[76] or indirectly by interacting with CD8+ (and possibly CD4+) lymphocytes with the stimulation of cytokine secretion[77] and other mediators like phospholipases and nitric oxide, as previously reported. Although such a mechanism as that proposed in the paper by Polakos *et al*[73] might explain the hepatitis observed in measles, SARS and CMV infection (where the virus is not identified in the liver), a similar mechanism could well operate in the pathogenesis of hepatitis due to hepatotropic viruses like HBV, HCV and HEV. The only difference would be that the generation of CD8+ virus specific cells would take place in either the portal tracts or the sinusoids per se, with Kupffer cells and dendritic cells being the antigen presenters.

### *Kupffer cells and hepatocellular carcinoma*

The liver is a frequent site of hematogenous metastasis particularly for cancers of the gastrointestinal system. Isolated Kupffer cells were found to be cytotoxic against human colon adenocarcinoma cells and this cytotoxicity was increased significantly when the KC were stimulated with INF-γ and endotoxin[78,79]. It has been suggested that this effect is related to TNF-α expression by Kupffer cells as it is inhibited by anti-TNF-α[80,81]. Other studies have demonstrated that Kupffer cells induce Fas expression in colon cancer cells[82] and malignant glioma cells[83] leading to Fas-mediated apoptosis and death in the presence of tumour infiltrating lymphocytes or TNF-α.

Data from *in vivo* studies show that the degree of activation or repression of Kupffer cells influences the number and the size of hepatic metastases following injection of colon carcinoma cells in portal circulation[84]. Administration of $GdCl_3$, which is reported to deplete and block the function of Kupffer cells, resulted in increased size of metastases, while activation of Kupffer cells with Zymosan and *Corynebacterium parvum* decreased the size of

metastases[85].

*In vivo* microscopy has shown that Kupffer cells are attracted to tumour cells in the hepatic circulation and have the ability to phagocytose these cells[86]. Nitric oxide produced by Kupffer cells after stimulation with endotoxin, TNF-α and prostaglandin $E_2$[16,87] may also be an effective weapon of the Kupffer cell machinery against tumor cells[88]. Moreover, an indirect mechanism of defence by Kupffer cells against hepatic tumours is the induction of natural killer cell (NK-cell) cytotoxicity *via* the production of IL-12[84] and a possible anti-tumour effect of octreotide in hepatocellular carcinoma[89,90] might, in part, be explained by its antiapoptotic effect on Kupffer cells[91].

### Alcohol-related liver disease and Kupffer cells

Alcohol-related liver disease is a chronic inflammatory disease of the liver parenchyma due to chronic ethanol ingestion with the end result being alcoholic fibrosis and cirrhosis. Kupffer cells have been suggested to participate in this process mainly through the increased production of inflammatory mediators. Indeed, increased circulating levels of pro-inflammatory cytokines like TNFα and IL-6, and chemokines like IL-8, MCP-1 and MIP-1α have been detected in patients with alcoholic liver disease, which could potentially be related to Kupffer cell activation[92-95]. Increased numbers of Kupffer cells in the portal tracts have been observed in patients with acute alcoholic hepatitis or chronic alcoholic liver disease[96].

Animal studies have shown that acute or chronic ethanol administration is associated with an increase in numbers of Kupffer cells that exhibit morphologic signs of cell activation[97], up regulation of CD14 expression[98] and increased production of inflammatory mediators such as IL-1, TNF-α[99] and oxygen free radicals[100]. Kupffer cell depletion with $GdCl_3$ has been found to prevent early alcohol-induced liver inflammation and necrosis[101].

One of the current hypotheses about the pathophysiology of alcohol induced liver damage is that ethanol increases the proportion of Gram negative bacteria in the bowel flora and therefore the intraluminal production of LPS. Concurrently, the increase in the intestinal permeability due to alcohol-induced alterations of the epithelial barrier function results in portal vein endotoxinemia. This activates Kupffer cells leading to production of inflammatory mediators, which in turn activate the endothelium and induce neutrophil and mononuclear cell recruitment and infiltration resulting in liver damage. Furthermore, it has been suggested that ethanol may also have a direct effect on Kupffer cell activation by altering cell membrane calcium channels[102].

A synergistic effect of LPS with ethanol has been described. Recent evidence indicates that chronic ethanol administration decreases the cellular cAMP levels of Kupffer cells and this leads to enhanced NF-κB activation by LPS and TNF-α production[95]. Interestingly an increase in cAMP does not affect NF-κB activation but it decreases its transcription capability.

### Kupffer cells and liver transplantation

There is indirect evidence indicating that Kupffer cells may play a role in the process of graft rejection following liver transplantation mainly though their ability to act as antigen presenting cells (APC). Kupffer cells express MHC class Ⅱ and have been found to be effective APC *in vitro*[103]. Animal studies have shown that following liver transplantation Kupffer cells up-regulate MHC class Ⅱ expression and this has been associated with the initiation of the rejection process[104]. In humans the rate of reconstitution of the graft with recipient-derived Kupffer cells has been found to increase during the rejection phase[104]. Finally, graft rejection and the vanishing-bile duct syndrome occur more frequently in cases of MHC class I incompatibility accompanied by a MHC class Ⅱ partial or complete match, which suggests that presentation of MHC I antigens of the billiary epithelium by donor Kupffer cells may also take place[105].

Ischemia-reperfusion injury during the extracorporal preservation of the graft may often result to primary graft dysfunction[106]. There is accumulating evidence to suggest a major role for Kupffer cells during this process through the activation and production of oxygen free radicals resulting in alteration of the microcirculation of the graft[107]. Kupffer cell inactivation using $GdC_{13}$ has been found to prevent ischemia-reperfusion injury, whereas administration of latex particles that induce Kupffer cell activation through phagocytosis, accelerates ischemia-reperfusion injury of the graft[108]. Kupffer cell derived TNF-α, MIP-2 and keratinocyte chemoattractant chemokine have also been found to play a role in the microcirculatory failure that accompanies ischemia-reperfusion. Increased expression of TNFα, MIP-2 and keratinocyte chemoattractant both systemically and in the liver parenchyma have been observed in animal models during the reperfusion phase injury, and they have been associated with endothelial activation and β2-integrin up-regulation[109] and infiltration of the graft by neutrophils[110] respectively.

### Kupffer cells and portal hypertension

Kupffer cells have been shown to be the main source of thromboxane A2 production in the liver and this production is mediated by COX-1 and COX-2[111]. Recently it was demonstrated that the infusion of endothelin-1 significantly increased portal pressure in animal models. This increase was mediated by the production of thromboxane A2 by the Kupffer cells[112], since both thromboxane synthase inhibition and thromboxane A2 receptor antagonists blocked the effect of endothelin-1 on portal pressure[113]. Whether this is relevant to the situation in humans remains to be established.

### Kupffer cells and non alcoholic steatohepatitis

Recently a connection between Kupffer cells and the progression of non alcoholic steatosis to steatohepatitis and fibrosis was reported[114]. Interestingly, this report is one of the few that are based on human data. The enzyme chitotriosidase (CHIT), a member of the chitinase family, was found exclusively expressed in Kupffer cells in liver biopsies from patients with NASH. The levels of this enzyme were significantly higher in NASH than in simple steatosis and CHIT overexpression influenced hepatic stellate cell activation. A significant correlation was also observed between CHIT, TNF-α and lipid peroxidation in both

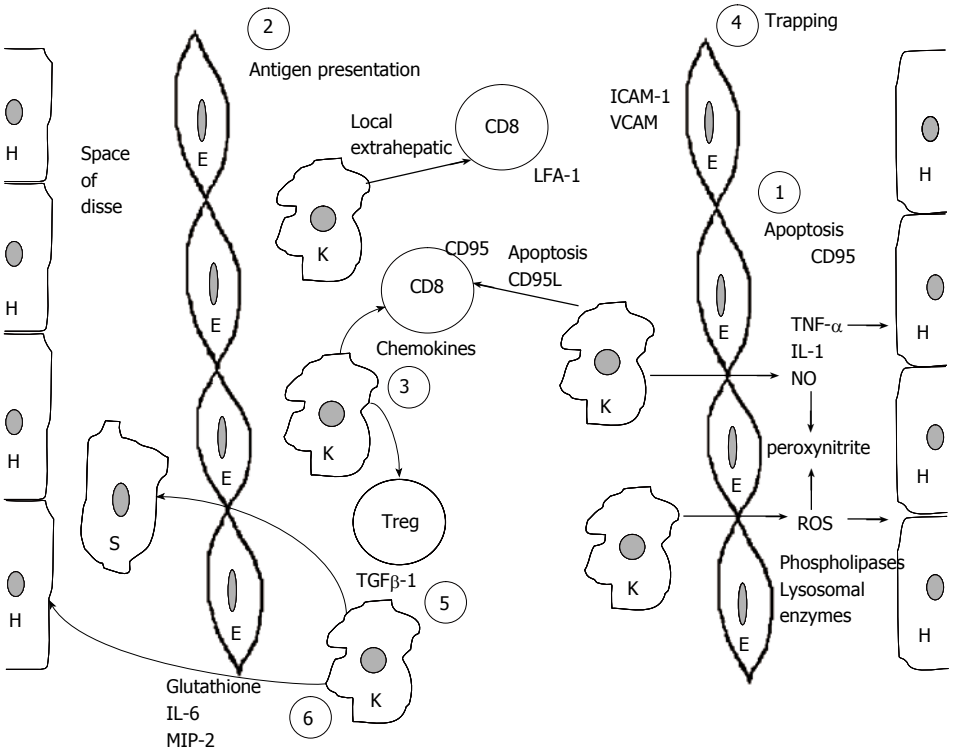


**Figure 1** Schematic representation of the proposed model for the role of Kupffer cells in the pathogenesis of liver disease. H: hepatocytes; E: endothelial cells; K: Kupffer cells; S: stellate cells. For numbers (1-6), please see text explanation.

NASH and simple steatosis. Since CHIT is increased in the liver in other forms of lipid storage disease it is postulated that Kupffer cells are implicated in the pathogenesis of NASH. Another study using an animal model has shown an enhancement of the TNF-α/TNFR mediated signalling pathway *via* activation of Kupffer cells in an autocrine or paracrine manner which might be critically involved in the pathogenesis of liver fibrosis in this NASH[115].

### *Kupffer cells and intrahepatic cholestasis*

Recently Kupffer cells have been implicated in the pathogenesis of intrahepatic cholestasis following hepatic ischaemia-reperfusion injury. Many hepatic canalicular transporters were reduced in parallel to the production of cytokines by Kupffer cells in an experimental model. Moreover, depletion of Kupffer cells abolished the reduced expression of transporters[116]. However, the role of Kupffer cells in cholestasis remains controversial. Recently, in bile duct ligated rats, selective anti-inflammatory blockade of Kupffer cells increased fibrosis and deposition of collagen I and Ⅲ[117]. More recently, in a bile duct ligated mouse model, depletion of Kupffer cells by intravenous inoculation of dichloromethylene diphosphonate resulted in high serum alanine transaminase levels and serious histologic portal inflammation and hepatocellular necrosis, indicating that Kupffer cells abrogate cholestatic liver injury in mice[118]. Moreover it seems that the abrogation of liver injury in this model might be cytokine dependent, mostly through the production of IL-6 by Kupffer cells[118].

## A PROPOSED MODEL FOR THE INVOLVEMENT OF KUPFFER CELLS IN THE PATHOGENESIS OF LIVER DISEASE

Based mostly on the presented data from experimental animals, we propose a model to demonstrate the role of Kupffer cells in the pathogenesis of various liver diseases. According to this model Kupffer cells are responsible for six major functions that are vital for the development of liver disease. Kupffer cells are the main effector cells, killing hepatocytes in various forms of hepatitis. This is achieved by the production of proinflammatory cytokines, reactive oxygen species, nitric oxide, phospholipase and lysosomal enzymes. Kupffer cells may harm hepatocytes by initiating their apoptosis through the CD95L-CD95 pathway (1). This effect is possibly accentuated by CD8 positive antigen restricted T cells and is stopped by $CD4^+CD25^+$ regulatory T cells. In this respect, Kupffer cells are acting as antigen presenting cells of either extrahepatic viruses like influenza[10,73] or intrahepatic viruses like HBV and HCV (2). Following antigen presentation Kupffer cells attract both CD8+ T cells and regulatory T cells by producing chemokines (3). T cells expressing LFA-1 are trapped as a result of endothelial cell overexpression of adhesion molecules like ICAM-1 and VCAM (4), while CD8 positive cells might be driven to apoptosis by direct contact with Kupffer cells. Moreover, TGF-β1 production by Kupffer cells drives stellate cells to be transformed into myofibroblasts eventually leading to fibrosis (5). Finally, by producing glutathione, IL-6 and MIP-2 Kupffer cells may protect hepatocytes from further damage (6). One vital question remains. Are all these six different functions mediated through the same Kupffer cells or are there different Kupffer cell subpopulations in the liver? A schematic presentation of this model is presented in Figure 1.

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*TOPIC HIGHLIGHT*

**Parimal Chowdhury, Professor,** *Series Editor*

# Pathophysiology of alcoholic pancreatitis: An overview

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## Abstract

Use of alcohol is a worldwide habit regardless of socio-economic background. Heavy alcohol consumption is a potential risk factor for induction of pancreatitis. The current review cites the updated literature on the alcohol metabolism, its effects on gastrointestinal and pancreatic function and in causing pancreatic injury, genetic predisposition of alcohol induced pancreatitis. Reports describing prospective mechanisms of action of alcohol activating the signal transduction pathways, induction of oxidative stress parameters through the development of animal models are being presented.



**Key words:** Alcohol; Metabolism; Pancreatic function; Pancreatitis; Mechanism of action

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## INTRODUCTION

Alcohol abuse and dependence are the major cause of morbidity and mortality in the United States. About three-fourths of individuals of 18-26 years of age and two-thirds of those 26 and older are current drinkers. The age related pattern for concurrent alcohol and tobacco dependence was similar to that found for tobacco dependence[1,2]. In a recent survey it was found that approximately 17.6 million adult Americans abuse alcohol or is alcoholic. Alcohol related problems cost society approximately $185 billion per year. In a study conducted at the population level, mortality from pancreatitis due to alcohol addiction was reported[3]. The mechanism of induction of alcoholic pancreatitis is not well understood.

Pancreatitis due to alcohol abuse is a very painful and potentially fatal condition. About one-third of acute pancreatitis cases in the United States are alcohol induced and 60%-90% of pancreatitis patients have a history of chronic alcohol consumption. It is estimated that drinking more than 80 gm of alcohol/d or about 10-11 standard U.S. drinks for a minimum of 6-12 years is required to produce symptomatic pancreatitis[4]. The risk of developing the disease increases with both amount and duration of alcohol consumption. Only 5% of clinically documented alcoholics develop disease but at autopsy only 5%-10% of alcoholics are found to have evidence of chronic pancreatitis[5-7]. Chronic pancreatitis is mostly caused by heavy alcohol consumption and is characterized by onset of symptoms in the 4$^{th}$ or 5$^{th}$ decade[8]. Due to this discrepancy in data of alcoholic and diseased patients, it is thought that other factors like environmental, genetic, race and concomitant risk factors are also involved.

Cigarette smoking might have an additive effect with alcohol in inducing pancreatitis. In the rat model of alcohol-induced pancreatitis, ethanol induces pancreatic ischemia while cigarette smoke potentiates the impairment of pancreatic capillary perfusion caused by ethanol[9]. Cigarette smoking accelerates progression of alcoholic chronic pancreatitis[10,11]. A dietary component may also interact and modify effects of alcohol on the pancreas. A protein and fat rich diet along with continued consumption of alcohol exacerbate the course of chronic pancreatitis[12,13]. African Americans are affected more than Caucasians and this could be due to differences in diet, type or quantity of alcohol consumption[14]. It can be due to differences in metabolism of alcohol in liver and pancreas. Alcohol consumption at intoxicating concentrations induces pancreatic cellular injury that may involve class Ⅲ isoenzymes of ADH[15].

## ALCOHOL METABOLISM

It is well known that alcohol is metabolized *via* an oxidative and a non-oxidative pathway in the liver. Various studies have been conducted to demonstrate alcohol metabolism in isolated pancreatic acini and cultured acinar cells. Haber *et al*[15] have studied oxidative metabolism of alcohol using cultured pancreatic cells. His findings corroborate a study reported by Gukovskaya *et al*[16], conduced with isolated pancreatic acini. In the cytosol of acini, ethanol is oxidized to acetaldehyde by alcohol dehydrogenase. At physiological

blood alcohol concentrations, cytochrome P-450 accounts for approximately 20% of ethanol metabolism[17,18]. The presence of cytochrome P-450 CYP2E1 has been demonstrated in rat pancreas[19] as well as human pancreas[20]. Chronic use of alcohol has been shown to induce the expression of CYP2E1 in rat pancreas[19] similar to that in liver[21].

The non-oxidative pathway of ethanol metabolism involves the formation of FAEE using FAEE synthase[22]. Gukovskaya *et al*[16] have confirmed the presence of FAEE synthase activity in pancreas that may explain the accumulation of FAEE in pancreas after ethanol exposure. Werner *et al*[23,24] studied correlation between oxidative and non-oxidative pathways of ethanol metabolism in the pancreas. Their studies show that ethanol causes a dose-dependent injury to pancreas due to a shift to non-oxidative metabolism following inhibition of the oxidative pathway. This results in an increase of FAEE. Carboxyl ester lipase (CEL) has been known to catalyze FAEE synthesis from fatty acids and ethanol. CEL gene polymorphism, especially an increase in the frequency of the L-allele, was found to be associated with alcohol induced pancreatitits[25]. The aspect that inhibition of the oxidative pathway can cause an increase in flux of ethanol metabolism *via* the non-oxidative pathway needs to be clarified further.

## GENETIC PREDISPOSITION OF ACUTE AND CHRONIC PANCREATITIS: ROLE OF ALCOHOL

In alcoholic pancreatitis, mutations of the cationic trypsinogen gene and of pancreatic secretory trypsin inhibitor (SPINK-1) have been implicated in its pathogenesis[26]. The discovery of genetic cause of hereditary pancreatitis has renewed interest in genetic predisposition to alcoholic pancreatitis. Various gene mutations like cationic trypsinogen (especially at codon 29 and 1220), SPINK1 mutation, and CFTR gene mutation are associated with development of chronic pancreatitis[27]. In idiopathic pancreatitis, both variants of SPINK1 (N 291 and R 122H) and CFTR were identified. Alteration of both genes was found in patients with alcoholic chronic pancreatitis and the increase of those genes is related to higher levels of alcohol consumption[27]. The SPINK1 inhibits the autoactivation of premature trypsinogen within the pancreas[28]. The most frequent mutation in the SPINK1 gene was the N34S mutation in Exon-3. Kuwata *et al*[29] detected intronic polymorphism in the SPINK1 gene in 4 alcoholic patients but found no differences when the data were compared with other patients. Similar findings have been reported in five other alcoholic chronic pancreatitis patients with an N34S mutation[30]. Truninger *et al*[31] and Monaghan *et al*[32] screened 58 and 46 alcoholic pancreatitis patients respectively for cationic trypsinogen gene mutation activation but no mutations have been found.

Alcohol metabolizing enzymes such as aldehyde dehydrogenase (ADH), metabolizing alcohol to acetaldehyde, exist as different isoenzymes. ADH polymorphism has been identified in ADH-2 and ADH-3[33]. Increased prevalence of ADH-1 isoenzyme in patients with alcoholic pancreatitis has been reported[34]. With regard to ADH-2 isoenzyme, results are not conclusive and further studies need to be done. Kimura *et al*[35] and Frenzer *et al*[36] found no correlation between ADH-2 polymorphism and chronic pancreatitis in Japanese and Austrian patients respectively while Maruyama *et al*[37] demonstrated elevated risk for chronic alcoholic pancreatitis for different genotypes of ADH-2. Cytochrome P-450 is involved in metabolizing alcohol in endoplasmic reticulum. Several studies have been done to evaluate the association between polymorphism of enzyme Cyto-P4502E1 (CYP2E1) and alcoholic pancreatitis with no success[36,38,39]. Verlaan *et al*[40] analyzed DNA samples from chronic pancreatitis -alcoholic, idiopathic and hereditary. They observed that the frequency of ADH-3 and CYP2E1C1C2 genotypes did not differ between chronic pancreatitis patients, alcoholic and healthy controls. But they conclude that the presence of CYP2E1 intron 6DD genotype might confer a higher risk of alcoholic chronic pancreatitis. Kim *et al*[41] compared the genotype and allele frequencies of ADH-2, ADH-3, ALDH-2, Cyto P450-2E1, IL-1, IL-6, IL-8 and TNF-alpha in patients with chronic pancreatitis and alcoholic liver cirrhosis with those from controls. No difference in frequencies of genotype and allele of enzymes and cytokines amongst three groups were found. Frequency of ADH-2 was significantly higher and those of CYP2E1 and ALDH-2 were significantly lower than control.

Polymorphism of other enzymes involved in free radical stress and acinar cell damage, such as glutathione -S-transferase (GST) family has also been investigated[42]. Bartsch *et al*[43] found a moderate increase in susceptibility of pancreatitis due to polymorphism of GSTM1 but Frenzer *et al*[36] and Scheider *et al*[44] were not able to confirm it. The enzymes GSTM1, GSTT1, GSTP1, CYP1A1 and CYP2E1 are involved in bioactivation and detoxification of a variety of xenobiotics present in smoke, alcoholic drink among others. A higher frequency of the Val/Val genotype in alcoholics and pancreatitis in comparison to alcoholics without the disease was found[45]. In the same study the investigators found an association between the occurrence of Val/Val GSTP1 genotype and chronic pancreatitis and also an association between m2/m2, CYP1A1 and alcoholic liver cirrhosis suggesting that these genotypes are genetically more prone to the development of alcoholic pancreatitis and alcoholic cirrhosis respectively.

## EFFECT OF ETHANOL ON GASTROINTESTINAL AND PANCREATIC FUNCTION

Ethanol had a stimulatory effect on gastric acid secretion[46]. Singer *et al*[47] found that the effect of ethanol on gastric acid secretion is concentration dependent. Gastric instillation of 1.4% and 4.0% (v/v) of ethanol has a small stimulatory effect while higher concentrations (up to 40% v/v) have either no effect or an inhibitory one. Different studies under controlled conditions have been conducted to determine the action of pure ethanol on gastric acid secretion but results were very different from each other[48-54]. Alcohol is oxidized by ADH enzyme

present in all parts of the gut[55,56]. Ethanol has a direct toxic effect on the mucosal epithelium leading to loss of epithelium and hemorrhagic erosions in the duodenum[57,58]. In large bowel, gut flora plays an important role in ethanol metabolism[59]. Due to increased amounts of conversion of ethanol to acetaldehyde, than to further oxidize to acetate, the toxic acetaldehyde accumulates thereby damaging colonic mucosa and after being absorbed into the portal blood may contribute to liver injury. Alcohol when taken orally is known to increase mucosal perfusion[60] and also to stimulate production of secretin[61]. Both can affect pancreas microcirculation indirectly. Ethanol is also known to affect pancreatic blood flow when given intravenously[62,63] and *via* intragastric route[64]. McKim *et al*[65] investigated the effect of chronic intragastric ethanol administration which induced pancreatic hypoxia and oxidative stress *in vivo*. Pancreatic hypoxia induced by chronic alcohol appears to be secondary to increase in oxygen consumption by pancreas or to decrease in local blood supply without alteration of hemodynamic patterns. Chronic ethanol ingestion was associated with dose related inhibition of basal pancreatic protein secretion which was reversed upon alcohol withdrawal[66]. Increased susceptibility to chronic alcoholic pancreatitis may be through a hyperstimulation mechanism due to combination of neurohormonal factors. In exocrine pancreas, alcohol induces secretory alteration which varies by manner and duration of alcohol exposure. Ethanol effects on pancreatic secretion appear to be primarily caused by systemic cholinergic mechanisms of the vagus nerve[67].

## ROLE OF ETHANOL METABOLISM IN PANCREATIC INJURY

Various studies have been done to understand the mechanism of ethanol induced pancreatic injury but till now the exact mechanism is not clear. Earlier it was thought that the Sphincter of Oddi spasm induced by alcohol may be one of the mechanisms responsible but due to a lack of consensus, the later proposal includes the Ductal-Plug hypothesis by Sarles and his colleagues[68]. The secretion of pancreatic juice rich in protein may "plug" the small ductules leading to acinar atrophy and fibrosis. It was not clear whether protein precipitation within pancreatic ducts precedes acinar damage. So protein plugs may be a cause or an effect of pancreatic injury. Saluja and Bhagat[69] investigated the mechanism by which alcohol may induce pancreatitis in an animal model. Ethanol administration resulted in a transient increase of pancreatic amylase output and plasma cholecystokinin (CCK) levels. This phenomenon was mediated by a trypsin-sensitive CCK-releasing factor from the duodenum. The studies suggest that ethanol-induced stimulation of pancreatic digestive enzyme secretion plays an important role in the development of pancreatic injury. Chronic ethanol exposure alone in animals inhibits apoptosis through an intrinsic pathway and the downstream apoptosis executor caspase-3 when compared with the controls[70]. Alcohol exposure accelerates pancreatic necrosis in response to endotoxin. The results from this study showed that the pancreas exposed to alcohol is more sensitive to LPS-induced damage because of increased sensitivity to necrotic cell death rather than apoptotic cell death suggesting that this mechanism may occur in acute alcoholic pancreatitis patients[70].

Due to inability to explain the pathogenesis of alcoholic pancreatitis by theories as mentioned above the focus was directed to pancreatic acinar cells. It is now believed that acinar cells are capable of metabolizing alcohol and the toxic effect may predispose the gland to injury in the presence of an appropriate triggering factor. The characteristics of pancreatic stellate cells showing the involvement of acinar cells in pancreatic fibrosis may be another possible link[71] . It is speculated that metabolites of ethanol like acetaldehyde and FAEEs have direct effects on acinar cells/or induce metabolic alterations within cells indirectly. Acetaldehyde is believed to interfere with the binding of secretagogue to their receptors[72] and thereby inhibit stimulated secretion from isolated pancreatic acini[72]. It also causes microtubule dysfunction thereby affecting exocytosis from acinar cells[73].

During oxidation of ethanol, hydrogen ions and reducing equivalents are released[74]; increase NADH, thereby leading to an imbalance between free radicals and antioxidant defense mechanism. It leads to a loss of mitochondrial glutathione and inactivation of GPx and other respiratory complexes[75]. Also, chronic ethanol ingestion upregulates CYP2E1[19] and catalase[76] for metabolism These pathways will require increased oxygen that will compete with mitochondrial electron transport system leading to localized and transient hypoxia in tissues. These transient conditions of hypoxia and re-oxygenation would further enhance ROS formation through the respiratory chain.

FAEEs, products of non-oxidative ethanol metabolism, have been shown to induce pancreatic injury *in vivo*[77] and *in vitro*[78]. FAEE undergo hydrolysis to FFA which impairs mitochondrial function by uncoupling of mitochondrial and oxidative phosphorylation[79]. Also its direct binding to the intracellular membrane leads to alteration in function and permeability of cell membrane[80]. The generation of cholestryl esters is responsible for the increase in lysosomal fragility releasing hydrolase's which act on the zymogen granule membrane and increase release of trypsin[81,82].

Impairment of blood flow to pancreas by ethanol causes hypoxia without any change in hemodynamic parameters. McCord[83] explained reoxygenation induced injury following hypoxia. Hypoxia can decrease the ability of cells to detoxify free radicals[84] and secondarily, hypoxia/reoxygenation causes more free radical formation leading to formation of α-hydroxyethyl radical and subsequent tissue damage and functional impairment. McKim *et al*[65] found 4-hydroxy nonenal protein adduct accumulation and increasing hypoxia in the pancreas following chronic intragastric alcohol administration in rats. These studies support the hypothesis that hypoxia contributes to oxidative stress caused by alcohol.

## EFFECT OF ALCOHOL ON CELL SIGNALING PATHWAY

The mechanism of acute and chronic ethanol mediated

pancreatic injury is unclear in the literature. Feeding alcohol to animals could not reproduce pancreatitis, suggesting that alcohol alone is not sufficient to induce pancreatitis. It sensitizes pancreas to other risk factors, thereby injuring pancreas[85]. Studies using CCK or its analog are done to induce pancreatitis both *in vivo* and *in vitro*[86]. CCK at supra-physiological doses causes pancreatitis with increased blood levels of amylase and lipase and acute inflammatory response along with parenchymal cell damage[87-89]. Katz *et al*[90] showed that ethanol with low dose CCK-8 caused 6-fold more zymogen conversion than that caused by CCK alone**.** To evaluate a mechanism for the development of alcoholic pancreatitis, Pandol *et al*[91] fed animals intragastrically with ethanol diet followed by infusion of CCK-8. Ethanol exposure in the presence of CCK-8 resulted in activation of pro-inflammatory transcription factors, NF-κB, AP-1 and other cytokine and inflammatory molecules thereby resulting in increased trypsin release. On the other hand alcohol when given alone causes less activation of NF-κB as compared to that given with CCK alone indicating that ethanol has inhibitory effects on the inflammatory response alone. $Ca^{2+}$ and PKC contribute to NF-κB activation induced by CCK-8 in acinar cells. Ethanol differentially affects the Ca $(2^{+})$/calcineurin- and PKC-mediated pathways of NF-kappaB activation in pancreatic acinar cells[16,92]. These effects may play a role to sensitize pancreas to the inflammatory response and pancreatitis. Acute oxidative stress modulates secretion and repetitive $Ca^{2+}$ spiking in rat pancreas[93]. Thus perturbations in $Ca^{2+}$ signaling do not fully explain the secretory block caused by oxidative stress in acute pancreatitis.

## CIGARETTE SMOKING AND PANCREATITIS: EFFECT OF ALCOHOL

Cigarette smoking is a known risk factor for alcoholic and chronic pancreatitis. About 80%-95% of people who abuse alcohol also smoke while 25%-30% of smokers do not drink alcohol[94]. The incidence of alcoholism is 10 times more likely in smokers than nonsmokers. Cigarette smoking accelerates the progression of alcohol induced pancreatitis[10,11]. Blomqvist *et al*[95] reported that intermittent nicotine administration to rats enhances ethanol intake and preference to ethanol in a free choice between ethanol and water. He suggested that subchronic nicotine doses increased the responsiveness of mesolimbic dopamine neurons to both nicotine and alcohol. Potthoff *et al*[96] found similar results in their experiments in rats administering chronic nicotine. Ericson *et al*[97] reported the involvement of nicotinic acetylcholine receptors (nAChR) in nicotine induced increased uptake of ethanol. He gave antagonist to peripheral nAChR to mice and rats subchronically for 15 d and after stopping drug, ethanol intake and preference as well as ethanol induced locomotor stimulation increased. This may be due to compensatory enhanced autonomic ganglionic and/or muscarinic neurotransmission. The mechanism (hormonal or metabolic) by which increased peripheral neuronal activity affects the brain dopaminergic system in the brain is not known.

The mechanism by which alcohol causes the pancreatic injury is not entirely clear. Metabolic as well as microcirculatory changes and other theories were proposed to explain this phenomenon. Cigarette smoking is known as a potentiating factor in the development of alcohol induced pancreatic injury. Hartwig *et al*[9] investigated the effect of cigarette smoke on alcohol induced pancreatic injury. They gave cigarette smoke alone or with ethanol intravenously to rats. Ethanol alone impairs pancreatic blood flow without any change in systemic hemodynamic parameters and inflammatory change. Cigarette smoke potentiates pancreatic microcirculatory impairment by ethanol and also induces leukocyte aggregation and adhesion.

Nicotine is metabolized by cytochrome P450 into cotinine[98,99]. Tissue distribution of $^{3}$H-nicotine in rats demonstrated that nicotine is distributed and accumulated significantly in the pancreas and parts of the gastrointestinal tract[100]. But nicotine metabolism in pancreas has not been reported yet. In liver, low doses of nicotine and ethanol induces CYP2E1 activity as reported by Howard *et al*[101]. The study suggests that nicotine may increase CYP2E1-induced toxicity and contribute to cross-tolerance in smokers and people treated with nicotine. It may be possible that nicotine might have some effect on pancreatic CYP2E1 induction leading to increased metabolism of ethanol in pancreas by cytochrome and thereby potentiate the damage caused by ethanol.

## CONCLUSIONS

Alcohol abuse/alcoholism are a major cause of pancreatitis. Combining alcohol abuse with smoking aggravates the condition further. Despite numerous reported studies the pathogenesis of alcoholic pancreatitis remains obscure. During recent years it has been possible to evaluate the mechanism of development of alcoholic pancreatitis in the animal model and in *in-vitro* acinar cell cultures. The summary of events relating to alcohol exposure that may lead to induction of alcoholic pancreatitis is shown in the flow diagram below (Figure 1).

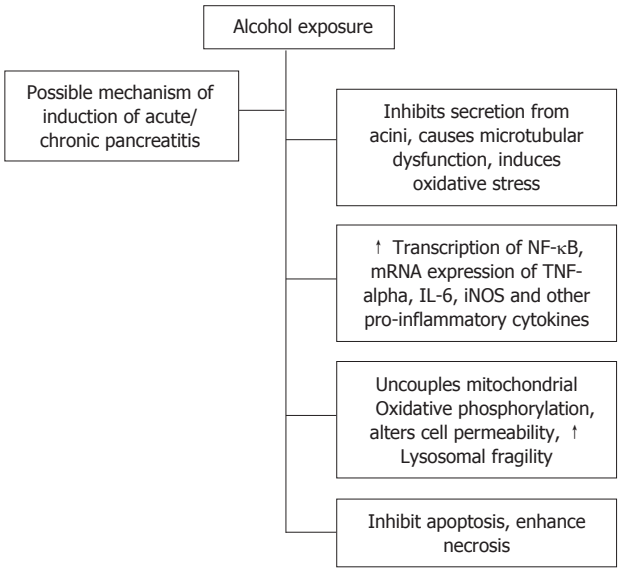


**Figure 1 The summary of events relating to alcohol exposure that may lead to induction of alcoholic pancreatitis.**

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*TOPIC HIGHLIGHT*

**Parimal Chowdhury, Professor,** *Series Editor*

# Nicotine as a mitogenic stimulus for pancreatic acinar cell proliferation

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## Abstract

Cell proliferation is an important process in life for growth of normal and cancer cells. The signal transduction pathways activated during this process are strictly regulated. This editorial focuses on the role of nicotine, a mitogen, in the induction of signaling pathways resulting in proliferation of pancreatic tumor cells and compares these events with those in normal acinar cells isolated from the rat pancreas. The data shows striking similarities between these two cellular systems. In addition, the editorial reviews very recent literature of the contribution of MAPK signaling in cell lines associated with human diseases. A prospective cellular model of nicotine induced activation of MAPK cascade is presented.



**Key words**: Cell Proliferation; Nicotine; AR42J cells; Pancreatic acinar cells; ERK activation

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## INTRODUCTION

Cell proliferation is a strictly regulated event. The rate of cell division depends upon instructions by which the cell responds to mitogenic or other stimuli in order to enter the cell cycle substantiated by cyclin dependent kinases (CDKs) which are activated by cyclins. In a normal resting cell the intracellular signaling proteins and genes, activated by extracellular growth factors, are quiescent. However, when stimulated by growth factors, the cells undergo proliferation. In cancer cells, intracellular signaling proteins and oncogenes activated by the growth factors remain constitutively active[1]. In response to growth factors or other mitogenic stimuli, three major types of signal transduction pathways are activated in a cell: mitogen activated protein kinase (MAPK) pathways, protein kinase C (PKC) pathways and JAK/STAT pathways. Activation of each of these pathways requires a phosphorylation step that regulates cell proliferation and differentiation[1].

Understanding of the complex molecular mechanisms involved in the regulation of cell proliferation in normal and cancer cells will provide us with tools to intervene in the regulation and control of the cell cycle in the presence of a mitogen. Qualitatively speaking, the biochemistry of growth of tumor and normal cells appear similar[2]. The fundamental difference most probably lies in a relaxation of regulation of cell growth[3,4]. Here we will discuss the role of nicotine in cell proliferation in reference to AR42J cells (a rat pancreatic tumor cell line) and look further into extended observations from our recent findings using primary cells derived from the normal rat pancreas when exposed to nicotine in culture.

Nicotine is a major component of cigarette smoke and is a known risk factor for the development of numerous diseases[5-9]. The role of nicotine/smoking as a risk factor for the induction of pancreatic inflammation and pancreatic cancer has been reported recently[10,11]. The mechanism by which nicotine induces such pathologies is as yet unknown. Understanding of the proliferative potential of nicotine in primary and tumor cells of the pancreas will allow us to develop measures that will ultimately lead to intervention, prevention and treatment.

## AR42J CELLS: CHARACTERISTICS AND ASSOCIATION WITH MAP KINASES

AR42J is a stable, rat pancreatic tumor cell line derived from the hyperplastic pancreatic nodules of male rats following the administration of azaserine[12]. Because of

their unique properties of stability, secretory capacity, and growth potential[13], these cells have been extensively used as an *in vitro* model for studying exocytotic secretory processes and activation of signal transduction pathways[14-16]. In response to regulatory peptides, AR42J cells can be stimulated to secrete enzymes and induce proliferation[13,17,18].

MAPK enzymes play critical roles in the regulation of cell proliferation, differentiation, and apoptosis, and are comprised of a ubiquitous family of tyrosine/threonine kinases that include the extracellular signal-regulated kinases (ERK1/2), c-jun $NH_2$-terminal kinases (JNK1/2), and p38 MAPK. Cytokines and mitogens trigger signaling cascades that lead to the activation of MAPK, as reported in studies with different cell lines[19-24].

Activation of ERK has been primarily implicated in cell proliferation and survival, whereas activation of JNK and p38 are associated with growth arrest and apoptosis[25-27]. Induction of MAPK's leads to the phosphorylation and activation of a variety of proteins, including a number of transcription factors involved in regulating the expression of genes controlling cellular proliferation[28-30]. ERK1/2 is found in most tumors and is involved in gastric carcinogenesis[31]. Differential activation of MAPK by cholecystokinin (CCK) and bombesin has been reported in AR42J cells[17].

## ACTIVATION OF SIGNALING PATHWAYS BY NICOTINE

Nicotine is known to activate several MAPK signaling pathways in a variety of tissue and cell types[32-37]. It also behaves like a growth factor promoting survival of human lung cancer cells[38]. Studies show that nicotine can increase the cell numbers of certain cancer cell lines[39-41]. This suggests that nicotine exposure can lead to the disruption of the dynamic balance between cell death and proliferation, which is required for normal functioning of cells.

In pulmonary neuroendocrine cells, nicotine binds to nicotinic receptors, resulting in the phosphorylation of ERK and stimulation of DNA synthesis[32,42]. Furthermore, nicotine induces $Ca^{2+}$ influx and stimulates the Ras/ERK cascade that promotes cell survival in neuronal cells[43]. Thus, ERK1/2 is one of the possible signaling pathways involved in nicotine-induced cell proliferation. Studies by Bose *et al*[44] on the mitogenic effect of nicotine on AR42J cells show that nicotine activates ERK1/2 in AR42J cells and induces proliferation without affecting basal and stimulated enzyme secretion. These data suggest that MAPK signaling by nicotine in AR42J cells is independent of the secretory response.

## ACTIVATION OF SIGNALING PATHWAYS BY NICOTINE IN PRIMARY CELLS: EFFECTS ON PROLIFERATION

Primary cells are normally derived from intact rat pancreas. The functional status of the primary acinar cells exposed to nicotine under basal and stimulated conditions has been reported[45,46]. However, the ability of nicotine to activate MAPK signaling pathways and subsequent proliferation has not yet been reported. It has been shown that p42 MAPK is fully activated at 5 min by cholecystokinin (CCK) in freshly isolated pancreatic acini, and JNK is activated maximally at 30 min and remains significantly elevated at 60 min[47,14]. Unpublished studies from our laboratory show that exposure of primary cells to nicotine in short term culture activates ERK1/2 MAPK with very little or no effect on JNK and p38 MAPK. The results on primary cells from this study and the studies cited above thus demonstrate that some parallelism in the time-dependent activation of ERK by CCK and nicotine exists, although these two secretagogues are completely different from one another in terms of their biological actions on the pancreas[15,14,48]. Further, cell proliferation studies conducted and monitored with three independent methods (MTT assay, BrdU assay and flow cytometry) confirmed the proliferative ability of primary cells in the presence of nicotine. This response is reversed in the presence of the ERK1/2 inhibitor UO126, suggesting that the proliferation induced by nicotine in primary cells is MAPK-dependent. Activation of these signals and application of their inhibitors in the presence of nicotine had no effect on the stimulated enzyme secretion by these cells (unpublished observations). The proliferation of primary cells by nicotine thus appears to be independent of the stimulus-secretion coupling response of amylase secretion.

## ACTIVATION OF MAPK SIGNALING PATHWAYS IN CELL LINES DERIVED FROM HUMAN DISEASES

MAPKs are known to exert several complex functions, such as regulation of cellular growth, proliferation, and differentiation[29,49,50]. It has also been shown that activation of ERK1/2 MAPK is associated with cell proliferative signals whereas activations of c-jun $NH_2$-terminal kinases 1/2 (JNK1/2) and p38 MAPKs are associated with stress-response signaling[51,52]. Zhao *et al*[53] have shown that in the human T lymphoma cell line Molt-4, ERK and p-38 mitogen activated protein kinase (MAPK) signaling are induced in response to hepatitis C virus E2 envelope protein resulting in alterations in cell behavior. In human squamous cell carcinomas (SCC) of the larynx, the potential derangement of MAPK pathways which showed decreased activity of ERK1/2 p44/42 reflecting alterations in tumor suppressing activity, has been reported[54]. Application of low power laser irradiation (LPLI) has been shown to promote cellular proliferation of human dental pulp derived fibroblast-like cells (dental pulp cells) inducing the activation of ERK 1/2 with no induction pf p38 MAPK or c-Jun N-terminal kinase (JNK) phosphorylation[55]. Hepatocyte growth factor (HGF) also enhanced proliferation and differentiation of dental pulp cells by partial activation of the ERK/MAPK pathway[56].

Recently Li *et al* have shown that anti-apoptotic human phosphatidylethanolamine-binding protein (hPEBP4) silencing, promotes tumor necrosis factor

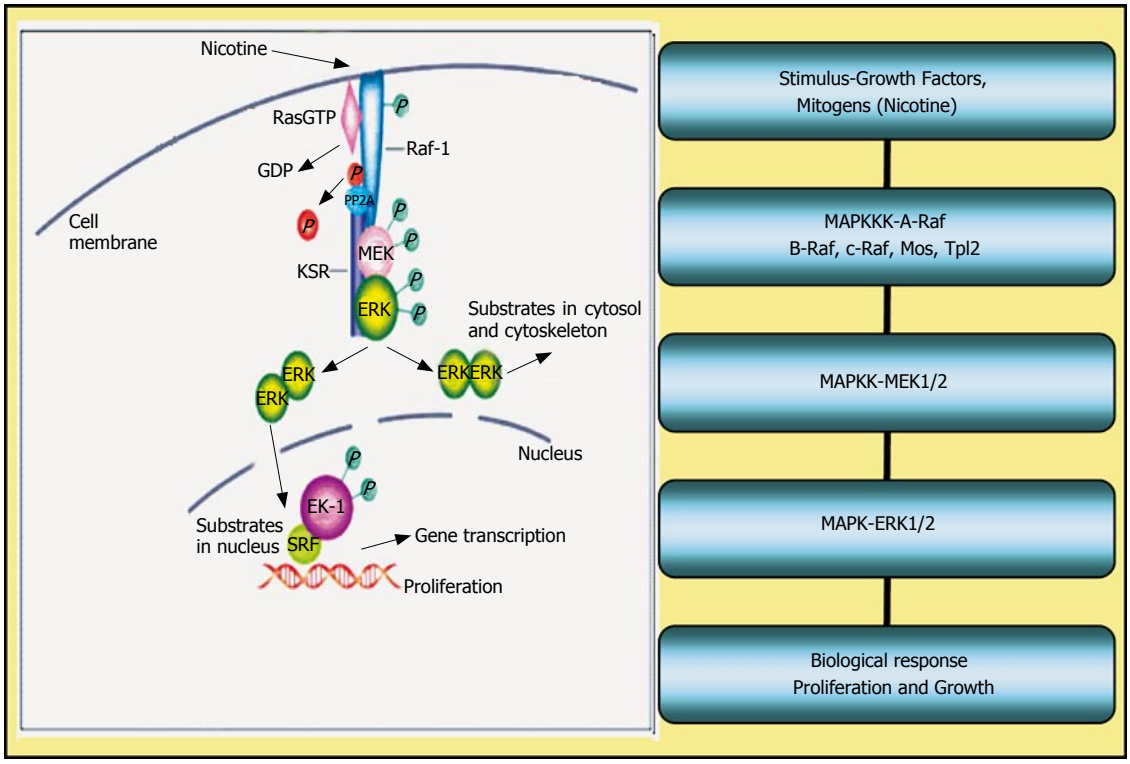


**Figure 1** The possible organisation of the Ras-Raf-MEK-ERK pathway by nicotine.

related apoptosis-inducing ligand (TRAIL)-induced apoptosis of human ovarian cancer cells by activating ERK and JNK pathways[57]. Mutant huntingtin, a protein derived from Huntington disease (HD) affects signaling at upstream points activating ERK and JNK, suggesting that pharmacological intervention of MAPK pathways may be an appropriate approach to HD therapy[58]. ERK 1 expression has been shown to be an early marker of cervical carcinogenesis[59]. In human glomerulopathies, activation of ERK pathways has been correlated with cell proliferation, histologic lesions, and renal dysfunction[60]. In human neutrophils, Rac/Cdc-dependent activation of MAPK/ERK is a critical event in the immediate phagocytic response of PMNs to microbial challenge[61]. IL-1 beta stimulated human airway smooth muscle cells demonstrate activated p38 MAPK, JNK kinase and p42/p44 ERK suggesting their role in the inflammatory process in asthma[62]. In the human myeloma cell line SKO-007, activation of ERK in the Ras/MAPK signaling pathway has been shown to play important differences in their responsiveness to IFN-alpha[63]. Signaling through SAPK/MAK pathways is shown to be a typical feature of chronic synovitis in rheumatoid arthritis, but not in degenerative joint disease. SAPK/MAPK signaling is found at distinct sites in the synovial tissue and is induced by proinflammatory cytokines[64].

## CONCLUSIONS

The possible signaling pathways leading to cell proliferation by nicotine are shown in Figure 1 below. The schematic shows that nicotine enters the cell either by diffusion or *via* a calcium regulated pathway as demonstrated earlier[65]. Entry of nicotine induces the activation of Ras-Raf-MEK-ERK pathways inducing phosphorylation of the MAP kinase cascade. Substrates of ERK in the cytosol include tyrosine kinase receptors among others. Substrates of ERK in the nucleus include transcription factors such as ELK-1 and others. The endpoint of ERK phosphorylation leads to the assembly of transcription factors which stimulate the production of proteins causing cells to proliferate and grow.

The parallelism observed in nicotine-induced cell proliferation studies conducted in a mutant pancreatic cell line and freshly isolated pancreatic acinar cells suggest the possibility that this stable mutant line can be used for extensive evaluation of signal transduction pathways mediating oncogenesis. The data gathered from these studies can be extended to assess the mechanisms of development of pancreatic diseases induced in animal models exposed to chronic/sub-chronic exposure to nicotine or cigarette smoking.

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*TOPIC HIGHLIGHT*

**Parimal Chowdhury, Professor,** *Series Editor*

# The genetics of nicotine dependence: Relationship to pancreatic cancer

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## Abstract

Smoking of tobacco products continues to be a major cause of worldwide health problems. Epidemiological studies have shown that tobacco smoking is the greatest risk factor for the development of pancreatic cancer. Smokers who are able to quit smoking can reduce their risk of pancreatic cancer by nearly 50% within two years, however, their risk of developing pancreatic cancer remains higher than that of non-smokers for 10 years. Nicotine is the major psychoactive substance in tobacco, and is responsible for tobacco dependence and addiction. Recent evidence suggests that individuals have genetically based differences in their ability to metabolize nicotine, as well as genetic differences in the psychological reward pathways that may influence individual response to smoking initiation, dependence, addiction and cessation. Numerous associations have been reported between smoking behavior and genetic polymorphisms in genes that are responsible for nicotine metabolism. In addition, polymorphisms in genes that encode neurotransmitters and transporters that function in psychological reward pathways have been implicated in differences in smoking behavior. However, there is a large degree of between-study variability that demonstrates the need for larger, well-controlled case-control studies to identify target genes and deduce mechanisms that account for the genetic basis of inter-individual differences in smoking behavior. Understanding the genetic factors that increase susceptibility to tobacco addiction may result in more effective tobacco cessation programs which will, in turn, reduce the incidence of tobacco related disease, including pancreatic cancer.

## INTRODUCTION

The use of tobacco products constitutes the most preventable cause of premature death worldwide. It is estimated that half of all Americans who continue to smoke will die of smoking-related diseases[1], including cancer, emphysema and heart disease. The U.S. Surgeon General reported in 2004 that cigarette smoking has caused more than 12 million premature deaths in the United States since the publication of the Surgeon Generals report on smoking and health in 1964[2]. Although the dangers inherent in smoking are well known, the use of tobacco continues because these products are delivery systems for nicotine, an extremely addictive drug. Long term smoking is the most established risk factor for lung cancer, however smoking also increases the risk of developing cancers of the esophagus, uterine cervix, kidney, bladder, stomach and pancreas[2]. This review will briefly discuss the effects of cigarette smoke components on the pancreas, and then focus on the genetic basis for nicotine addiction that is responsible for tobacco product use.

Pancreatic cancer is a relatively rare tumor; the lifetime risk of dying from pancreatic cancer is between 1% to 2% of the general population. However, pancreatic tumors are extremely aggressive, with a 5-year survival rate of less than 5% and a mortality rate of nearly 100%[3,4]. Since there are presently no tests for early screening, and once detected, therapeutic choices are limited, the best hope for reducing mortality from pancreatic cancer is prevention. Smoking of tobacco products is the most important risk factor for developing pancreatic cancer[3-6], with risk increasing with higher levels of tobacco use and more years of exposure. A 40 year epidemiological study of British physicians

determined that the pancreatic cancer rate for smokers was 35 per 100 000 person years, while the risk for ex-smokers and non-smokers was reduced to 23 and 16 per 100 000 person years respectively[7]. A large prospective study by Fuchs *et al*[8] determined that current smokers had a 2.5 times greater relative risk of developing pancreatic cancer compared to study subjects who had never smoked. They also found that although smoking cessation reduces the risk of pancreatic cancer by 48% within 2 years, smokers who quit have an increased risk for approximately 10 years compared to non-smokers. Smoking, as a single risk factor or in combination with alcohol, increases the risk of idiopathic pancreatitis, a precancerous condition[3,5,9,10]. Clearly, one way to reduce the risk of pancreatic cancer is to develop more effective smoking cessation strategies; however, smoking is a complex behavior, which is likely influenced by both environmental and genetic factors. There is wide inter-individual variation in the risk of becoming dependent on nicotine. Genetic factors that influence nicotine metabolism and the psychological reward system of nicotine use are both thought to be important in the development of nicotine dependence.

## MECHANISMS OF TOBACCO INDUCED PANCREATIC CANCER

Cigarette smoke is a complex mixture of carcinogenic compounds and nicotine, many of which have deleterious effects on the exocrine pancreas. Nicotine forms carcinogenic *N*-nitroso compounds during processing[11-14], which induce pancreatic cancer in the Syrian golden hamster model of pancreatic carcinogenesis[15,16]. A particular component of tobacco smoke, 4-(methylnitrosamino)-1-butanone (NNK), is implicated in pancreatic carcinogenesis by its ability to form DNA adducts[17] which have been associated with activating RAS mutations that are found in most human pancreatic adenocarcinomas[18]. NNK also induces proliferation in pancreatic ductal epithelial cells by stimulating EGF mediated signal transduction pathways through binding to β-adrenergic receptors[19,20].

Nicotine, although not carcinogenic, exerts toxic effects on the pancreas. Exposure of rat pancreatic acini to nicotine results in increased protein secretion[21]. We have demonstrated that nicotine induces cytoplasmic vacuolization, cellular edema and increases the cellular amylase content in the exocrine pancreas[22]. We also demonstrated that the increase in pancreatic enzymes in nicotine treated rats was accompanied by reduced CCK mediated enzyme secretion, which may be the causative factor in nicotine induced pancreatic cell pathology. Exposure of rats to tobacco smoke produced fibrosis and scarring of pancreatic acinar structures, characteristic of chronic pancreatitis[23]. In humans, nicotine inhibits the secretion of bicarbonate and affects the composition of pancreatic secretions[24,25], and in patients with pancreatitis, nicotine exposure resulted in increased pancreatic enzyme secretion, including amylase[26] and lipase[27].

## THE GENETICS OF NICOTINE DEPENDENCE

Nicotine is the major psychoactive component of tobacco that is responsible for dependence through a nicotine stimulated reward system that is thought to be mediated by the dopaminergic system of the brain. Recent research has identified behavioral, environmental and genetic factors that influence the various stages of smoking behavior, including smoking initiation, development of addiction and smoking cessation. Early evidence for the influence of heredity on tobacco dependence came from studies of tobacco use among families[28], adopted siblings and among mono and dizygotic twins[29,30]. In a study of mono and dizygotic twins, True *et al*[31] found that 50% of the risk of smoking initiation and 70% of the risk for continuing to smoke were due to genetic factors.

## POLYMORPHIC GENES INVOLVED IN NICOTINE METABOLISM

Individuals with increased tolerance to nicotine because of a greater capacity to metabolize the drug may experience fewer adverse reactions to their first encounter with nicotine, and therefore may have a greater propensity to continue using tobacco products. Conversely, slow metabolizers of nicotine would be expected to smoke less and would be less likely to become nicotine dependent. Smokers tend to adjust their smoking behavior in order to maintain a certain level of nicotine in the brain[32], so that an individual's capacity to metabolize nicotine will influence their intake and exposure. Polymorphic expression of genes that are responsible for nicotine metabolism may be responsible for the wide variability in nicotine tolerance between individuals.

The hepatic enzymes cytochrome P450 2A6 (CYP2A6) and cytochrome P450 2D6 are the major isoforms responsible for the metabolism of nicotine to cotinine, however hepatic CYP2A6 is responsible for 90% of the first pass metabolism of nicotine[33,34]. Both of these enzymes are polymorphic in the human population, with genetic differences that are responsible for high and low activity alleles.

## CYTOCHROME P450 2A6 (CYP2A6)

The CYP2A6 gene is polymorphic in the human population, with large inter-individual differences in the levels of hepatic CYP2A6 protein and enzyme activity[34-36]. A deletional allele was found to be responsible for low or non-existent CYP2A6 activity[36]. A number of studies have found that individuals with genetically determined slow or absent CYP 2A6 activity have a reduced risk of becoming smokers, and those who do smoke tend to smoke fewer cigarettes per day and have a higher smoking cessation success rate[37-40]. Pianneza *et al*[41] reported that a tobacco dependent population had an under representation of low activity CYP2A6 alleles, and that those smokers who had low activity alleles tended to smoke fewer cigarettes per week, suggesting a role for CYP2A6 in nicotine tolerance and dependence. However, other studies have failed to detect an association between genetically low CYP2A6 activity and nicotine use or dependence[42,43]. One reason for this discrepancy was the use in early studies of a

genotyping protocol that overestimated the number of low activity alleles in the study population[44,45]. A meta-analysis by Carter *et al*[46] also failed to find a significant association between CYP2A6 genotype and smoking behavior. In another meta-analysis, Munafo *et al*[47] concluded that the influence of individual genes on smoking behavior may be subtle, and that larger studies that have the power to detect the effects of multiple genes on smoking behavior will be necessary. However, in a number of studies, they found strong evidence that the reduced activity allele of CYP2A6 was associated with smokers who were able to quit smoking.

Recently, the use of CYP2A6 inhibitors has been explored as a chemoprevention strategy for smoking cessation. A study by von Weymarn *et al*[48] reported that the benzyl and phenylethyl isothiocyanates that are found in cruciferous vegetables such as broccoli and cabbage, were effective competitive inhibitors of both CYP2A6 and CYP 2A13. CYP2A13 is the enzyme responsible for the activation of the tobacco procarcinogen NNK to its ultimate carcinogenic form. This report suggests that inhibition of CYP2A6 may convert the phenotype of smokers to one which confers less metabolic tolerance to nicotine, leading to fewer cigarettes smoked per day. The resultant reduction in nicotine metabolism may possibly increase success with smoking cessation. At the same time, inhibition of CYP2A13, which is found primarily in the lung, may result in less activation of NNK, potentially protecting individuals who continue to smoke from developing lung cancer. Sellers *et al*[49] reported that another CYP2A6 inhibitor, methoxsalen, was effective in increasing the bioavailability of nicotine in smokers, resulting in a decrease in the number of cigarettes they smoked per day. These studies demonstrate that the use of CYP2A6 inhibitors may be a useful strategy to reduce tobacco exposure and may have the potential to increase the success rate of smoking cessation programs.

## CYTOCHROME P450 2D6 (CYP2D6)

Caporaso *et al*[50] determined inter-individual differences in CYP2D6 phenotype by measuring the metabolism of dextromethorphan, a CYP2D6 substrate. They concluded that polymorphisms in CYP2D6 were not major determinants of nicotine metabolism in smokers except in ultrametabolizers. These are individuals who have a duplication of the CYP2D6 gene that is present in 3%-8% of Caucasians and up to 30% of other ethnic groups[51]. This duplication results in the production of high levels of functional CYP2D6 protein and results in the increased metabolism of CYP2D6 substrates, including nicotine. In a case-control study of lung and larynx cancer, the CYP2D6 gene duplication was found in 13% of cancer patients compared to 6% of healthy control subjects. The frequency of a genetic polymorphism that codes for a high activity CYP2D6 allele called CYP2D6*9 was also higher in cases compared to controls[52]. Approximately 3%-10% of Caucasians are CYP2D6 poor metabolizers, due to inheritance of two defective alleles. Saarikowski *et al*[53] found the same proportion of poor metabolizers in groups of smokers and never-smokers, however among men, a trend toward more poor metabolizers in the non-smoking group was observed. They also found twofold more ultrametabolizers among heavy smokers compared to non-smokers. Overall, the results of these studies suggest that CYP2D6 affects nicotine metabolism among individuals who have high activity due to gene duplication, however the influence of the low activity allele remains controversial.

## GENES INVOLVED IN NICOTINE DEPENDENCE

Nicotine is thought to induce a euphoric state in users and by that is thought to be the result of activation of the mesolimbic dopaminergic reward system in the nucleus accumbens of the brain[54-56]. Nicotine binds to nicotinic receptors that, when activated, enhance dopamine release in areas of the brain that are thought to be involved in reward[55,57]. The involvement of the dopaminergic system in the reinforcement activity of nicotine may be related to the highly addictive properties of the drug[56]. Genetic polymorphisms in genes that affect this reward system, including dopamine receptors and transporters, nicotinic receptors and serotonin receptors may modulate an individual's risk of becoming nicotine dependent.

## DOPAMINE RECEPTOR GENE POLYMORPHISMS

The human dopamine $D_2$ receptor (DRD2) has a *Taq*I polymorphism with two minor alleles termed the *Taq*IA allele ($A_1$ and $A_2$) and the *Taq*IB allele ($B_1$ and $B_2$). The *Taq*I*A1 allele has been shown to be associated with reduced expression of dopamine $D_2$ receptor in the striatum[58-61]. It has been hypothesized that subjects with reduced numbers of dopamine receptors may compensate for this deficiency by using nicotine to increase brain dopamine levels. The presence of the DRD2 *Taq*I allele has been associated with an earlier age of smoking initiation[62], increased risk of being a current smoker, and reduced duration of smoking abstinence[63]. Spitz *et al*[64], conducted a case control study of lung cancer patients and found that a greater percentage of chronic smokers had the $B_1B_2$ genotype compared with non-smokers, whether they were cancer cases or controls. The least common $A_1$ or $B_1$ alleles were associated with individuals who were younger when they started smoking and had attempted to quit smoking fewer times compared with smokers with the more common DRD2 alleles. Other studies have failed to find an association between the DRD2 *Taq*I allele and smoking behavior. Bierut *et al*[65] analyzed a family study by the transmission disequilibrium test and found no difference in the frequency of DRD2 alleles transmitted to habitual smokers. In a small British study, Singleton *et al*[66] found no increase in the DRD2 *Taq*I allele in smokers compared to non-smokers, and Johnstone *et al* duplicated these findings in a larger study[67]. Munafo *et al*[47] conducted a meta-analysis of the genetic basis for smoking behavior and concluded that there is some evidence for an association between the DRD2 *Taq*I*A1 allele and smoking behavior, but larger, better designed

studies in a variety of populations are needed to confirm this relationship.

Other dopamine receptors are genetically polymorphic, but have received less attention than the DRD2 gene with respect to smoking behavior. A 5' polymorphism of no known function, located in the dopamine $D_1$ receptor gene (DRD1), has been associated with smoking[68]. The DRD3 receptor is genetically polymorphic and is highly expressed in the nucleus accumbens, however, no association with smoking behavior has been reported[69].

A polymorphism characterized as a variable number of tandem repeats has been reported in the dopamine D4 receptor (DRD4)[70,71]. The receptor containing 7 repeats has been characterized as having a reduced response to dopamine binding[72]. Shields *et al*[73] compared DRD4 genotype for a population of smokers compared to non-smokers and found that African American smokers had a higher incidence of the DRD4 allele containing 7 repeats than African American non-smokers. In addition, African American smokers with this allele had an earlier age of smoking initiation and less success at smoking cessation than African Americans with shorter repeat sequences. The same analysis of a Caucasian population showed no association with smoking status. This data suggests that individual genotypes may be a factor in the success of smoking cessation strategies, and more effective strategies may need to be tailored to an individual's genetic background. The human DRD5 gene has at least 4 missense or nonsense polymorphisms, however, no association with smoking behavior has been reported[74].

## DOPAMINE TRANSPORTER POLYMORPHISMS

The dopamine transporter gene (*SLC6AC*) has a variable number of tandem repeats polymorphism in the 3' noncoding region of the gene[75] that is associated with reduced transporter levels in the brain[76]. The role of this polymorphism in determining smoking behavior is not clear, however, in some studies, the polymorphism containing 9 repeats was found to be associated with greater levels of cigarette craving among African American smokers[77]. Other studies found that individuals with the *SLC6AC* 9 repeat allele were less likely to be smokers, especially if they also had the DRD2 Taq1 $A_1$ allele[78]. These individuals were less likely to start smoking at an early age, and those who did smoke had greater periods of smoking cessation. Sabol *et al*[79] confirmed these findings in a diverse population of smokers, non-smokers, and ex-smokers, however Vandenbergh *et al*[80] failed to replicate these results in spite of another report on the function of the 9 repeat sequence as a transcriptional enhancer[81, 82].

## CATABOLISM OF DOPAMINE

The action of dopamine is terminated by the action of catabolic enzymes, primarily catecholamine-*O*-methyltransferase (COMT) with lesser roles for monoamine oxidase A and B (MAO) and dopamine β hydroxylase (DBH). Genetic polymorphisms have been discovered in these enzymes, and the effects on smoking behavior have been tested in a number of studies. In a study of smokers, McKinney *et al*[83] reported that polymorphisms in DBH and MAO were related to maintenance of nicotine levels and predicted the quantity of cigarettes smoked. No association between the amount of tobacco consumed and the functional COMT A1947G single nucleotide polymorphism (SNP), which results in the substitution of a methionine for valine at codon 108 (Met108Val) in the COMT protein, was detected in this study. These results were unexpected in light of a report that the G allele at this locus results in a three to fourfold increase in COMT activity[84], and a reported association of this allele with addiction to other drugs[85]. The lack of association between this SNP and smoking initiation, persistence and cessation was replicated in a larger study of current smokers, ex-smokers and lifetime non-smokers[86]. More recent studies include the finding of a positive association between the high activity COMT allele and nicotine dependence in a Caucasian population, however, these results were not replicated in a second independent study by the same researchers[87]. In a case-control study of women, those who were homozygous for the lower activity Met allele were more likely to be ex-smokers rather than current smokers, and in a nicotine replacement clinical trial reported by the same group, Met homozygotes at the COMT locus had more success at smoking cessation[88]. Bueten *et al*[89] analyzed five allelic variants in the COMT gene, including the Met108Val SNP, and found a significant association with nicotine dependence. The lack of reproducibility of this data, even when the same researchers analyze independent study populations, may be due to the lack of power to detect relatively small effects of COMT on smoking behavior. These results demonstrate the need for large, adequately powered replication studies to determine the genetic basis of smoking behavior and nicotine addiction.

## SEROTONIN TRANSPORTER PROMOTER POLYMORPHISMS

Nicotine increases the secretion of serotonin in the brain[90] therefore, the serotonergic system may have a function in determining smoking behavior. Lower serotonin reuptake has been associated with an increased risk of depression as well as increased impulsive or aggressive behavior[91]. This combination of behavioral traits is termed neuroticism, and has been associated with increased incidence of smoking, nicotine dependence, and difficulty in quitting smoking[92]. Evidence for a genetic link to neuroticism and smoking behavior centers around a 44-bp deletion/insertion polymorphism that corresponds to short (S) and long (L) versions of the serotonin transporter gene (5-hydroxytryptamine transporter or 5-HTT) promoter[93]. Functional characterization of this polymorphism has demonstrated that the short promoter variant reduces the transcriptional activity of the gene and results in decreased 5-HTT expression and decreased serotonin uptake[94]. Hu *et al*[92] found a relationship between the genotype for the 5-HTT promoter polymorphism and degree of

neuroticism and smoking behavior. This finding was confirmed by another study which reported that smokers who were heterozygous or homozygous for the 5-HTT S-allele were more likely to be dependent on nicotine than subjects who were homozygous for the L allele[95]. However in a Japanese population, the presence of the S allele was associated with non-smokers or ex-smokers, indicating that individuals who were homozygous for the S allele were less likely to begin smoking, or were more successful at smoking cessation[96]. Other studies were unable to detect any association between the 5-HTT promoter polymorphism and smoking behavior in Caucasian or African American populations, in spite of significant differences in the distribution of 5-HTT promoter alleles between racial groups[97].

## CONCLUSIONS

The variability of results reported for most candidate genes that are hypothesized to affect smoking behavior demonstrates the need for larger, well controlled studies designed to define the genetic basis for inter-individual differences in nicotine and dopamine metabolism as they relate to smoking behavior and nicotine dependence. Results of these future studies will be useful in identifying individuals who are at increased risk of becoming dependent on nicotine and will also facilitate the development of smoking cessation strategies that are targeted to individual differences in nicotine and neurotransmitter action and metabolism. Considering that smoking is the greatest risk factor for pancreatic cancer, reduction of tobacco use through both abstinence programs and successful smoking cessation is the best hope for reducing the risk of developing this devastating disease.

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*TOPIC HIGHLIGHT*

**Parimal Chowdhury, Professor,** *Series Editor*

# Emerging therapies in gastrointestinal cancers

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## Abstract

Members of the receptor tyrosine kinase family, that include EGFR, ErbB-2/HER-2, ErbB-3/HER-3 and ErbB-4/HER-4, are frequently implicated in experimental models of epithelial cell neoplasia as well as in human cancers. Therefore, interference with the activation of these growth factor receptors represents a promising strategy for development of novel and selective anticancer therapies. Indeed, a number of inhibitors that target either EGFR or HER-2, with the exception of a few that target both; have been developed for treatment of epithelial cancers. Since most solid tumors express different ErbB receptors and/or their ligands, identification of inhibitor(s), targeting multiple EGFR family members may provide a therapeutic benefit to a broader patient population. Here we describe the significance of an ErbB family of receptors in epithelial cancers, and summarize different available therapeutics targeting these receptors. It also emphasizes the need to develop pan-ErbB inhibitors and discusses EGF-Receptor Related Protein, a recently isolated negative regulator of EGFR as a potential pan-ErbB therapeutic for a wide variety of epithelial cancers.



**Key words:** Gastrointestinal cancers; Carcinogenesis; Targeted therapies; Pan-ErbB family; EGF-Receptor Related Protein

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## EPIDEMIOLOGY

Gastrointestinal cancers account for 21% of all cancers incidences and 25% of the cancer mortality in the United States[1]. Despite recent advances in diagnosis and treatment, gastric carcinoma, ductal carcinoma of the pancreas and colorectal cancer are the leading causes of cancer-related deaths worldwide. The pathogenesis of these cancers remains elusive and the available treatment options are limited. Among the GI cancer-related deaths, carcinoma of the stomach ranks seventh in the USA. However, stomach cancer is the leading cause of cancer-associated mortality in Japan, China and India[2]. Gastric cancer ranks number 2 worldwide, second to lung cancer and thus is a major international health concern[2]. Colorectal cancer is the third most common malignancy among men and women in the USA and ranks second for cancer related deaths[3]. In terms of global incidence, colorectal cancer ranks third in frequency, but fourth in cancer related mortality[2]. Adenocarcinoma of the pancreas is the second most common gastrointestinal malignancy in the USA[4]. However, pancreatic cancer is the fourth leading cause of cancer-related mortality among American men and women[5]. GastroIntestinal Stromal Tumor (GIST) is a rare stomach and intestinal cancer with unknown global incidence that spreads rapidly and the survival rate for patients is quite low. GIST is resistant to most known therapies and the available ones have much harsher side effects.

## CURRENT TREATMENT MODALITIES

Different treatments are available for gastrointestinal (GI) cancers. These may be employed alone or in combination with other therapeutic/adjuvant therapy. The available treatments for GI tract cancers are (1) Surgery: resection of the solid tumor whenever possible (2) Chemotherapy: employing cytotoxic drugs to kill cancers cells (3) Radiation therapy: to treat localized solid tumors, (4) Hormonal therapy: systemic treatment that targets cancer cells through out the body. Different analogs of GI hormonal peptides and endogenous growth factors are utilized to inhibit the progression of tumor. Such analogs target gastric releasing peptide (GRP), bombesin, somatostatin, as well as peptide receptors and antagonists of growth hormone releasing hormone (GH-RH)[6]. However, the successful treatment of cancer often requires the combination and coordination of several different

treatment approaches. This is referred to as multi-modality treatment and may consist of surgery, chemotherapy, radiation therapy, and/or hormonal therapy. It is important to emphasize that surgery is a local treatment and is only capable of removing cancer cells from a defined area. By the time a cancer is diagnosed, many patients will already have experienced spread of cancer cells through the blood and lymph system to other locations in the body. They are referred to as micrometastases. Currently available tests cannot always detect micrometastases. Information obtained during surgery and from other tests determines the likelihood of the cancer having spread and whether additional treatments with chemotherapy, radiation, or hormonal therapy is necessary.

## TARGETED THERAPIES

With the recent advances towards understanding of the molecular basis of carcinogenesis, different cell signaling pathways have been implicated in aberrant growth of cells. Direct or indirect interference with signaling pathways often results in modulation of cellular growth. The modalities that target single or multiple signaling pathways, referred to as "Targeted therapies" have been developed and are currently being utilized in clinics as anti-cancer therapeutics. A targeted therapy is designed to treat the cancer cells and minimize damage to normal, healthy cells. Treatments that "target" cancer cells specifically offer the advantage of reduced treatment-related side effects and improved outcome. Conventional cancer treatments such as radiation therapy, do not distinguish between cancer cells and healthy cells. Consequently, healthy cells are commonly damaged in the process of treating the cancer, which results in side effects. Chemotherapy damages rapidly dividing cells, a hallmark trait of cancer cells. In the process, healthy cells that are also rapidly dividing (such as blood cells and the cells lining the mouth and GI tract) are also damaged. Many chemotherapy drugs, which damage DNA of both malignant cells and normal cells, frequently cause toxicity. Treatment-related damage to healthy cells leads to complications of treatment, or side effects. These side effects may be severe, reducing a patient's quality of life, compromising their ability to receive their full, prescribed treatment, and sometimes, limiting their chance for an optimal outcome from treatment.

A number of genes/proteins have been identified to be abnormally expressed in tumors and thus serve as targets for therapeutic intervention. Among the known targets for treatment of GI cancers, Epidermal Growth Factor Receptor and its family members (EGFRs) form the most attractive candidate. EGFR sits across the outer membrane of the cell, receiving and transmitting growth signals from the cell surface to the nucleus. EGFRs have been implicated in variety of epithelial cancers and are generally overexpressed or aberrantly activated in one third of all the cancers. The overexpression of the receptor has been reported in cancers of the stomach (33%-74%), colorectum (25%-77%), esophagus (43%-89%), and pancreas (30%-50%)[7]. Increased EGFR or some of its family members expression is normally associated with poor prognosis with more advanced disease, increased metastasis and decreased survival[8-10]. For the sake of simplicity, the current article will focus on different clinically approved and ongoing developing therapeutics that target EGFR or its family members.

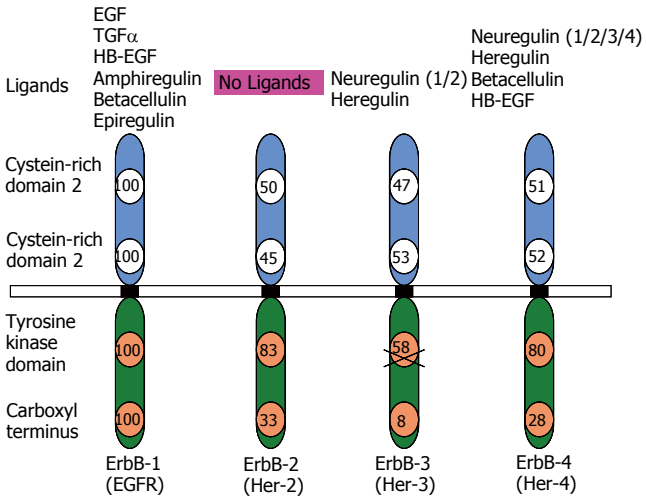


**Figure 1** Schematic representation of the four ErbB family members and their respective ligands. The numbers depict percentage homology of each domain relative to EGFR/ErbB-1. There are no known ligands for ErbB-2 and the tyrosine kinase domain is non-functional in ErbB-3, as marked with a cross.

### *ErbB family of tyrosine kinase receptors*

The EGFR family of RTKs is comprised of four members in mammals but containing one member of the EGFR family in *C.elegans* and *D. melanogaster*. The four members are called: EGFR (also ErbB-1 or HER-1), ErbB-2/HER-2, ErbB-3/HER-3 and ErbB-4/HER-4[11,12]. The signaling transduced by these members is crucial for the development of mice, fruitflies and nematodes[13-17]. The best-characterized functions of the ErbB family of receptors are regulating aspects of replication, migration and survival of cells. In this regard, these receptors interact with multiple signaling molecules and pathways, transmitting and receiving both stimulatory and inhibitory signals[18-20].

All members of the ErbB family are cell surface allosteric enzymes consisting of a single transmembrane domain that separates the extracellular ligand binding domain from the intracellular kinase domain (Figure 1). Under normal physiological conditions, activation of ErbB receptors is regulated by the specific spatial and temporal expression of their ligands which are members of the EGF family of growth factors[11,20]. Ligand binding initiates homo/hetero dimerization of the receptors, leading to auto and trans- tyrosine phosphorylation of the receptors. Tyrosine phosphorylation/activation of the kinase domain leads to recruitment of different proteins initiating a downstream signal cascade. Protein phosphorylation and dephosphorylation, catalyzed by protein tyrosine kinases and protein phosphatases respectively represent the two fundamental biochemical events for downstream intracellular signal transduction[21]. Autophosphorylation of tyrosine residues within the C-tail terminus of EGFR in the cytoplasm following activation of the receptor initiates a cascade of intracellular signaling pathways[22,23].

The receptor tyrosine kinase signaling may be terminated by the endocytosis of the receptor-ligand complex[24]. The downstream signaling by ErbB receptors results in transcriptional regulation of various genes including proto-oncogenes like jun, fos and myc in addition to some zinc-finger containing transcription factors[25].

### Complexity of ErbB signal transduction

Signaling by ErbB receptors is quite diversified and finely tuned at two levels of regulation. These include the specific binding of the ligand to the receptor(s) and the ability of each receptor to form homo/hetero dimers[20,26]. The peptide ligands are produced as transmembrane precursors and their ectodomains are processed by proteolysis, which leads to shedding/secretion of the soluble form of growth factors[27]. There are several ErbB specific ligands, all sharing an EGF-like motif of 45-55 amino acids, including six cysteine residues that interact covalently to form three loops. Depending on the binding specificity conferred by this region, the ligands may be categorized into three groups (Figure 1). The first group includes EGF, amphiregulin and transforming growth factor α (TGF-α) that bind specifically to EGFR/ErbB-1. The second group includes betacellulin (BTC), heparin binding EGF (HB-EGF) and epiregulin that exhibit dual specificity for ErbB-1 and ErbB-4[24]. The third group includes neuregulins (Neu also called Neu differentiation factors or Heregulins). This group is further divided into two sub-groups depending on their ability to bind to ErbB-3 and ErbB-4 or only ErbB-4[28,29]. The second level of regulation depends on the homo-hetero dimerizing partners. Although, a total of nine possible homo- and hetero-dimeric receptor combinations can occur, EGFRs often display preference for their dimeric partners. In this network, ErbB-2 is the most preferred partner and thus plays a co-ordinating role[30,31]. The ErbB-2 containing dimers are known for their high signaling potency as ErbB-2 drastically reduces the rate of ligand receptor dissociation and allows for strong and prolonged activation of the downstream signaling pathways[32,33]. Also, each homo/hetero dimer has been shown to possess unique specificity for the ligand that would stimulate the ErbB activation[34-39]. Within the same heterodimer, the signaling properties of a receptor can be significantly modulated by specific ligand binding. In context of EGFR/ErbB-4 heterodimer, EGF induction is quite fast and recruits both Grb2 and p85 for downstream signaling. On the other hand, addition of NDF stimulation of the receptor heterodimer is relatively slow and recruits only p85 to activate downstream pathways.

### ErbB signaling and integration with other cell signaling pathways

Each of the seven ligands (Figure 2) has a preferred receptor homo/hetero dimer. The ligand-bound receptor homo/hetero dimer, in turn, has a different set of tyrosine phosphorylation sites, which serve as docking sites for specific SH2 containing proteins and recruit different combinations of intracellular signaling molecules[20,40,41]. This is despite the fact that there is a considerable overlap in the molecules recruited to the active receptors. For example, tumor cells that over express EGFR with kinase domain mutations preferentially activate the pro-survival PI3K-Akt pathway and Signal Transducer and Activator of Transcription (STAT) pathways. EGFR has no consensus sequence for the p85 adaptor subunit of PI3K; it couples to this pathway via GAB1. GAB1 in turn binds the Growth Factor Receptor Bound protein 2 (GRB2), which docks at the phosphorylated tyrosine of the kinase domain of the activated EGFR. Similarly, there is no evidence for direct binding of STAT to the EGFR. But it is proposed that this coupling is mediated via tyrosine 1068 and tyrosine 1086 of the EGFR kinase domain[42]. ErbB-2 couples to the Mitogen-Activated Protein Kinase (MAPK) pathway through GRB2, SHC, downstream of Kinase Related (DOK-R)[43] and CRK. PhosphoLipase C γ (PLCγ) binding has recently been implicated in transducing signals by EGFR[44]. Although ErbB-3 is able to bind neuregulins (NRGs), it has impaired kinase activity owing to substitutions in crucial residues in the tyrosine-kinase domain. Therefore, ErbB-3 gets phosphorylated and functions as a signaling entity only when it heterodimerizes with another ErbB receptor[45], ErbB-2 being the preferred partner. ErbB-3 contains six docking sites for the p85 adaptor subunit of PI3K[37,46] and couples very efficiently to this pathway[41].

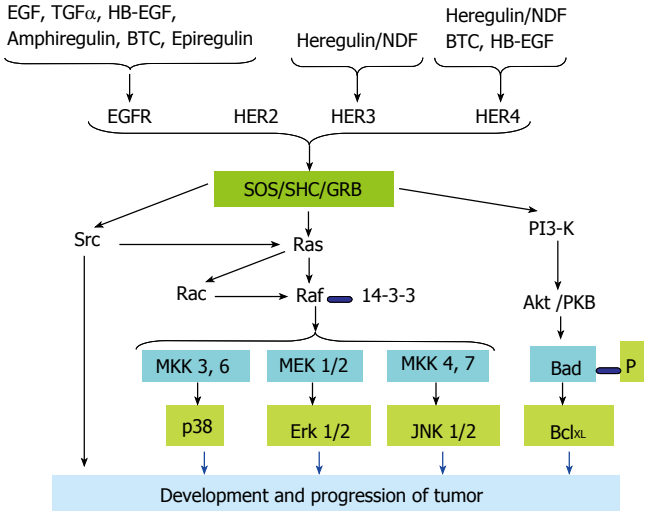


**Figure 2** Schematic representations of different ErbBs/EGFRs ligand-induced signaling pathways leading to development and progression of tumors. BTC: betacellulin; EGF: Epidermal growth factor; EGFR: Epidermal growth factor receptor; ERK: Extracellular signal regulated protein kinase; HB-EGF: heparin binding-EGF; Grb: Growth factor receptor receptor binding protein; JNK: c-Jun N-Terminal kinase; MEK: Mitogen activated protein kinase kinase; NDF: neu differentiation factors (also called neuregulins); PI3K: Phosphoinositide-3-kinase; PKB: Protein kinase B; SOS: Son of sevenless;TGF-α: transforming growth factor α.

### Functions of ErbB members

The ErbB family of RTK plays a crucial role in the development of the cardiovascular system, nervous system, mammary gland, and probably others[47]. Expression patterns of ErbB receptors and their ligands, as well as targeted inactivation of components of the ErbB signaling network highlight the importance of short-range ligand-receptor interactions, especially in mid-gestation processes. ErbB receptors regulate different developmental processes

by fine-tuning of apoptosis and proliferation. In light of data from *in vivo* and *in vitro* studies, it may be concluded that the primary role of ErbB receptor signaling is to promote growth and proliferation. This is achieved in two ways (1) suppressing apoptotic signals and (2) promoting pro- survival signals. ErbB receptor signaling has the ability to antagonize the activation of extrinsic apoptosis signals by Fas receptor and TNFR-1[48]. On the other hand, ErbB-1 signaling can increase Bcl-$X_L$ transcription through STAT-3[49,50] or the mitogen-activated protein kinase kinase (MEK) protein of the mitogenic Ras pathway[51]. Thus, ErbB receptors function at both the transcriptional and post-transcriptional levels to modulate the expression and localization of Bcl family proteins to promote cell survival.

### *Significance of ErbB receptors in oncogenesis*

**ErbB receptors and clinical studies:** In head and neck cancer, the vast majority of tumors are strongly EGFR-positive[10]. Studies have also reported EGFR overexpression in the following cancers: bladder, brain, breast, cervical, uterine, colon, esophageal, glioma, non-small-cell lung cancer (NSCLC), ovarian, pancreatic and renal cell[52-54]. ErbB-2 overexpression, generally attributable to gene amplification, occurs in 25%-30% of breast cancers and correlates with shorter time to relapse and lower overall survival[55]. Overall, about 30% of invasive ductal carcinomas overexpress ErbB-2, but no ErbB overexpression is seen in benign breast disease[56]. ErbB-3 overexpression was linked to several negative prognostic factors, including lymph node involvement, invasion, and patient survival[57]. Involvement of ErbB-4 in breast cancer remains controversial. Some reports indicate that increased ErbB-4 expression or signaling is associated with tumorigenesis. ErbB-4 overexpression has been observed in a variety of cancers, including tumors of the thyroid, breast, and gastrointestinal tract[58-61]. However, other reports indicate that increased ErbB-4 expression or signaling correlates with tumor cell differentiation and reduced tumor aggressiveness. ErbB-4 overexpression in breast tumors is associated with progesterone receptor and estrogen receptor expression and is often a favorable factor in prognosis[62,63]. In one study of common solid human cancers, the loss of ErbB-4 expression was seen in a significant percentage of breast, prostate, and head and neck malignancies[64,65]. These findings raise the intriguing possibility that ErbB-4 is unique to the ErbB family of receptors in that ErbB-4 expression and signaling may couple to reduced tumorigenesis or tumor cell proliferation. However, due to presence of the conflicting evidence, it remains unclear what general or specific roles ErbB-4 plays in differentiation, tumor suppression, or proliferation.

**Elevated Co-expression of partners and ligands:** In many cases EGFR is co-expressed with other members of the ErbB family, leading to the formation of highly transforming dimers such as EGFR//ErbB-2 and EGFR//ErbB-3. It is also well known that other dimers such as ErbB-2//ErbB-3 play a key role in various cancers such as breast carcinoma. ErbB-3 is frequently overexpressed than ErbB-2 in gastric cancers and was widely detectable, making it a potential marker for postgastrectomy recurrence. It was further demonstrated that ErbB-3 functions as an indispensable ErbB-2 dimerization partner and is required for proliferation of ErbB-2-overexpressing tumor cells in ErbB-2 overexpressing breast tumor cell lines[66]. There was a correlation between ErbB-3 expression and sensitivity to ErbB-2 directed inhibitors. For above reasons, the prognostic significance of any ErbB expression in tumors also depends on the expression of other ErbB members being co-expressed. For instance, in childhood medulloblastoma patients with tumors overexpressing both ErbB-2 and ErbB-4 have worse prognosis than patients with tumors that express either receptor alone[67]. Moreover, the levels of ligands like TGF$\alpha$ also significantly correlated with advanced disease, suggesting that elevated levels of ligand and its receptor might create an autocrine signaling loop[68].

**Deregulation of ErbB signaling:** Enhanced activity of the receptors resulting from overexpression, coexpression of the receptor, and their ligands, as well as activating mutations, is the hallmark of many human carcinomas[24]. However, the most common is the overexpression of the receptor along with the expression of the respective ligands like TGF$\alpha$, EGF, amphiregulin and HB-EGF leading to persistent autocrine stimulation. Another common occurrence is the activating mutation in the EGFR extracelluler domain; where the exons 2-/7 are deleted, leading to a persistently active receptor EGFR$\Delta$[69-74], that is persistently active in the absence of a ligand[73,74]. This activating mutation is the hallmark of many tumors that overexpress EGFR. Activation of the EGFR stimulates tumor growth and progression, including the promotion of proliferation, angiogenesis, invasion, metastasis and inhibition of apoptosis[18,75,76]. The emergence of this mutation represents the most aggressive form of the tumor. ErbB receptors appear to have the potential to acquire novel survival signaling pathways when overexpressed and/or mutated within certain tumor cell types. ErbB survival signals can also prevent tumor cells from responding to chemotherapeutic agents[77-81], which often function by activating apoptotic pathways within targeted tumor cells[82,83]. Studies by Yu *et al*[78,79] have demonstrated that overexpression of ErbB-2 permits the MDA-MB-435 breast cancer cell line to withstand ten times the usual dose of the chemotherapeutic agent Taxol before undergoing apoptosis. Similarly, studies by Nagane *et al*[77], indicate that expression of a mutated version of ErbB-1 in human malignant gliomas results in tumor cell resistance to the apoptosis-inducing chemotherapeutic drug cisplatin. Enhanced tumorigenicity and escape from cisplatin-induced apoptosis have been correlated with mutant ErbB-1 induced upregulation of the anti-apoptotic Bcl-$x_L$ protein.

### *Strategies for inhibition of ErbB receptors*

Over the years, different strategies have been developed that inhibit signaling by ErbB receptors. These agents with degrees of receptor specificity include monoclonal antibodies, tyrosine kinase inhibitors, immunotoxin conjugates, antisense oligonucleotides, and bispecific antibodies (Figure 3). Among the classes of agents targeting ErbB receptors, the monoclonal antibodies and tyrosine kinase inhibitors are furthest in development. In general, monoclonal antibodies against the extracellular domain target an individual ErbB receptor, whereas

**Table 1 Selected ErbB-targeted monoclonal antibodies**

| Drug/agent | Type | Target | Company/institution | Stage of development |
|---|---|---|---|---|
| Trastuzumab (Herceptin) | Humanized mAb | ErbB-2 | Genentech/Roche | Approved for ErbB-2 over expressing breast cancer in 1998 |
| Cetuximab (Erbitux/IMC-225) | Human-mouse Chimeric mAb | EGFR | ImClone/Merck KGaA Bristol-Myers Squibb | Approved for colorectal cancer, Phase Ⅲ trials ongoing for HNSCC and NSCLC in 2004 |
| Panitumumab (ABX-EGF) | Fully Human mAb | EGFR | Abgenix | Phase Ⅲ trials for renal cancer, prostate cancer, pancreatic cancer, colorectal and NSCLC, esophageal cancer |
| Pertuzumab (Omnitarg/2C4) | Humanized mAb | ErbB-2 | Genentech | Phase Ⅱ trials for ovarian cancer, breast cancer, prostate cancer and NSCLC |
| Matuzumab (EMD-72000) | Humanized mAb | EGFR | Merck KGaA | Phase Ⅱ trials ongoing for gynaecological cancer, pancreatic cancer and esophageal cancer |
| Thera CIM (hR3) | Humanized mAb | EGFR | YM Biosciences/CIM | Phase Ⅱ trials for HNSCC |
| HuMab-Mouse (MDX-447) | Humanized mAb | EGFR | Medarex/Merck KGaA | Preclinical trials ongoing. Phase Ⅱ trials ongoing for HNSCC |
| Mab 806 | - | EGFR(del 2-7)/ EGFR vⅢ | Ludwig Institute | Preclinical trials ongoing. |

EGFR: Epidermal growth factor receptor; mAb: Monoclonal antibody; NSCLC: Non-small-cell lung cancer; HNSCC: Head and neck squamous-cell cancer.

tyrosine kinase inhibitors that compete with the ATP binding site of the intracellular kinase domain[84-92]. The first ErbB-targeted compound approved for use in human malignancies was trastuzumab (Herceptin), a monoclonal antibody directed against ErbB-2. Subsequently, cetuximab (Erbitux), a monoclonal antibody directed against EGFR; and gefitinib (Iressa), an EGFR tyrosine kinase inhibitor were approved by the FDA for treatment of a number of epithelial cancers. Individually, they target a single ErbB receptor (Table 1). However, because members of the ErbB receptor family cooperate in signal transduction and malignant transformation, the efficacy of these agents has been limited. As mentioned earlier, the ErbB signaling is quite diverse due to different combinations of ligand binding and ErbB partners and the cross talk with other pathways. Preclinical studies have shown that cancer cells can escape the antiproliferative activity of an agent directed against one ErbB receptor by overexpressing ligand for another ErbB receptor. For these reasons there is an ever-increasing requirement for the development of therapies that can induce concurrent inhibition of two or more receptors/pathways. Using both *in vitro* and *in vivo* models, strategies that employ a dual ErbB approach seem to have a greater antitumor efficacy than agents targeting an individual ErbB receptor[93-99].

### *Therapies targeting inhibition of dual /pan Erb B*

Different multi-target therapies can be categorized in the following three groups:

**Combinations of agents that target individual ErbB receptors:** As an ErbB-targeted approach, the combination of a monoclonal antibody together with a small-molecule tyrosine kinase inhibitor uses two agents with different sites of action (Table 2). A phase II clinical study is currently underway to test the dual therapy with trastuzumab (mAb to ErbB-2) and EGFR tyrosine kinase inhibitors. The potential mechanisms of action of trastuzumab combined with an ErbB tyrosine kinase inhibitor include receptor down-regulation, signaling perturbation, angiogenesis inhibition, and antibody-dependent cell-mediated cytotoxicity[54,100,101].

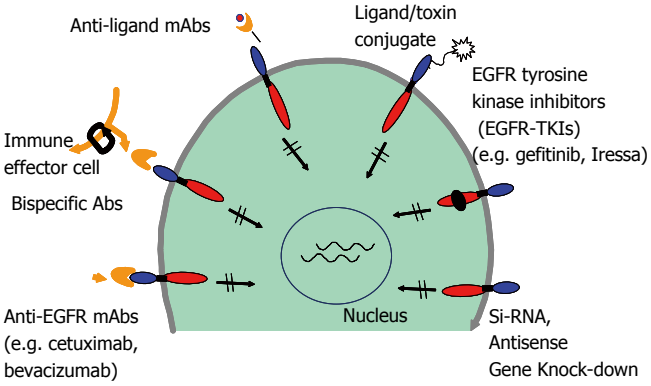


**Figure 3** Different strategies to inhibit EGFRs signaling. mAbs: monoclonal antibodies; siRNA: small interfering ribonucleic acid.

**Single agents that target multiple ErbB receptors:** They are either dual or pan-ErbB tyrosine kinase inhibitors (Table 2). Most tyrosine kinase inhibitors compete with the ATP binding site to inhibit phosphorylation. Among these agents, canertinib, targeting EGFR, ErbB-2, and ErbB-4, and lapatinib, targeting EGFR and ErbB-2, are the furthest in development. Lapitinib (GW2016) is a quinazoline derivative that functions as a reversible, dual ErbB tyrosine kinase inhibitor.

**Agents that interfere with ErbB receptor interactions:** Another approach to inhibiting multiple ErbB receptors is provided by pertuzumab (Omnitarg, 2C4), a monoclonal antibody against ErbB-2 that interferes with ErbB receptor interactions. Pertuzumab binds to a different epitope of the ErbB-2 extracellular domain than trastuzumab[102,103] and seems to differ from trastuzumab in its mechanism of action. Trastuzumab is only active in cells that overexpress ErbB-2, and it does not directly affect the ability of ErbB-2 to function as a coreceptor[104,105]. In contrast, pertuzumab is active in cells that do not overexpress ErbB-2, and it inhibits ligand-mediated signaling by preventing the recruitment of ErbB-2 into ligand/ErbB

**Table 2 Selected ErbB-targeted small molecule inhibitors**

| Drug/agent | Molecular properties | Target Selectivity | Clinical activity in cancer type | Company/Institution | Stage of development |
|---|---|---|---|---|---|
| Gefitinib (ZD 1839; Iressa) | Reversible TKI | EGFR inhibitor | NSCLC, HNSCC, colorectal cancer and breast cancer | AstraZeneca | Approved for NSCLC in 2003, ongoing Phase Ⅲ Trials for other cancers |
| Erlotinib (OSI-774; Tarceva) | Reversible TKI | EGFR inhibitor | NSCLC, HNSCC, colorectal cancer and pancreatic cancer | Genentech/ OSI pharmaceuticals | Approved for NSCLC in 2005, ongoing Phase Ⅲ Trials for other cancers |
| Canertinib (CI-1033)/(PD183805) | Irreversible TKI | EGFR/ErbB-2 inhibitor | NSCLC, HNSCC, Ovarian cancer, breast cancer | Pfizer | Phase Ⅱ |
| Lapatinib (GW2016) | Reversible TKI | EGFR/ErbB-2 dual inhibitor | Breast cancer | GlaxoSmithkline | Phase Ⅲ |
| EKB-569 | Irreversible TKI | EGFR inhibitor | Colorectal cancer, cancer, HNSCC, and NSCLC | Wyeth-Ayerst | Phase Ⅱ |
| AEE788 | TKI | EGFR/ErbB-2/VEGFR | Anti-proliferative effects in tumor cell lines and animal models of cancer | Novartis | Phase I |
| EXEL 7647/EXEL 0999 | TKI | EGFR/ErbB-2/VEGFR | | EXELIXIS | Phase I |
| PKI-166 | Reversible TKI | EGFR/ErbB-2 | Thyroid, Renal, colorectal, HNSCC, and NSCLC | Novartis | Phase I |
| PD 168393 | Irreversible TKI | EGFR | - | Calbiochem | Preclinical |
| AG-1478 | Irreversible TKI | EGFR | - | Calbiochem | Preclinical |
| CGP-59326A | Reversible TKI | EGFR | - | Novartis/ | Preclinical |
| BIBX 1382 | TKI | EGFR | - | Boehringer/Ingelheim | Preclinical |

EGFR: epidermal growth factor receptor; TKI: tyrosine-kinase inhibitor; NSCLC: non-small-cell lung cancer; HNSCC: head and neck squamous-cell cancer, VEGFR: vasculo-endothelial growth factor receptor.

receptor complexes[105,106]. In this manner, pertuzumab provides a unique opportunity to study the contribution of individual heterodimers to the activation of specific signaling pathways.

### *EGF-Receptor Related Protein (ERRP), a novel pan ErbB inhibitor*

Several strategies have been developed to inhibit EGFR and other members of the ErbB family receptors. As mentioned above, usually more than one ErbB member may be involved in a given type of cancer. Thus, in such a scenario it becomes imperative that strategies are developed to target multiple members of the EGFR family. EGFR Related Protein (ERRP) recently isolated from the rat gastro-duodenal mucosa, was found to be a pan-ErbB inhibitor that targets multiple members of the EGFR family. ERRP, a 53-55 kDa protein, possesses three of the four extracellular domains of EGFR, which are responsible for the ligand binding, and subsequent homo/hetero dimerization of various ErbB members. The ERRP cDNA shows 85%-90% homology to the external domain of EGFR and 50%-60% homology to ErbB-2, ErbB-3 and ErbB-4[107]. Though the human counter part of the rat ERRP remains to be isolated, the rat ERRP shows approximately 85% homology to the extracellular domain of human EGFR. Nevertheless, immunohistological analyses in conjunction with anti-rat ERRP polyclonal antibodies revealed that ERRP expression changes in the gastrointestinal tissues (as discussed below) of the rat and human during carcinogenesis as well as aging. These data suggest the presence of an ERRP like molecule in humans.

Garrett *et al*[108] has reported that a truncated EGFR, lacking the extracellular domain Ⅳ of the receptor that binds EGF and TGF-α with higher affinity than the full-length extracellular domain of EGFR. ERRP, a naturally occurring molecule, lacking most of the extracellular domain Ⅳ, also binds TGF-α and is expected to be effective in preferentially binding/sequestering other ligands of ErbBs. In addition, recent biochemical studies utilizing EGFR mutant lacking exons 2-7 of the receptor extracellular domain demonstrated intermolecular inhibitory function of EGFR extracellular domains[109]. Such mutants dimerize with EGFR and cause phosphorylation of wild type EGFR in the absence of ligands[108]. In the light of these findings and the available experimental data, it is suggested that loss of such subdomains is associated with constitutive activation of EGFR, while the truncated EGFRs containing only the extracellular domains serve as repressors of EGFR functions. Ectopic expression of recombinant ERRP causes increased binding/sequestration of EGFR ligand(s) resulting in decreased availability of the ligand(s) for binding to and activation of EGFR with a subsequent attenuation of EGFR signaling pathways. A schematic representation of our hypothesis is depicted in Figure 4. This is further supported by experimental data. Marcinaik *et al*, have demonstrated that exposure of HCT-116 cells to recombinant ERRP and TGF-α results in the formation of heterodimers of EGFR and ERRP with molecular weight of about 220 kDa[110]. TGF-α also induces the

formation of a 340 kDa homodimer of EGFR[110].

**ERRP and carcinogenesis:** In order to investigate the correlation between expression of ERRP protein and carcinogenesis, benign and neoplastic tissues from the pancreas, liver and gastric and colonic mucosa were examined. Expression of ERRP was found to be high in benign human colonic and gastric mucosa as well as in the liver and pancreas but low in the respective carcinomas of these tissues[110-118]. It was further observed, that in colorectal and pancreatic cancers, expression of ERRP decreases progressively with decrease in differentiation[112,113]. In the colon, ERRP expression became more attenuated in polyps with increasing grades of dysplasia. Expression of EGFR inversely related to ERRP in representative samples of normal and neoplastic colon[112]. In light of these observations it is speculated that the loss of ERRP may partly be responsible for induction of EGFR, and may play a causative role in the development of carcinogenesis.

To further evaluate the role of ERRP in the development of cancer, colonic mucosa from rats treated with the colonic carcinogen dimethylhydrazine (DMH) or vehicle (controls) was analyzed for ERRP and EGFR expression. ERRP expression was significantly lost in early stages of chemically induced colon cancer[113]. These observations suggest a potential role for ERRP in the development and progression of carcinogenesis. Supporting data for this inference was further obtained when EGFR and ERRP expression was analyzed in the gastric mucosa of rats during advancing age. In the rat model, it is generally accepted that aging is associated with increased proliferation of colonic mucosal cells that frequently involves the enhanced activity and expression of EGFRs. It has been postulated that age-associated enhanced EGFR activity is due in part, to loss of ERRP which acts as a negative modulator of EGFRs. Immunohistochemical studies revealed that ERRP expression decreased in the gastric mucosa with aging in contrast to increasing expression of EGFR[117].

**ERRP as a potential pan-ErbB inhibitor:** Our hypothesis that ERRP could be a potential therapeutic agent for epithelial cancers came from initial observation that transfection of ERRP cDNA into colon cancer cells inhibited proliferation in the matrix-dependent and -independent systems. This inhibition was associated with attenuation of tyrosine phosphorylation and tyrosine kinase activity of EGFR[107]. The similar phenomenon was also noted in prostate cancer cells (PC-3) following transfection with ERRP cDNA (unpublished data).

To further determine the therapeutic potential of ERRP, we generated and purified recombinant ERRP using the drosophila expression system (Invitrogen)[110]. The affinity-purified recombinant protein was utilized to investigate its effects on the growth of colon and other epithelial cancer cells *in vitro* and *in vivo*. ERRP was reported to inhibit proliferation of colon, and prostate cancer cell lines in a dose-dependent manner. These changes included the inhibition of EGFR signaling and attenuation of downstream signaling involving activation of Akt, mitogen activated protein kinase (MAPK) and nuclear factor (NF-κB)[110,118]. The similar effects were observed in other studies involving non-small cell lung cancer (NSCLC) cell lines,

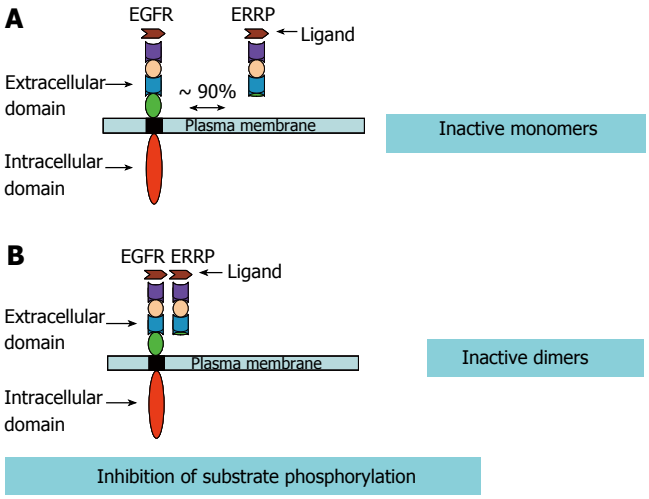


**Figure 4** Schematic representation of the comparison of ERRP structure with EGFR (**A**) and hypothetical mechanism of action of ERRP (**B**). EGFR: Epidermal growth factor receptor; ERRP: Epidermal growth factor receptor-related protein.

breast cancer and pancreatic cancer[119-121]. These epithelial cancer cells express varying levels of EGFR and other ErbB receptors. Thus, a pan-ErbB inhibitory role of ERRP was suggested. The results from efficacy trials using SCID mice have further shown tumor regression in some and arrested growth in other animals[110,122]. ERRP was effective at dose levels of 25 μg/kg and could be tolerated up to 100 μg/kg without producing signs of toxicity in the SCID mice. Although the withdrawal of ERRP administration leads to reappearance of the tumors, the growth rate was significantly reduced afterwards.

Immunohistochemical analysis of ERRP-treated tumors revealed that ERRP-induced inhibition of growth, accompanied by a marked stimulation in expression of active caspase-3 and reductions in phosphorylated (activated) forms of Akt and ERKs[122]. This suggests that ERRP inhibits tumor growth, in part by inducing apoptosis, which was further supported by *in vitro* experiments[122]. ERRP induced apoptosis follows arrest of the cells in the Go/G1 phase of the cell cycle[122]. In order to test the therapeutic value of ERRP, its immuno-reactivity in humans has been further investigated. *In vitro* studies in human peripheral blood lymphocytes have shown marginal activation of the immune response (unpublished data). In an attempt to understand the mechanisms underlying ERRP induced growth inhibition and apoptosis of the tumors, further *in vitro* studies have been conducted. Rishi *et al* observed that ERRP inhibits the processes of cell invasion and blood vessel formation by colon cancer cells. Further, ERRP also inhibited tubule formation by aortic endothelial cells and invasion by colon cancer cells through matrigel[123]. This finding suggests that ERRP inhibits different processes of invasion, metastasis and angiogenesis that are critical in the progression of carcinogenesis. Thus, ERRP, a novel pan-ErbB inhibitor, has a potential utility as a therapeutic for a wide variety of epithelial cancers.

## CONCLUSION

Limited success of chemotherapy/adjuvant therapies in

cancer treatment has necessitated the development of novel targeted therapies. A number of pharmacologic as well as biologic agents have been developed that target specific aspects of intracellular processes and interfere with development and progression of tumors. In light of the fact that the ErbB family of receptors plays an important role in epithelial cancers, a number of inhibitors that target these receptors have been developed. In particular, the monoclonal antibodies Cetuximab and Herceptin, as well as small molecule inhibitors gefitinib and tarceva have shown some promise in the treatment of cancer. However, these agents target EGFR or Her2 but not both. Since most cancers over-express multiple ErbBs, targeting a single receptor often leads to activation and signaling by other ErbBs resulting in development of resistance. In this regard, the agents/inhibitors that target multiple ErbBs are anticipated to display superior efficacy. In our pursuit to develop a pan-ErbB inhibitor, we have recently isolated and characterized ERRP. ERRP have been shown to inhibit proliferation and induce apoptosis of the prostate, colon, gastric, pancreatic, breast and lung cancer cells in *in vitro* models. ERRP attenuates the basal and ligand induced (TGF-$\alpha$, HB-EGF and heregulin) activation of EGFR and Her-2 in a variety of epithelial cancers, suggesting a pan-ErbB inhibitory property. Recombinant ERRP also inhibits growth of colon and pancreatic cancer cell-derived xenografts in SCID mice. ERRP, is unlikely to initiate an immune response as it is an endogenous, secretory protein. ERRP thus is a potential therapeutic for a wide variety of epithelial cancers.

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*TOPIC HIGHLIGHT*

**Parimal Chowdhury, Professor,** *Series Editor*

# Exploitation of the nicotinic anti-inflammatory pathway for the treatment of epithelial inflammatory diseases

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## Abstract

Discoveries in the first few years of the 21st century have led to an understanding of important interactions between the nervous system and the inflammatory response at the molecular level, most notably the acetylcholine (ACh)-triggered, α7-nicotinic acetylcholine receptor (α7nAChR)-dependent nicotinic anti-inflammatory pathway. Studies using the α7nAChR agonist, nicotine, for the treatment of mucosal inflammation have been undertaken but the efficacy of nicotine as a treatment for inflammatory bowel diseases remains debatable. Further understanding of the nicotinic anti-inflammatory pathway and other endogenous anti-inflammatory mechanisms is required in order to develop refined and specific therapeutic strategies for the treatment of a number of inflammatory diseases and conditions, including periodontitis, psoriasis, sarcoidosis, and ulcerative colitis.



**Key words**: α7-nicotinic acetylcholine receptor; Inflammation; Mucosa; Nicotine; Nicotinic anti-inflammatory pathway; Tobacco smoking

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## INTRODUCTION

Tobacco smoke appears to affect susceptibility to and the severity of various skin and mucosal diseases differently. For example, tobacco smoking is associated with an increased incidence and clinical severity of psoriasis[1-3] and Crohn's disease[4-6] but is associated with a lower incidence of pouchitis[6,7], celiac disease[6,8] and ulcerative colitis[6] as well as improved symptoms of ulcerative colitis[6]. While smokers are more susceptible to developing inflammatory periodontal diseases, smoking masks overt signs of gingival inflammation, which represents a clinical conundrum for dental professionals[9,10]. In order to explain these associations, and to harness any therapeutic potential of tobacco components, it will be necessary to better understand the cellular and molecular mechanisms by which tobacco smoke and smoke components influence epithelial inflammation in the skin and mucosa. This review describes the nicotinic anti-inflammatory pathway and provides some insight into the possible exploitation of this pathway for the treatment of epithelial inflammation and other inflammatory conditions.

In addition to observations of associations between tobacco use and specific inflammatory diseases, the use of nicotine delivery systems, for reasons other than tobacco cessation therapy, has received attention. However, such studies have been essentially limited to inflammatory bowel and neurodegenerative diseases. Clinical trials using transdermal nicotine have shown that nicotine can improve symptoms in individuals with ulcerative colitis[11-14]. Additionally, several studies suggest that nicotine treatment may be useful in improving learning and attention, but importantly, probably not memory function in subjects with Alzheimer's disease and other neurodegenerative diseases associated with a loss of neuronal nAChR protein or function[15-18]. However, an increasing understanding of the mechanisms by which nicotine interacts with the inflammatory system may soon open up further avenues for the therapeutic use of nicotine and other cholinergic agonists in the combat of several inflammatory disease processes.

## KEY INFLAMMATORY CELLS EXPRESS NICOTINIC ACETYLCHOLINE RECEPTORS

Monocytes and macrophages are key innate response cells that, when appropriately activated, potentiate inflammation. Lipopolysaccharide (LPS), a cell wall component of Gram negative bacteria, is a potent inducer of the inflammatory response and the best studied pro-inflammatory stimulus.

LPS recruits, activates, and promotes degranulation events in the most numerous inflammatory leukocyte; i.e., the neutrophil. LPS and neutrophil degranulation products each recruit monocytes and macrophages to the locus of infection. While neutrophils are, in relative terms, short-lived and transcriptionally quiescent, activated monocytes/macrophages are longer-lived cells that produce large amounts of pro-inflammatory cytokines *de novo*, including TNF, IL-1, IL-6, IL-12/IL-23 p40, IL-18, and HMGB-1, when stimulated by inflammatory mediators. Such macrophage-derived mediators amplify and direct inflammation and link the innate and adaptive immune responses. In addition to the pro-inflammatory cytokine functions of HMGB-1, the continued production of this protein is a requisite for survival in monocytes, with apoptosis occurring when HMGB-1 translation is suppressed[19-22].

The ability of the host's immune system to initially recognize and respond to bacteria and other insults is largely mediated by the innate immune system *via* the expression of a family of type I transmembrane receptors; i.e., the Toll-like receptors (TLRs)[23-26] that signal the production of pro-inflammatory cytokines when stimulated by their cognizant ligands. For example, LPS activation of TLR4 on monocytes and macrophages triggers the biosynthesis of diverse mediators of inflammation, such as TNF and IL1-β, and activates the production of costimulatory molecules (B7, CD40, MHCII) and the immunoregulatory cytokine IL-12 required for the adaptive immune response[23,27-29]. These inflammatory events are critical for clearing bacterial pathogens locally and surviving systemic infections. However, inflammation is a leading cause of morbidity and mortality in humans. Pro-inflammatory cytokines, such as TNF, have been found to be key mediators of chronic inflammatory diseases, including periodontitis[30]; rheumatoid arthritis[31]; and inflammatory bowel diseases[31,32]. Additionally, the onset of sepsis has been associated with a predominant production of multiple pro-inflammatory cytokines, including IL-1, TNF, IFN-γ and IL-12[33]. There is a subsequent set of cytokines, including HMGB1, that play a predominant role in mediating mortality in the latter phase of septic shock[32]. Therefore, there is great interest in learning how to control the production and activity of immune cell-derived inflammatory mediators[30,34] and the potential of their targeted suppression is enormous.

Cholinergic agonists act *via* either muscarinic (G-protein coupled) or nicotinic receptors. Nicotinic acetylcholine receptors are ligand-gated ion channels, but have additional functions unrelated to ion-channeling. Functional AChRs are pentameric, are composed of multiple combinations of a possible 16 monomer subtypes (α1-7; α9-10; β1-4; δ; ε; and γ), and exhibit divergent pharmacological behaviors[32,35,36]. Thus, identifying the exact type of nAChR involved in specific events can be difficult. It has been known for 25 years that phagocytic cells express nAChRs[37], yet this knowledge has not been significantly explored until recently. Neutrophils are known to express multiple nAChR subtypes. nAChR expression on monocytes and macrophages, in contrast, is much more restricted and may be limited solely to the α7 subtype in humans[35]. Certainly, of the α-bungarotoxin sensitive human nAChRs (α1, α7, and α9), monocytes and macrophages appear to express only α7 receptors that are functional[21,35,38,39]. nAChR expression on human macrophages is shown in Figure 1.

Of the known AChRs, α7 nAChR exhibits a number of unusual features[40]. First of all, it can assemble and function as a homopentamer[40,41]; the ion channel exhibits high permeability for calcium ions in preference to sodium[42]; and it is widely expressed in the central and peripheral nervous system[36] as well as on leukocytes[32]. The last few years have seen a great expansion of our knowledge of how nicotine interacts with α7 nAChRs on monocytes and suppresses pro-inflammatory activities in these cells. The most extensively studied signaling mechanism involved in nicotine-induced inflammatory suppression in monocytes is the cholinergic, or nicotinic, anti-inflammatory pathway.

## THE NICOTINIC ANTI-INFLAMMATORY PATHWAY

In order to limit self-damage, excessive inflammation is normally controlled by several endogenous anti-inflammatory mechanisms. One such mechanism is the nicotinic anti-inflammatory pathway. It has been known for some time that products of the central nervous system, such as adrenocorticotropic hormone, glucocorticoids, substance P, and melanocyte-stimulating hormone, are immunomodulatory[21,43,44]. In 2000, Borovikova *et al* first showed that synthesis of TNF by macrophages was under the control of the vagus nerve[45]. The vagus nerve is part of the parasympathetic system, is finely branched, and because it is composed of sensory (input) and motor (output) fibres can theoretically react to cytokines and suppress their production[31]. Furthermore, the vagus nerve is the longest of the cranial nerves and innervates most peripheral organs in humans. Recently, it has been shown that vagus nerve stimulation does not block TNF production in splenectomized animals dosed with LPS and the cholinergic pathway is functionally hard-wired to the spleen *via* the celiac nerve[46]. It has been dramatically shown that electrical stimulation of the vagus nerve prevents TNF production from macrophages and protects against death from LPS-induced shock[45]. The same authors have also shown that severance of the vagus nerve increases LPS-sensitivity in mice. Recently, it has been shown by using (1) α-bungarotoxin, an inhibitor of the α7 AChR, in wild type mice and (2) α7 AChR-deficient mice, that acetylcholine (ACh) or nicotine interaction with the α7 AChR is critical in the suppression of TNF release in response to LPS[32,39]. α7 AChR-deficient mice are not only hypersensitive to LPS and produce high amounts of TNF, but they also exhibit an exaggerated production of the pro-inflammatory cytokines IL-1 and IL-6[31,32]. It is currently known that the nicotine-dependent suppression of TNF release from primary macrophages is abrogated by α7 nAChR-specific, but not α1- or α10-specific, anti-sense oligonucleotides surrounding the translation-initiation codon of the α7 nAChR gene[32,39]. It is important to note that suppression of TNF and other cytokine release from macrophages

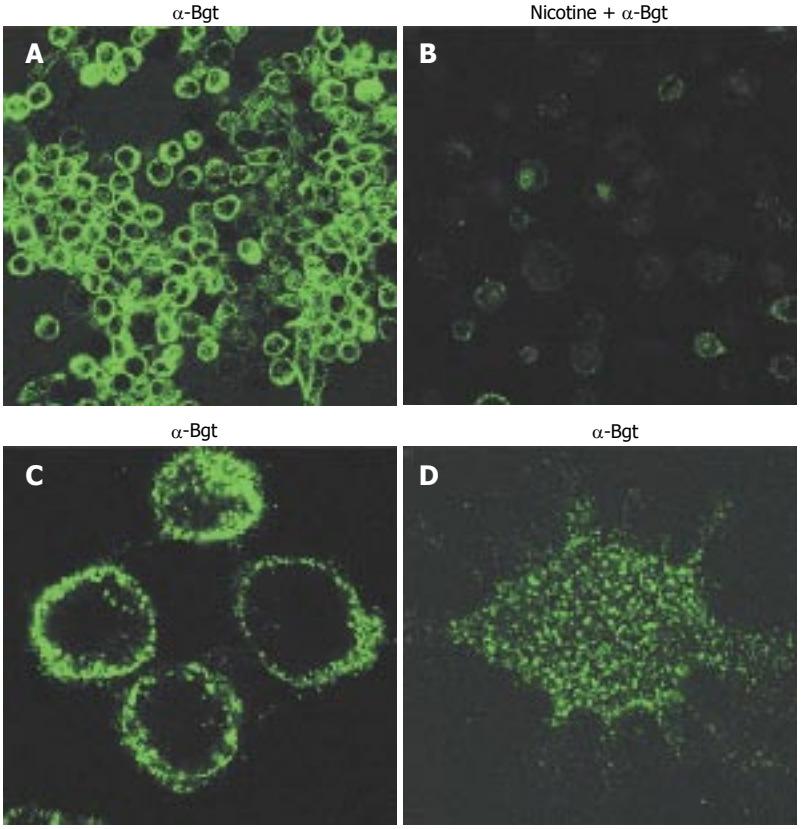


**Figure 1** α-Bungarotoxin-binding nicotinic receptors are clustered on the surface of macrophages. Primary human macrophages were stained with fluorescein isothiocyanate (FITC)-labelled α-bungarotoxin (1.5 μg /mL) and viewed by fluorescent confocal microscopy. **A**: Cells were stained with α-bungarotoxin alone; **B**: Nicotine was added to a final concentration of 500 μmol before addition of α-bungarotoxin. **C**, **D**: Higher magnification reveals receptor clusters. **C**: Focus planes are on the inside layers close to the middle (three lower cells) or close to the surface (upper cell) of cells; **D**: Focus plane is on the surface of the cell. Magnifications: **A**, **B**, x 50; **C**, x 200; **D**, x 450.

by the cholinergic anti-inflammatory system is extremely rapid, acting *via* a post-transcriptional mechanism[32,39,45,47,48], further enhancing the attractiveness of this pathway as a therapeutic target for mucosal inflammatory conditions, endotoxemia and sepsis, for example. The cholinergic anti-inflammatory pathway is presented in Figure 2.

A critical intracellular pathway involved in the production of pro-inflammatory cytokines in innate immune cells is the NF-κB pathway[19,20]. Nicotine prevents or inhibits the degradation of the inhibitory IκB protein that masks the nuclear localization signal of NF-κB and thus prevents NF-κB translocation and activation in monocytes/macrophages, in a dose-dependent manner[32,45,49], as may also be the case for other cell types, such as TNF-stimulated endothelial cells[50]. Therefore, the nicotinic anti-inflammatory pathway may not be limited to cells of monocyte lineage. As a further example, nicotine has been shown to inhibit LPS-induced TNF production by microglia cells[49,51]. Laan *et al* have shown that nicotine dampens the inflammatory response to LPS in bronchial epithelial cells and suggested that the down-regulation of the LPS-induced transcription factor, AP-1, may be of importance in regulating this phenomenon[52]. Thus, we are beginning to understand that, while tobacco smoke exerts a plethora of negative effects on the immune and inflammatory system, tobacco appears to have the potential to protect against highly specific pathological conditions; e.g. inflammatory bowel diseases[53], perhaps neurodegenerative diseases[54] and overt periodontal inflammation[9]. Obviously, the negative effects of smoking are likely to significantly outweigh any "positive" health impacts and given the enormous epidemiological and mechanistic data linking tobacco use and disease this must remain the primary public health message. Nevertheless, identification of mechanisms by which tobacco smoke components suppress certain aspects of inflammation may lead to the identification of novel therapeutic targets and may drive the future development of non-tobacco-derived agonists and antagonists. In this regard, much recent attention has focused on the translation of the anti-inflammatory agent, CNI-1493 (semapimod). CNI-1493 suppresses TNF production in multiple environments; e.g. retinopathy[55], ischemic heart failure and stroke[21,56], inflammatory bowel disease[57], Haemophilus influenza type b and LPS-induced sepsis[58-60]. While its precise mode of action is unclear, it now seems that CNI-1493 activates vagus nerve electrical activity and acts *via* the cholinergic anti-inflammatory pathway[21].

In macrophages, the nicotine-induced suppression of pro-inflammatory cytokine release may involve recruitment of the tyrosine kinase Jak2 to the α7 nACh receptor, the subsequent phosphorylation of the transcription factor STAT3, and the activation of STAT3 and SOC3 signaling cascade[61], which is known to interact with the NF-κB system[62-64], and to inhibit the expression of IL-1, IL-6, and TNF[62]. There is an obvious need to further elucidate the signaling mechanisms activated on interaction of nicotine with the α7 nAChR.

## A NON-α7nAChR-DEPENDENT ANTI-INFLAMMATORY PATHWAY

Matsunaga *et al*[65] have shown that a non-α7nAChR-dependent, nicotine-induced anti-inflammatory pathway may also function in macrophages. Nicotine-treated,

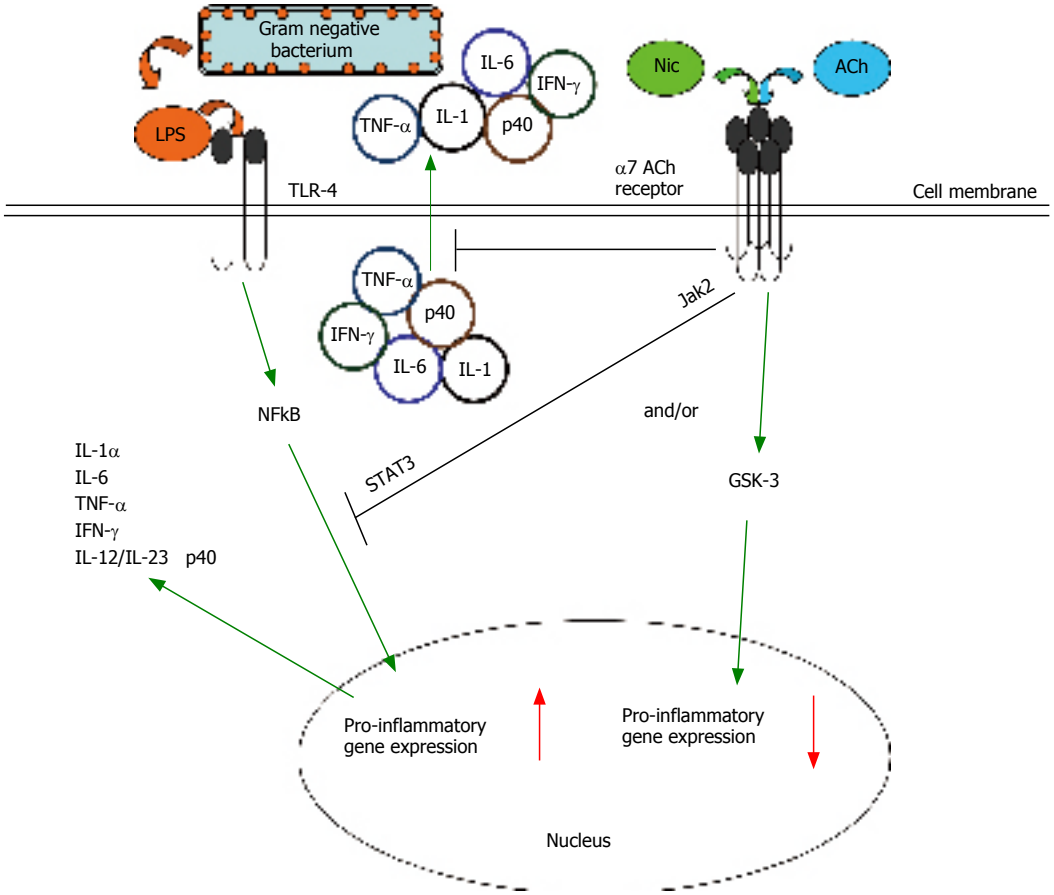


**Figure 2** The cholinergic anti-inflammatory pathway. Multiple inflammatory stimuli activate the NFκB system and lead to the release of pro-inflammatory cytokines from innate immune cells. For example, interaction of bacterial LPS with Toll-like receptors (TLRs) on the monocyte surface induces a pro-inflammatory response characterized by the production and release of several key pro-inflammatory cytokines[88]. The α7 nAChR-dependent cholinergic anti-inflammatory pathway, triggered endogenously by acetylcholine or exogenously by nicotine, can suppress the production of several pro-inflammatory cytokines in activated monocytic cells (see Figure 4)[21,22,32,39,45]. Such nicotine-mediated suppression of TNF *in vivo* protects mice from endotoxic shock[32,46]. The cholinergic anti-inflammatory pathway acts at both the transcriptional and post-translational levels. Engagement of the α7 nAChR results in the rapid suppression of the release of pre-formed pro-inflammatory cytokines[32,39,45-47]. Engagement of the α7 nAChR also results in inactivation of the NFκB system, preventing the upregulation of pro-inflammatory gene activity[32]. There is a need to further explore the signaling within the cholinergic anti-inflammatory pathway. In macrophages, the nicotine-induced suppression of pro-inflammatory cytokine release involves recruitment of the tyrosine kinase Jak2 to the α7 nACh receptor, the subsequent phosphorylation of the transcription factor STAT3, and the activation of STAT3 and SOC3 signaling cascade[61]. We have shown the potential convergence of the nicotinic anti-inflammatory and an endogenous, GSK-3-dependent anti-inflammatory pathway[88] in monocytes (see Figure 5).

*Legionella pneumophila*-infected murine alveolar macrophages exhibit enhanced intracellular bacterial replication and down-regulation of key pro-inflammatory cytokine release (IL-6, IL-12, and TNF) but not the anti-inflammatory cytokine IL-10. This inflammatory suppression was unaffected by a selective antagonist; i.e., α-bungarotoxin. Thus, the suppression of macrophage cytokine production in the pulmonary environment may help to explain the increased susceptibility for respiratory infections in smokers.

## THE NICOTINIC ANTI-INFLAMMATORY PATHWAY AND SPECIFIC DISEASES AND CONDITIONS

### *Periodontitis*

Smoking is the primary environmental factor associated with increased susceptibility and severity of periodontitis in western populations, and more than 50% of periodontitis cases in the USA can be attributed to tobacco use[66]. However, as we have previously reported, smoking reduces overt, clinically apparent, periodontal inflammation (edema; gingival index; bleeding on probing)[10,67], which is not due to any acute vasoactive effect of tobacco smoke (unlike the obvious tobacco-induced vasodilatation and vasoconstriction known to occur in forehead skin and the thumb, respectively)[68], but rather is a chronic tobacco-induced angiogenic suppression[69] that is reversed within weeks of tobacco cessation[10]. A representative case of chronic periodontitis in a smoker is shown in Figure 3.

While conflicting data has been presented for TNF[70,71], several studies have shown reduced gingival crevicular fluid (GCF) levels of major pro-inflammatory mediators, such as IL-1[72-74] in smokers with periodontitis compared to non-smokers with periodontitis, whereas anti-inflammatory cytokines are increased in the GCF of smokers, including IL-10 and TGF-β1[72,75]. This agrees well with recent and exciting data showing that nicotine activates the nicotinic anti-inflammatory pathway and suppresses pro-inflammatory cytokine production by monocytes and macrophages at the transcriptional and/or post-translational levels[21,39,45,56,61]. Thus, the inflammatory response to plaque bacteria is altered in periodontitis, and

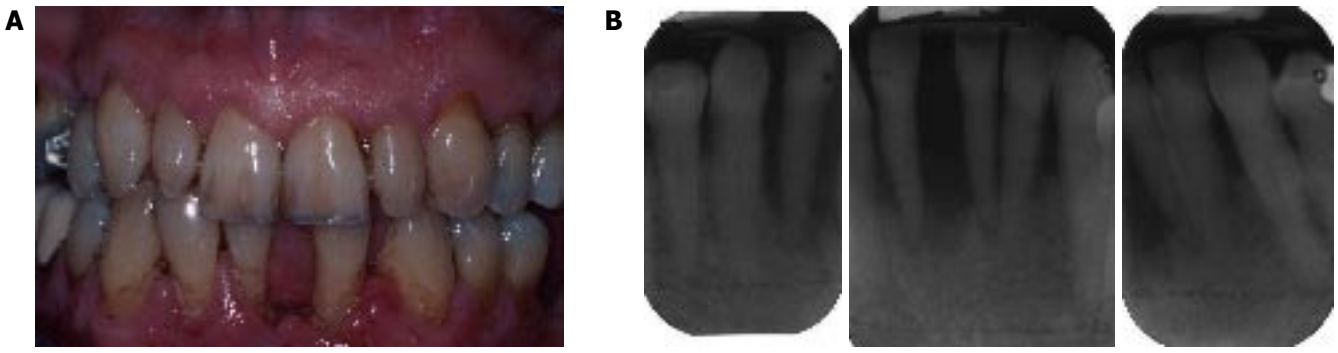


**Figure 3** Periodontitis in a male smoker, age 55. **A**: An anterior view of the mouth of a male smoker, age 55. The teeth have some staining and visible plaque. The gingivae are receded and some root surfaces are exposed. The gingivae in the upper jaw are relatively uninflamed and appear pink and fibrous in contrast to the red and swollen appearance in the lower anterior jaw; **B**: Radiographs of the lower anterior teeth. One tooth exfoliated a few months before. The remaining incisor teeth have advanced bone loss almost to the apices. Loss of these teeth is almost inevitable.

further understanding of the interactions between tobacco components, the immune system and the development of periodontitis are needed.

### *Psoriasis*

Like periodontitis, tobacco smoking is associated with an increased clinical severity of psoriasis, with up to 95% of subjects with a genetically-determined localized variant of psoriasis; i.e., palmoplantar pustulosis, being smokers[1-3]. Smoking can influence nAChR expression in skin epidermis and, in palmoplantar pustulosis, skin epidermal $\alpha$7-nAChR expression is abolished, whereas $\alpha$7-nAChR staining of the endothelium is stronger, compared to controls. Such findings have led to the hypothesis that there is an abnormal inflammatory response to nicotine, or other tobacco smoke constituents, in subjects with palmoplantar pustulosis[3]. These findings suggest that patients with palmoplantar pustulosis may not be able to activate the endogenous nicotinic anti-inflammatory pathway due to a lack of $\alpha$7nAChR, and thus treatments that activate this pathway, or other anti-inflammatory pathways, may prove efficacious in such subjects.

### *Sarcoidosis*

Sarcoidosis is a systemic granulomatous disease that can present in any organ but primarily involves the lungs and can lead to respiratory failure. In sarcoidosis, macrophages release multiple inflammatory mediators favoring an initial accumulation of Th1 cells and the generation of a polarized Th1-type environment (IL-12, TNF and IFN-$\gamma$), which has led to the targeting of specific cytokines as potential therapeutics with which to prevent the reduction in pulmonary function that accompanies granuloma formation[76-78]. A higher frequency of sarcoidosis in non-smokers than in smokers has been reported by several authors[79-83]. Thus, therapeutic activation of the nicotinic anti-inflammatory pathway represents a theoretical intervention with which to prevent progression of sarcoidosis.

### *Ulcerative colitis*

While smokers may be at increased risk of Crohn's disease[13], increasing evidence suggests that the risk for ulcerative colitis is significantly reduced in smokers, and that smoking itself may reduce disease symptoms[21]. This has led to clinical trials that show potential therapeutic efficacy in nicotine treatment for this specific inflammatory bowel disease[11-14,84]. Thus, the suppression of cytokine production and/or release by nicotine appears to contribute to a dampening of intestinal inflammation and an improved disease course. There is some evidence that ulcerative colitis may be a Th2-type inflammatory disease[6], and it is known that nicotine suppresses the production of the IL-12/IL-23 sub-unit p40, which is critical in promoting Th1 responses (our unpublished data, see Figure 4). This implies that refined targeting of the nicotinic anti-inflammatory pathway may allow the development of therapeutic interventions that are as successful in reducing the symptoms of ulcerative colitis as nicotine but that may not induce the side-effects, such as nausea, lightheadedness, headache, tremor, sleep disturbance, contact dermatitis, nausea and acute pancreatitis[11-13,84], that compromise the attractiveness of nicotine delivery as a treatment for ulcerative colitis.

### *Crohn's disease*

Tobacco smoking is associated with an increased incidence and clinical severity of Crohn's disease[4-6]. Considering that pro-inflammatory cytokines, particularly TNF, are considered to be key mediators of Crohn's disease[85], it seems counter intuitive to suggest that activation of the nicotinic anti-inflammatory pathway may exacerbate this disease. It has, however, been hypothesized that tobacco-induced suppression of the normal inflammatory response of macrophages, by $\alpha$7 and non-$\alpha$7 nAChR-dependent mechanisms, impairs the macrophage response to intestinal bacteria, leaving smokers more prone to developing Crohn's disease[6,65]. Therefore, a better understanding of the nicotinic anti-inflammatory pathway may allow pharmaceutical manipulation of this pathway, the counteraction of nicotine-dependent inflammatory suppression, and the recovery of the inflammatory response to a level sufficient for rescue macrophage effector function.

### *Septic shock*

Severe sepsis, the organ dysfunction that occurs during

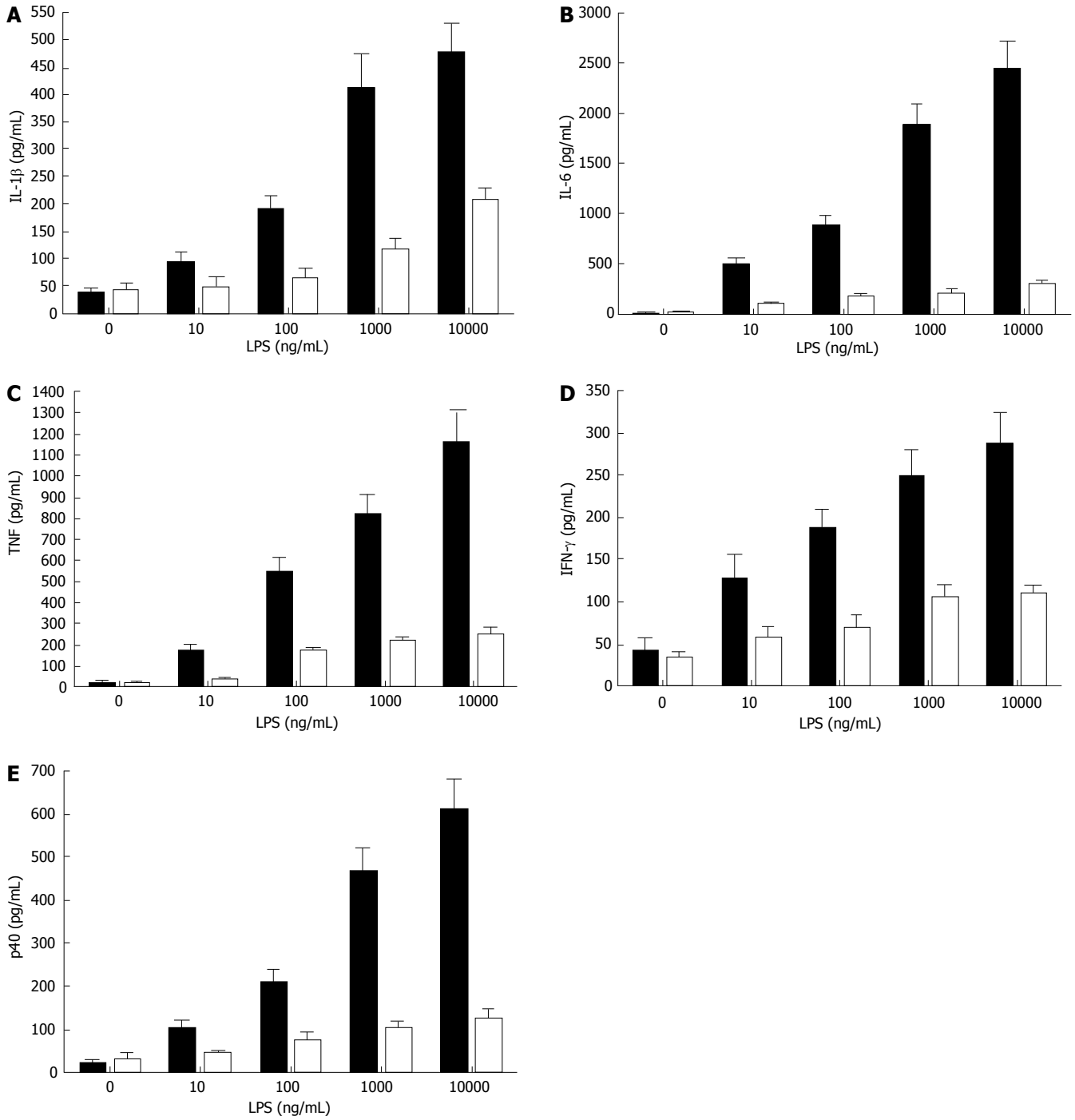


**Figure 4** Nicotine inhibits the release of multiple cytokines (TNF, IFN-γ, IL-1β, IL-6, and the common IL-12/IL-23 subunit, p40) under the control of the NF-κB pathway. Cells were pre-treated with nicotine (100 ng/mL) for 2 h then stimulated with purified LPS (0 to 1 x $10^4$ ng/mL) for 24 h. Cell-free supernatants were harvested by centrifugation and levels of pro-inflammatory cytokines were determined by ELISA. Data represents the mean (SD) of triplicate experiments.

systemic inflammation, is a major cause of mortality in developed nations, contributing to 9.3% of the total annual deaths in the United States. It is characterized by the rampant production of multiple pro-inflammatory cytokines, including TNF, IL-1β, HMGB-1, and IL-12[21,86,87]. Therapies designed to combat individual pro-inflammatory mediators in order to prevent septic shock have not been as successful as hoped and it has thus been hypothesized that inhibition of several or all pro-inflammatory mediators may be required[21]. Alternatively, it is recognized that HMGB-1, which is a late mediator of sepsis, is critical in the disease process and can be inhibited by nicotine[21,22]. Therefore, there is now a great deal of interest in using nicotine as an inducer of the nicotinic anti-inflammatory pathway as a potential treatment for severe sepsis[21,22,31,32,39].

## FUTURE DIRECTIONS

Cholinergic strategies have been suggested as potential treatments for multiple diseases. In this review we have largely limited ourselves to the discussion of the relevance

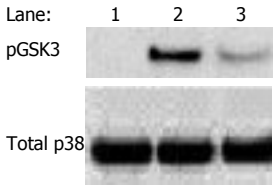


**Figure 5** Nicotine-treated human monocytes exhibit augmented levels of phosphorylated (Ser9) GSK3-β in monocytes stimulated with the Gram negative bacterium, *Porphyromonas gingivalis*. Monocytes were pre-treated for 2 h with 100 ng/mL of nicotine and stimulated for 60 min with *P. gingivalis* (MOI = 10). Western blot was performed using whole-cell lysates (20 μg) and probing for GSK3 using a phospho-specific GSK3-β (Ser9; denoted pGSK3) antibody. Blot was stripped and re-probed for total p38 to ensure equivalent loading. Lane 1: Nonstimulated; Lane 2: *P. gingivalis* + Nicotine; Lane 3: *P. gingivalis*. Data are representative of three experiments.

of the nicotinic anti-inflammatory pathway to skin and mucosal pathologies.

It is essential to point out that the use of nicotine as an anti-inflammatory agent, while supported by the current evidence, nevertheless represents a "sledgehammer" strategy. It must be envisaged that non-nicotinic (and, indeed, non-tobacco-derived) cholinergic agonists will be developed and explored in order to avoid the psychoactive, vascular, and other actions of nicotine on multiple nAChR-initiated pathways and to avoid the side-effects and adverse reactions associated with nicotine delivery. As recently pointed out by Ulloa, better structural characterization of nAChRs will be crucial in designing such nicotinic agonists[21].

Furthermore, it must be expected that targets downstream of the nAChRs will be identified allowing therapeutic refinement and an avoidance of blanket targeting of nAChRs.

Finally, the nicotinic anti-inflammatory pathway is unlikely to exist as a single, self-contained entity, but rather it is anticipated that the nicotinic anti-inflammatory pathway interacts and converges with multiple other pathways, including the NF-κB pathway and others. For example, we have shown the convergence of the nicotinic anti-inflammatory and an endogenous GSK-3-dependent anti-inflammatory pathway[88] in monocytes (our unpublished data, see Figure 5). As our knowledge of these signaling interactions increases, we are likely to identify further attractive anti-inflammatory targets and refined selectivity. In conclusion, the manipulation of nAChR-initiated signaling pathways likely represents a potentially fruitful area for inflammation research in the coming years and the currently expanding literature suggests that the number of diseases in which the pathway is relevant, for example, pancreatitis[47] and various vascular pathologies will increase[50,89,90].

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*TOPIC HIGHLIGHT*

**Parimal Chowdhury, Professor,** *Series Editor*

# Functional significance of erythropoietin receptor on tumor cells

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## Abstract

Erythropoietin (Epo) is the regulator of red blood cell formation. Its receptor (EpoR) is now found in many cells and tissues of the body. EpoR is also shown to occur in tumor cells and Epo enhances the proliferation of these cells through cell signaling. EpoR antagonist can reduce the growth of the tumor *in vivo*. In view of our current knowledge of Epo, its recombinant forms and receptor, use of Epo in cancer patients to enhance the recovery of hematocrit after chemotherapy treatment has to be carefully evaluated.



**Key words:** Erythropoietin; Tumor cell; Receptor; Cell signaling; Proliferation; Erythroid cell

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## INTRODUCTION

Erythropoietin (Epo) is a hormone which regulates the red blood cell formation. Epo is produced in the kidney and delivered to the target organ, bone marrow, *via* circulation[1]. The circulatory levels of Epo is usually at a very low level but in response to the loss of red blood cells or during hypoxic exposure its level is increased within a few hours due to the enhanced production in the kidney. The elevated Epo level acts on the bone marrow erythroid cell precursor population to augment the erythroid cell production resulting in the increase of red blood cell mass. Thus EPO acts in a tightly regulated system for the maintenance of red blood cell volume. Action of Epo on the responding cells is mediated through its receptor, EpoR.

The signal provided by Epo and its receptor, EpoR are essential for erythroid cell proliferation and differentiation[2]. Studies have revealed that there is an unique mechanism of Epo binding and activation of EpoR-Jak2 kinase complexes. Erythroid cell populations collected from humans or mice and enriched by various techniques have been used for these studies[3]. Purified human blood erythroid burst forming units, mice fetal liver cells or spleen cells from mice made anemic by two consecutive injections of phenyhydrazine have been used for such investigations[3]. Studies indicated that Epo binds to EpoR and induces a signal for proliferation and survival similar to other cells.

## STRUCTURE AND CHARACTERISTICS OF Epo

Epo is a glycoprotein with a molecular weight of approximately 34 000 daltons[4]. The protein backbone of the mature hormone consists of 165 amino acids. The alpha form of the hormone consists of 31% carbohydrate while the beta form consists of 24% carbohydrates. These two forms of Epo have similar biologic and antigenic properties. The carbohydrate moiety of Epo plays an important role in the mediation of its overall effect *in vivo*. Sialic acid, which comprises 30% of carbohydrates, represents the critical component of the carbohydrate moiety for *in vivo* biological activity. The desialation of Epo, while fully functional *in vitro*, results in significant loss of *in vivo* activity due to the rapid clearance by the liver of disialated Epo[5]. The importance of glycosylation of the Epo molecule in the mediation of its *in vivo* effect also became evident when it was found that the recombinant Epo produced in *E. coli* was not effective *in vivo*. Expression of the Epo gene in mammalian cells that provided sugars was needed to attain its full biological effect *in vivo*. Epo is a single chain polypeptide and is resistant to denaturation by heat, alkali or reducing agents. The Epo gene is located on chromosome 7 and unlike other hormones like insulin or ACTH, it is fully synthesized in its active form prior to secretion into circulation.

## Epo AND EpoR FUNCTION

Effect of Epo on erythroid cells is mediated through the EpoR similar to other hormones. One single class of EpoR with an apparent molecular weight of about 100 000 daltons occurs and it binds to the hormone with a dissociation constant of about 300 pM[6]. Erythroid cells display between 1000 and 3000 EpoR sites per cell. Only a small percentage of these receptor sites have to be populated with Epo to initiate cell division and this process is dependent largely on $Ca^{2+}$.

Previously it was thought that EpoR and Epo are required for the survival and proliferation of erythroid cells alone. However, recent investigations have indicated the presence of EpoR in normal cells of other tissues as well. Epo is now known to be important in function and development of the brain. Furthermore the brain makes its own Epo which is slightly smaller in size than Epo produced by the kidney[7]. Epo is needed for the development of new blood vessels in the muscles of athletes training at high altitude. A greater oxygen demand by the exercised muscle tissue is supplemented by the presence of Epo. Requirement of Epo is also shown in ovary, oviduct, uterus, and testis and in all of these organs the presence of EpoR has been shown[4].

## PRESENCE OF Epo AND EpoR ON TUMOR CELLS

Presence of EpoR is shown in the breast cancer cells while adjacent normal cells have no such receptors[8]. Authentic EpoR transcripts and protein have been detected in human renal-cell carcinoma and the cell lines derived from these are shown to enhance proliferation by the presence of Epo[9]. Presence of Epo and EpoR has also been shown in breast carcinoma but not in normal breast, benign papilloma or fibrocystic tissues[10]. EpoR is also found in epithelial ovarian carcinoma as detected by Western blot analysis[11]. Using the immunoblot technique, Belenkov *et al* have confirmed the presence of EpoR in the human malignant glioma cell line, U87 and the primary cervical cancer cell line, HT100[12]. Examination of breast cancer biopsies has revealed a high level of EpoR expression in cancer cells in 90% of tumors while Epo expression has been found in 60% of tumors[13]. Wollman *et al* have demonstrated the presence of EpoR in the human neuroblastoma cell line using the RT-PCR technique[14]. Occurrence of EpoR in human prostatic epithelial cells and prostate cancer cells has been demonstrated and in this instance Epo serves as a growth factor for these cells[15]. Yasuda *et al* have reported that signaling pathways of Epo and EpoR are involved in the tumorigenesis of ovarian and uterine cancers[16]. Hepatic tumors, chemically induced in rats, showed the presence of Epo while in normal cirrhotic liver tissues Epo was not detected[17]. EpoR was present in such tumors. Table 1 summarizes the publications which have looked into the occurrence of EpoR and Epo in various tissues. Our own observation on rat pancreatic tumor cell line, AR42J cell, has indicated the presence of EpoR on the surface of these cells and Epo induces proliferation of these cells (unpublished observation). Hence it is now well established that EpoR is present in most of the cancer cells and Epo has a significant effect upon these cells.

**Table 1 Presence of Epo and EpoR in the tumors of different tissues of the body. Symbol + in the columns indicate the presence of particular item in the tumor and - indicate either it is not tested or it is absent**

| Tissue of origin | EpoR | Epo | References |
|---|---|---|---|
| Breast cancer | + | + | [8, 10, 13] |
| Glioma cell line, U87 | + | - | [12] |
| Cervical cancer cell line, HT100 | + | - | [12] |
| Epithelial ovarian carcinoma | + | - | [11] |
| Renal carcinoma | + | + | [9] |
| Nuroblastoma | + | - | [14] |
| Prostate cancer cells | + | - | [15] |
| Glioblastoma | + | + | [16] |
| Gastric cancer | + | + | [16] |
| Stomach choriocarcinoma | + | + | [16] |
| Lung small cell carcinoma | + | + | [16] |
| Pancreatic cancer | + | + | [16] |
| Head & neck squamous cell carcinoma | + | + | [18] |
| Hepatic tumor | + | + | [17] |

## FUNCTIONAL SIGNIFICANCE OF EpoR ON TUMOR CELLS

Question naturally arises what is the function of the EpoR present in cancer cells? Attempts have been made in many investigations to find the role of EpoR in light of induction of signal transduction and cell growth. Some studies, as described earlier, have noted the induction of proliferation of tumor cell lines in the presence of Epo. Transplanting several tumor cell lines into nude mice Yasuda *et al* have confirmed the presence of Epo-responsive sites in xenografts in which phosporylation of the STAT5 (signal transducer and activator of transcription) is detected[16]. In these nude mice when Epo signaling is blocked by EpoR antagonist, angiogenesis and tumor cell survival are inhibited leading to the destruction of tumor mass and the disturbance of phosphorylation of STAT5. Epo mimetic peptide, on the other hand, has promoted angiogenesis and tumor cell survival. Hence Epo is indispensable for the growth and viability of malignant tumor[16].

EpoR on prostate cells is functional and exhibited a dose-dependent proliferative response to Epo and triggered STAT5 phosphorylation[15]. Epo mediated invasion of head and neck squamous cell carcinoma cells in Epo-treated head and neck cancer patients has been shown to be due to induction of limited proliferation effect by the pharmacological dose of Epo[18]. Epo activates the mitogen activated protein kinase, extracellular signal regulated kinase (ERK), and promotes migration in breast cancer cells. This migration can be inhibited by the inhibitor MEK[19]. Further, hypoxia induced Epo mRNA and EpoR expression in breast cancer cells is followed by the activation of ERK and cell migration[19]. When rat mammary adenocarcinoma cells were implanted into rats

in a tumor Z-chamber model and administered with a neutralizing anti-EPO antibody or soluble Epo receptor or an inhibitor of Jak2, all resulted in a delay in tumor growth with 45% reduction in tumor depth in a dose dependent manner[13]. Renal carcinoma cells have been shown to have increased proliferation in the presence of Epo[9]. Several effects of Epo described above with various tumors and carcinoma indicates a compelling proof that cancer cells have EpoR and Epo has an effect on these cells despite the fact that a recent study has indicated that all anti-EpoR antibodies do not always predict the EpoR expression[20]. An exception to this is an early study by Wollman *et al*[14] who have found no proliferative effect of Epo on nerve tumor cells in spite of the presence of EpoR on these cells.

## CONCLUSIONS

The reports on studies of Epo and EpoR indicate that they are not specific to erythroid cell proliferation and differentiation alone. They have an active role in other cells as well, normal as well as tumor cells. Use of Epo in cancer patients has to be monitored carefully for its side effects related to its proliferation potential of the tumor for which chemotherapy is given. It is important as more and more patients are being treated with Epo for a rapid hematocrit recovery after chemotherapy treatment. Knowledge is emerging in this regard and further studies are needed to sort out the beneficial effect of Epo from its harmful effect.

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*TOPIC HIGHLIGHT*

**Parimal Chowdhury, Professor,** *Series Editor*

# Apoptosis in skeletal muscle and its relevance to atrophy

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## Abstract

Apoptosis is necessary for maintaining the integrity of proliferative tissues, such as epithelial cells of the gastrointestinal system. The role of apoptosis in post mitotic tissues, such as skeletal muscle, is less well defined. Apoptosis during muscle atrophy occurs in both myonuclei and other muscle cell types. Apoptosis of myonuclei likely contributes to the loss of muscle mass, but the mechanisms underlying this process are largely unknown. Caspase-dependent as well as -independent pathways have been implicated and the mode by which atrophy is induced likely determines the apoptotic mechanisms that are utilized. It remains to be determined whether a decrease in apoptosis will alleviate atrophy and distinct research strategies may be required for different causes of skeletal muscle loss.



**Key words:** Skeletal muscle; Apoptosis; Disuse atrophy; Aging

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## INTRODUCTION

Apoptosis, or programmed cell death, is an important process in multicellular organisms both during development, where it ensures the elimination of superfluous tissues, and in adulthood, where it is critical for maintenance of tissue homeostasis. The early stage of apoptosis involves death-inducing signals, such as reactive oxygen and nitrogen species, ligands for the death receptors, imbalances in calcium regulation, and alterations in the composition and abundance of B-cell lymphoma (Bcl)-2 family proteins, such as Bax, Bad, Bcl-2, Bcl-xl (see[1] for review). After this induction phase, nuclear activators, cell surface receptors, or mitochondrial pathways become activated in the commitment to cellular death followed by cytoplasmic and nuclear events. During apoptosis, protein-cleaving enzymes, i.e., caspases (cysteine-dependent aspartate-directed proteases), become activated in the cytosol and are responsible for proteolytic cleavage of a broad spectrum of cellular targets[2]. Caspase-independent mechanisms also exist, such as the release of apoptosis-inducing factor (AIF) and endonuclease G (EndoG) from mitochondria (Figure 1), inducing large scale DNA fragmentation and apoptosis after translocation to the nucleus[3-5]. In the nucleus, DNA fragmentation caused by activated endonucleases, chromatin condensation, and the breakdown of the nuclear envelope occurs and eventually the cell itself disintegrates into apoptotic bodies and is phagocytosed by surrounding cells or macrophages[6]. Both extrinsic or intrinsic stimuli can be responsible for the induction of apoptosis, with some cross-talk between signaling pathways[1,7,8].

## ROLE OF APOPTOSIS

In highly proliferative tissues, such as the intestinal epithelium, apoptosis serves to maintain a constant number of cells and consistent tissue architecture, counterbalancing the rapid proliferation. Indeed, cell loss in the normal intestine can largely be explained by apoptosis[9]. In addition, in the small intestine apoptosis serves to stabilize the stem cell population by removing excess, or perhaps compromised, stem cells[10]. Decreased rates of apoptosis have been observed during tumor progression in colon carcinomas[11] and the overall decrease in apoptosis in proliferative tissues may predispose the cells in those tissues to accumulate genetic changes characteristic of tumorigenesis.

In contrast, in post mitotic tissues such as skeletal muscle, the role of apoptosis is less clear. Also, with respect to cell death, skeletal muscle is a unique tissue because muscle cells, i.e., myofibers, are multinucleated. This aspect of skeletal muscle has led to the concept of the myonuclear domain, which is defined as the theoretical amount of cytoplasm supported by a single muscle fiber nucleus, which is called a myonucleus (protein/DNA)[12].

Even though muscle size can vary considerably under different conditions, the myonuclear domain size remains relatively constant, implying a fairly strict regulation of myonuclear number (for review[13]). Myonuclear number decreases in muscles undergoing atrophy in a variety of experimental conditions, such as spinal cord isolation and transection, microgravity, hind limb suspension, and chronic denervation[14-20] and the process by which nuclei are eliminated from muscle fibers resembles apoptosis. Since destruction of the entire cell does not necessarily follow the elimination of a nucleus, as occurs with apoptosis in mononucleated cells, this process is called 'apoptotic nuclear death'. The mechanisms underlying apoptotic nuclear death in muscle are likely distinct from those involved in apoptosis in mononucleated cells.

The fact that apoptosis plays an important role in skeletal muscle atrophy can be deduced from the observation that it is increased in skeletal muscle in a number of pathological and under some physiological circumstances. Chronic heart failure, motor neuron disorders, skeletal muscle denervation, spinal cord injury, muscular dystrophy, and skeletal muscle atrophy due to hind limb suspension or immobilization are all associated with an increase in apoptosis in affected skeletal muscles, as measured by terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end-labeling (TUNEL) or by DNA fragmentation in gel electrophoresis[16,21-26]. In addition, exercise was shown to increase apoptosis when assayed acutely after a bout of exercise[24], but by contrast, exercise training for a period of 8 weeks decreased apoptosis[27]. Interestingly, we and others have shown that exercise training attenuated the apoptosis induced by disuse (spinal cord injury or hind limb suspension)[16,26]. This exercise-associated decrease in apoptosis under atrophy-inducing conditions may depend on the mode or intensity of exercise, since recently we found that gravity-independent resistance exercise did not decrease apoptosis during hind limb suspension[28]. Therefore, apoptosis in skeletal muscle seems to be a highly regulated process that may serve distinct functions under different physiological and pathological conditions and a better understanding of pathways involved in the apoptotic response in muscle is warranted.

## APOPTOTIC PATHWAYS IN SKELETAL MUSCLE

Caspases are thought to be the main enzymes involved in both the initiation (caspase-8, -9, -12) and execution of apoptosis (caspase-3, -6, -7) (Figure 1). Internal as well as external signals can activate caspases and this is under the control of the balance between pro-and anti-apoptotic proteins of the Bcl-2 family, heat shock proteins, and inhibitors of apoptosis proteins (IAPs)[29]. However, apoptosis can ensue in the absence of caspase activation, whereas caspase activation does not always necessarily trigger cell death[30]. Caspase-3 is often used as a surrogate for apoptosis, but in muscle, in particular, this may not be justified. We and others found that caspase-3 activity did not increase with atrophy induced by hind limb suspension[31,32], even though apoptosis was increased concurrently, indicating that other pathways, besides caspase-3 activation, may be involved. In contrast, denervation-induced atrophy was associated with an increase in caspase-3 activity[33] as well as caspase-8[34], indicating that the activation of caspases may serve a different role in distinct models of muscle atrophy or that the mode by which atrophy occurs determines the involvement of different pathways. Indeed, caspases have been found to play non-apoptotic roles in pathways such as the inflammatory response, immune cell proliferation and differentiation of various cell types (e.g. skeletal muscle)[35]. In skeletal muscle, caspase-3 was found to be involved in protein degradation, in particular of filamentous actin[36], and it contributed to muscle weakness in response to endotoxin[37]. Similarly, in cardiac muscle caspase-3 was shown to be involved in post-ischemic contractile dysfunction (cardiac stunning), apparently independent of apoptosis[38]. Therefore, classical apoptotic pathways, as observed in mononucleated cells, may not be as important in muscle and other molecules may take on the role of apoptosis inducers.

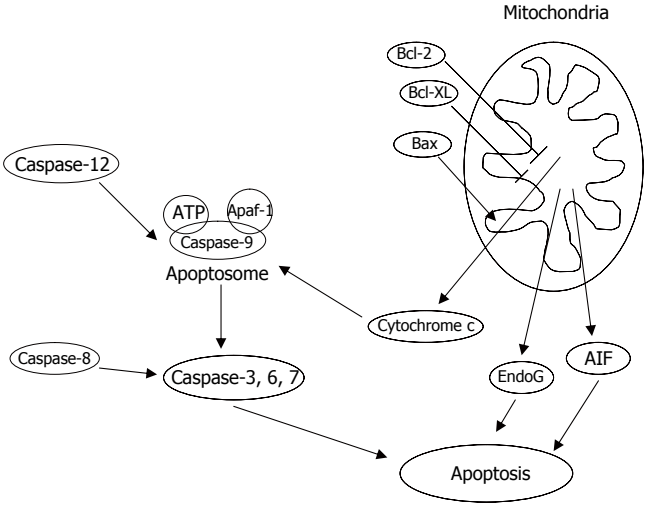


**Figure 1** Pathways involved in apoptosis in skeletal muscle. Caspase-dependent and -independent pathways are likely involved in apoptotic nuclear loss during atrophy in skeletal muscle. Caspase-3 activation and subsequent apoptosis occurs through the release of cytochrome-c from mitochondria in response to changes in the ratio of pro- and anti-apoptotic members of the Bcl-2 family of proteins, or through the activation of initiator caspases, such as caspase-8 or -12. Caspase-independent pathways such as the release of EndoG and/or AIF may be more important in skeletal muscle as they directly induce DNA fragmentation.

In this light, we have investigated the role of EndoG during skeletal muscle atrophy[31,39]. EndoG is a protein released from mitochondria upon pro-apoptotic stimulation and is capable of inducing DNA fragmentation independent of caspase activation[40-42] (Figure 1). We found that EndoG co-localization with nuclei was increased in muscles atrophied in response to both hind limb suspension and age[31,39] and that EndoG protein was increased in muscles of aged rats undergoing disuse-induced atrophy[31]. Moreover, EndoG translocation was very specific for myonuclei and did not seem to be involved in the apoptosis of interstitial cells, which also occurs during muscle atrophy[39]. In contrast, caspase-3 activation was observed more in interstitial cells and therefore different cell types within

the same tissue may be undergoing apoptosis through different mechanisms. Another protein released from mitochondria upon pro-apoptotic stimulation and capable of inducing apoptosis independent of caspases is apoptosis inducing factor (AIF)[43]. Siu and Alway[33] showed that AIF release was elevated in denervated muscle, in concert with cytochrome-c, Smac/DIABLO and a subsequent upregulation of caspase-9 and -3. Therefore, the atrophy-inducing stimulus, the time point after onset of disuse, or the different cell types undergoing apoptosis may be important in the activation of distinct apoptotic pathways. Finding interventions to counteract the increase of apoptosis with muscle atrophy will be challenging, considering the many different pathways used to induce apoptosis in skeletal muscle.

## INHIBITION OF APOPTOSIS

The question arises whether inhibiting apoptosis in skeletal muscle will also decrease atrophy induced by disuse or due to aging. Recently, Siu and Alway[44] showed that inhibition of apoptosis somewhat attenuated muscle atrophy induced by denervation in a Bax knock out mouse model. Interestingly, DNA fragmentation after denervation was much lower in the knock out mice compared to the wild type and caspase activation was also lower. However, mitochondrial AIF release was not decreased, possibly implying that caspase-independent mechanisms were not affected. In this study, the effect on different cell types was not investigated, but it is plausible that the minimal effect on muscle mass could be due to the fact that myonuclear apoptotic loss was not affected by the Bax-/- phenotype. Therefore, it remains to be determined whether inhibition of myonuclear apoptosis will decrease muscle atrophy.

## CONCLUSION

Recently a number of studies have implicated an important role for apoptosis in the development of skeletal muscle atrophy. It is likely that atrophy induced by different conditions, such as denervation, microgravity, aging, cachexia, and spinal cord injury, initiates different apoptotic signals and indeed the role of apoptosis may be distinct among the conditions. It will be important to investigate, at the cellular level, which signals are responsible for the loss of myonuclei, interstitial cells, or stem cells in skeletal muscle in order to develop strategies to decrease apoptosis and atrophy.

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*REVIEW*

# Treatment of hyperbilirubinemia with blood purification in China

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## Abstract

The incidence of hyperbilirubinemia is high clinically, which is difficult to cure by medication, surgery or interventional therapies. Non-bioartificial liver is the main alternative in the blood purification for hyperbilirubinemia, which includes plasma exchange, hemoperfusion, hemodialysis, molecular adsorbent recycling system and so on. The research results and clinical experiences in China show that these methods are effective in lowering high levels of bilirubin with fewer side effects. The hyperbilirubinemias of different causes, with different complications or accompanying different diseases can be treated by different methods. Bioartificial liver, hybrid artificial liver support system and adsorbent membrane material have also been studied and their development in reducing hyperbilirubinemias has been achieved. This article gives a brief overview on the actuality and research improvement in blood purification for hyperbilirubinemia in China.



**Key words:** Hyperbilirubinemia; Blood purification; Treatment; China; Review

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## INTRODUCTION

Hyperbilirubinemia, namely jaundice, is a common clinical manifestation, and it may be life threatening. Many diseases result in hyperbilirubinemias, some are refractory, and cannot be cured by medication or surgery. Though some obstructive jaundice related to the common bile duct can be relieved quickly and temporarily with the recent development of interventional therapies, there is a paucity of effective methods for those lesions occurring in small or capillary bile ducts such as primary biliary cirrhosis of the liver, and for some other hyperbilirubinemias that need to be cured in a short time such as an obstructive jaundice that cannot be relieved or does not have an indication for operational or interventional therapy temporarily, an acute or chronic hepatic failure, a portal hypertension associated with serious icterus in liver cirrhosis and a quite severe jaundice before liver transplantation. So, how to reduce the high concentration of bilirubin in the blood of these patients has always been a focus of study for many researchers. Blood purification has been used for treating hyperbilirubinemia in the recent two decades, and it is also called non-bioartificial liver (non-BAL) for distinguishing bioartificial liver (BAL), a recent research hotspot. Non-bioartificial liver could temporarily improve the status of patients with end-stage liver diseases and help by extending time and increasing opportunity for further treatment despite lack of synthesis and metabolic function. Especially at present when research of the artificial liver is still at its initial phase in China, non-bioartificial liver is applied widely and further research is being performed for higher efficacy. In this paper, reports by Chinese researchers on these aspects are reviewed.

## APPLICATION AND CURATIVE EFFECT OF BLOOD PURIFICATION FOR DEPRESSING HIGH LEVEL OF BILIRUBIN

Non-bioartificial blood purification applied in China currently includes plasma exchange (PE), hemoperfusion (HP), hemodialysis (HD), molecular adsorbent recycling system (MARS)[1], of which PE is used most frequently.

### *Plasma exchange (PE)*

In the process of PE, plasma is separated and discarded, while cells, as well as some supplied albumin, plasma and balance solution are reinfused back so as to remove the causative agents. PE not only removes bilirubin, endotoxin and complement activator, but also replenishes albumin, coagulation factor and hepatic regenerative stimulating substance, which can correct metabolic disorder.

Shang *et al*[2] compared treatments in two different groups: 116 patients with chronic severe hepatitis and

hepatic failure were categorized as a therapy group with 142 instances of blood purification using an artificial liver support system (ALSS, 800 PE) and medication therapy; 118 patients with the same illnesses as mentioned above were categorized as a control group which was given only supportive care and observed. All cases were consistent with virus hepatitis diagnostic criteria enacted at the China Virus Hepatitis Conference in 2000 (2000CVHC). After treatment, total bilirubin (TBIL) decreased 62.96% in the therapy group, and the clearance rate was much higher than that in the control group. The statistical difference was significant. Ming *et al*[3] also treated 32 cases of severe hepatitis diagnosed according to 2000CVHC by PE (HP200 blood purification equipment, YT-50PP PE, Yatai Company, Ningbo, China; plasma separation rate, 20-30 mL/min; blood flow rate, 60-100 mL/min; infused fresh plasma, 2000 mL), and the result showed TBIL descended 25.17%, icterus faded and symptoms improved. PE could alleviate hyperbilirubinemia, decrease mortality, and prolong survival time. However, by use of PE, large amounts of fresh plasma are consumed and there is a paucity of safety issues such as hepatitis, HIV, anaphylaxis and other complications addressed for regulatory approval. Zou *et al*[4] utilized Plasauto IQ PE instrument (OP-08W plasma segregator) for 116 times in 93 patients with severe hepatitis (consistent with 2000CVHC). After treatment, the short-term total effective rate was 80.2% (93/116), 51 were cured, 18 died, and 24 were discharged from hospital. During the treatment, adverse reactions occurred in 64 patients including erythema in 24 (22.41%), hypotension in 21 (1.72%), anesthesia accompanied hemifacial spasm in 15 (12.93%) and alimentary tract hemorrhage in 2, both of whom died, one died of intracranial hemorrhage. Besides, the drop of serum bilirubin level after PE treatment might rise again. Even so, PE is still a relatively mature method. Yang *et al*[5] reported that in the treatment of viral hepatitis hyperbilirubinemia, fewer amounts and more numbers of albuminpheresis could decrease hyperbilirubinemias more effectively in refractory hepatitis, especially in Types non-B and non-C. Meanwhile it could avoid blood contamination, providing a relative security. Long-term effects with low incidence of adverse reaction were confirmed.

### HP

The accurate meaning of hemoperfusion is blood adsorption, i.e., pathogenic medium dissolved in the blood is adsorbed to a solid matter with an ample surface for removing various toxins in a perfusion apparatus. Based on blood or plasma flowing through the perfusion apparatus, blood perfusion or plasma perfusion was named. Blood perfusion is rarely used clinically because the adsorbent may hurt or activate blood cells, so usually plasma perfusion is used for hemoperfusion. There is no incidence of cross infection, protein susceptibility or other side effects because fresh plasma, albumin and other blood products do not need to be supplied in the process of hemoperfusion. There are mainly two kinds of adsorbents used in hemoperfusion, activated charcoal and resin. Activated charcoal, is a kind of broad-spectrum adsorbent, which can effectively clear endotoxin, leukocyte inhibitory factor, cytotoxin of inhibition liver cell regeneration agent, aromatic amino acid, hydroxybenzene, indole and short chain fatty acids, and also can availably adsorb bilirubin in the blood[6]. Clinically resin is used more frequently than activated charcoal. Sorbents are macroreticular resins including uncharged resin and ion exchange resin. Although the adsorption capacity of resin is inferior to that of activated charcoal, it has a higher clearance rate towards all kinds of lipophilic and hydrophobic substances such as bile acid, bilirubin, free fatty acid and amine. Du *et al*[7] treated 2 cases of severe obstructive jaundice with carcinoma in pancreas head by HB-H-6 resin (Liver and Gall Disease Research Center, the Third Center Hospital of Tianjin, China) before surgery. TBIL, direct bilirubin (DBIL) and indirect bilirubin (IBIL) decreased more than 340 μmol/L, 230 μmol/L and 98 μmol/L, respectively. Bilirubin adsorption in the perfusion was at the highest speed from 15 to 45 min. HB-H-6 resin consisted of macroporous anion exchange resin and cation exchange resin. Anion adsorbed IBIL while cation adsorbed DBIL. Two patients were bridged successfully to surgery on the following day. It indicated that perfusion by HB-H-6 is safe and effective as a bridging option for malignant obstructive jaundice during the waiting time for transplantation. Zhang *et al*[8] identified bilirubin binding capacities of ten different kinds of resin (Tianjin Jinlin Biochemistry Development Company, China) and found that the clearance rates of both resins No.5 and No.9 towards DBIL were more than 70%; however, resin No.9 was superior to resin No.5 in regard to IDBIL. It indicated that No.9 had the best bilirubin removal efficiency among the ten kinds of resins, and No.5 was next to it. Resin No.9 had polyvinyl alcohol as a matrix and amino acid as a ligand, while No.5 was constituted by polystyrene resin and polar reactive groups coupled on it. Among the ten kinds of resin with pretreatment for the experiments of bilirubin adsorption in a solution, only one kind was selected, which had a higher capacity for bilirubin removal, constituted by hydrophilic crosslinked polyvinyl alcohol matrix, and amino acid as a ligand. The clearance rates of TBIL and DBIL in the solutions were 68.16% and 58% respectively, and those of TBIL and DBIL were 68% and 70% in the sera of patients. Furthermore, its blood compatibility was superior. In addition, resin could adsorb molecules with a molecular weight between 500 and 5000 Daltons, which covered the molecular weight of bilirubin, 548.67 Daltons. Accordingly, HP could treat hyperbilirubinemia effectively, and showed an applicable foreground[9].

### HD

As a safe and simple technique of blood purification, HD has been applied extensively in renal failure. Dialysis means the solute passing through a semipermeable membrane from high concentration to low in solution, which includes the movement of solute and water. A routine application of HD cannot effectively clear a high concentration of serum bilirubin. It has been reported that the clearance rate could be raised using plasma as the dialysate or associated with other purification methods. In the report by Liu *et al*[10], 21 cases of

hyperbilirubinemias received different alternatives: high volume hemofiltration, HE (HP200 blood purification equipment, YP-50PP membrane type plasma separator, Yatai Company, Ningbo, China; total amount of plasma exchange, 3000-3600 mL; plasma exchange rate, 25-30 mL/min; displacement with homeotype fresh refrigerated plasma), MARS (ALSS-MARS, Germany; blood flow, 150 mL/min; time of treatment, 6 h) and hemodialysis with plasma-based dialysate (HD-PBD) (AV600 filter, Diapact CRRT, CVVHD mode; blood flow, 250 mL/min). After treatment, the clearance rates of TBIL were 19.1%, 39.9%, 36.6% and 38.2%, respectively, while that of DBIL were 2.7%, 41.0%, 33.9% and 28.5%. The results indicated that there were no significant differences among HD-PBD, PE and MARS in removals for TBIL, DBIL, serum ammonia and bile acid. Lin *et al*[11] treated 26 severe hepatitis patients with a combination of PE and high-flow hemodiafiltration (HDF), there was obvious improvement of symptoms, with 49.46%, 48.77% and 47.98% decrease in levels of TBIL, DBIL and ammonia with low incidences of severe hemorrhage, shock or other complications. The common adverse effect was still hypersensitivity to plasma. You *et al*[12] treated 8 cases of severe hepatitis complicated with renal failure by a combined therapy of hemoperfusion and hemodialysis (330 mL HA blood perfusion equipment coupling F6 blood dialyzor; F-4008E Hemodialysis machine, Germany; carbonic acid dialysate; blood flow, 200-220 mL/min; dialysate flow, 500 mL/min; time of treatment, 4-5 h), and the patients had good tolerance with a decrease of TBIL by 15.6%-45.8% and no severe complications occurred.

#### *Molecular adsorbent recycling system (MARS)*

MARS is composed of an albumin recycling system, activated charcoal or resin, dialysis and so forth. It combines blood purification techniques of HD and a molecular adsorbent to selectively clear lipophilic albumin-bound toxin and hydrosoluble toxin. It can also adjust water and electrolyte disturbance, as well as acid-base disequilibrium. Yang *et al*[13] treated 30 patients with chronic severe type B hepatitis by MARS (MARS equipment, AKLIN Company, Germany; AK95 hemdialysis machine, Jinbo Company, China). The diagnosis of each patient was consistent with revised criteria at a Beijing academic conference in 1995, and their TBIL and DBIL were 369.77 ± 112.17 μmol/L and 114.75 ± 38.98 μmol/L, respectively. The levels lowered to 293.19 ± 99.16 μmol/L and 95.00 ± 31.41 μmol/L and symptoms of patients were improved after treatment of MARS. Chen *et al*[14] reported 28 patients with acute or chronic liver failure, who received 56 intermittent MARS treatments (Teraklin, Germany; time of therapy, 6-8 h) based on medicinal treatment, the TBIL, DBIL and total bile acid decreased by 31.47%, 29.15% and 35.91%, respectively after a single treatment of MARS, and the process was smooth except one ended having acute upper gastrointestinal bleeding; 8 cases of hypokalemia were corrected in time by infusing kalium intravenously. In Sun's study on treatment of 27 patients with severe hepatitis (the diagnosis was consistent with 2000CVHC) by MARS, he evaluated the degree of severity of disease by the MELD system[15,16], and reported that MARS could effectively alleviate hyperbilirubinemia, cure hepatorenal syndrome and decrease mortality obviously. Anyhow, MARS is an effective alternative for acute or chronic liver failure and their complications.

## COMPARISON AND SELECTION OF BLOOD PURIFICATION METHODS FOR REDUCING BILIRUBIN

The research of non-bioartifical liver is relatively mature and it is safe and easy to gain ground. Based on medicinal treatment, the hyperbilirubinemias caused by different pathogenic factors should be treated by different blood purification methods, or by a combination of these methods according to the accompanying symptoms, complications, concomitant diseases, existing equipment and conditions as well. Liu *et al*[1] treated 161 cases of severe hepatitis or liver failure with different blood purifications. The results indicated that PE(variant homeotype plasma exchange; 2000-2500 mL plasma; plasma segregating rate, 25-30 mL/min; blood flow, 30-40 mL/min), HP(microcapsule activated charcoal perfusion column; total time, 1.5-3 h) and MARS (NCU-11 blood dialysis machine and MARS mainframe, Germany) could reduce TBIL by 46.53%, 21.20% and 37.69% respectively, and blood ammonia by 19.04%, 15.35% and 44.13%. PE showed a superior ability in removing bilirubin, and at the same time it could supply albumin, coagulation factors and other biological active substances. MARS reduced blood ammonia effectively, so it would be a good option for patients complicated with hepatic encephalopathy to reduce icterus and correct hepatic coma simultaneously. Patients who had ascites and renal failure could be treated by HD. HP was able to correct alkalosis and hypokalemia, so it could be applied to patients with these complications. In addition, combination of MARS, PE, HP and HD can treat abdominal dropsy and renal inadequacy[11,12]. Recently it was reported that PE (SP1 or SP2, Fresenius Company), HP (BR-350, Asahi Company; Japan) and HD could be used in combination. Firstly, PE was performed (blood flow ⩽ 100 mL/min), subsequently bilirubin was adsorbed. Excessive water was dialysed out in those patients with complicating hepatorenal syndrome or being infused with redundant water during PE[17].

## RESEARCH FOCUS AND ADVANCEMENT OF BLOOD PURIFICATION FOR CLEARING BILIRUBIN

The study of bioartificial liver is a new hotspot recently. The so-called bioartificial liver is loaded with a combination of liver, tissues, cells and other things that are obtained from homologous or heterogeneous animals with special materials and equipment. It simulates the main functions of the liver: detoxification, synthesis and regulation[18]. Liver cells form a crucial part of BAL, and the cell type that can be used on humans is limited. It includes normal adult hepatocytes, fetus stem cells, liver tumor cells, swine hepatocytes, human stem cells,

reversible immortalized hepatocytes and so on[19]. Several liver support systems have been described, but no system has gained widespread clinical acceptance[20]. Chen *et al*[21] used a high concentration of L-02 human hepatocytes (Shanghai Institute of Biochemistry and Cell Biology, China) into hollow fiber bioreactor( YT-PP/F50 grate Ⅱ plasma separator, Yatai Company, Ningbo, China) and constituted extracorporeal BAL with another assisting circulation device. An extracorporeal-circulation test was performed later and the effect on bilirubin was observed simultaneously. The result showed that TBIL decreased by 35.78% after 4 h of treatment. Heterogenic or heterologous hepatocytes and tumor cells of liver cultured *in vitro* are used in BAL, so it has risks of xenogenic rejection, potential xeno-zoonosis and morbigenous. The hollow fiber bioreactor was also given attention because of its vital function for containing hepatocytes in BAL. The limitation of cultured cells substituting natural livers and the culture techniques, as well as the limitation of large-scale production, preservation and transportation of biologic materials make it difficult for BAL to be popularized clinically.

Recently, the combination of non-BAL and BAL was proposed, namely hybrid ALSS. It consisted of plasma separator, activated charcoal adsorber, level detector, attemperater and hollow fiber bioreactor and so on[22]. Li *et al*[23] used hybrid ALSS (Chinese experimented small porcine, Experiment Animal Research Center of Beijing Agriculture University; D-hanks perfusate and TECA- Ⅰ hollow fiber bioreactor, USA) to treat 15 chronic severe hepatitis patients (the diagnosis was consistent with 2000CVHC) and found that hybrid ALSS had an effect of reducing bilirubin. The TBIL decreased 240 μmol/L while DBIL decreased 110 μmol/L after treatment. Ten patients improved and were discharged from the hospital and one case was bridged to transplantation successfully and is living till now. Although 4 cases died, cure rate and survival rate of severe hepatitis had already increased without severe complications. Hybrid ALSS will be improved constantly with the development of ALSS. Consequently, for the time being the application of non-BAL is still the main alternative as a clinical treatment method for hyperbilirubinemia.

Though the technology of blood purification has improved gradually, a great deal of problems still exist. The main problems include low removal efficiency, long therapeutic time and expensive treatment costs. One key limitation of PE is bulk supplement of plasma and costly price, while the single application of HD is rare because of its low clearance. Further improvement and study on HP and MARS was brought into a focus by modified adsorbent materials. The ideal method of solving these vital problems is using the best membrane material and an optimally modified method, and increasing removal efficiency to the best advantage. Many scholars in the world are further groping for membrane modification for specific adsorption based on investigating bead affinity adsorbents. Yu *et al* immobilized guanidine on cross-linked polyvinyl alcohol gel, and coated cyclodextrin and glucose to polymer for adsorbing bilirubin in the blood[24-26]. At present, the most advanced affinity membrane is CA/PEI affinity membrane. The basic component of the membrane-cellulose acetate (CA) has favorable blood compatibility. Wei *et al*[27] used cellulose acetate as a compatible membrane matrix and made polylysine and quaternary ammonium salt as a ligand to prepare two kinds of modified affinity membranes after grafting. It was used to remove bilirubin from the HSA solution. Polyethyleneimine (PEI) has been widely used in the field of biochemistry and medicine now. CA/PEI membrane could be used directly to clear endotoxin or adsorb heavy metal[28,29], to prepare a metal chelating affinity membrane, to further covalently link other specific ligands on the membrane, and to work out a modified affinity membrane that could possess better adsorption performance for bilirubin in serum. Otherwise, favorable immune compatibility, i.e., ability to reduce immunoreaction was a precondition of the biomaterial being applied in clinics[30]. Now it is known that structure character of material is related to activating rejection of body fluid, cell and host[31]. Peng *et al*[32] evaluated the immunocompatibility of propylene-acidamide grafted polypropylene membrane (PP-g-AAm) *in vitro* on peripheral blood mononuclear cells (PBMCs) and confirmed that PP-g-AAm had preferable immunocompatibility.

Blood purification for hyperbilirubinemia is not an etiological therapy but strives for the opportunity to cure. So many patients can obtain surgery and liver transplantation finally. With the development of medicine, molecular biology, biochemistry and chemical engineering, blood purification will improve continuously and will make new breakthroughs for treatment of hyperbilirubinemia.

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*ESOPHAGEAL CANCER*

# Down-regulation of Bcl-$X_L$ by RNA interference suppresses cell growth and induces apoptosis in human esophageal cancer cells

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## Abstract

**AIM:** To determine the inhibitory effect of the vector-generated small interfering RNAs (siRNAs) on the expression of the Bcl-$X_L$ gene in established human esophageal cancer cells, and to investigate the effect of the Bcl-$X_L$ siRNAs on cell growth and apoptosis in esophageal cancer cells.

**METHODS:** Three siRNA-expressing vectors targeting different sites of the Bcl-$X_L$ gene were constructed from pTZ-U6+1 vector. Cultured esophageal cancer cells were transfected with the siRNA-expressing vector (or the control vector) using lipofectamine 2000. Bcl-$X_L$ gene expression was determined with semiquantitative RT-PCR assay and Western blotting. Among the three siRNA-expressing vectors, the most highly functional vector and its effect on cell growth and apoptosis in esophageal cancer cells was further analyzed.

**RESULTS:** Of the three siRNA-expressing vectors, siRNA-expressing vector No.1 was the most potent one which suppressed Bcl-$X_L$ mRNA production to 32.5% of that in the untreated esophageal cancer cells. Western blotting analysis showed that siRNA-expressing vector No.1 markedly down-regulated the expression of Bcl-$X_L$ in human esophageal cancer cells. Treatment of esophageal cancer cells with siRNA-expressing vector No.1 resulted in inhibition of cell growth and induction of apoptosis.

**CONCLUSION:** Down-regulation of Bcl-$X_L$ by vector-generated small interfering RNAs can suppress cell growth and induce apoptosis in human esophageal cancer cells.



**Key words:** Esophageal cancer; Bcl-$X_L$; RNA Interference; Apoptosis

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## INTRODUCTION

Esophageal cancer is one of the most common malignant tumors of mankind. About 300 000 people died of esophageal cancer each year in the world. The incidence and mortality of esophageal cancer are unusually high in China, especially in the areas of Henan, Shanxi, Hebei and Sichuan provinces[1]. Even though a small number of esophageal cancer patients survive longer than 5 years after initial surgical treatment, over 60% of patients still die of metastasis and local recurrence[2]. It is therefore imperative to investigate new therapeutic strategies in the treatment of esophageal cancer. Due to genetic abnormalities observed in esophageal cancer cells, the application of gene therapy has attracted the attention of many researchers. Activation of the cellular apoptotic program is a current strategy for the treatment of human cancer. It has been demonstrated that radiation and standard chemotherapeutic drugs kill some tumor cells through induction of apoptosis[3,4]. Upon apoptosis stimulation, several key events occur in mitochondria, including the release of cytochrome c. The mitochondrial cytochrome c release can be inhibited by expression of an antiapoptotic Bcl-2 family member (such as Bcl-2 or Bcl-$X_L$) and induced by expression of a proapoptotic member of the Bcl-2 family (such as Bax or Bid)[5]. In fact, an increased expression of Bcl-$X_L$ has been found in a variety of cancers[6-9]. In many neoplastic cells, high expression of Bcl-$X_L$ also correlates with resistance to conventional chemotherapy[10-13]. Bcl-$X_L$ is considered to be a highly promising molecular target to design new molecular targeted anticancer therapies.

Small interfering RNAs (siRNAs) are double-stranded RNA molecules that induce sequence-specific degradation of homologous single-stranded RNA. It has been verified as a powerful tool to knock down the expression of a

target protein in mammalian cells. siRNA technology has several major advantages over other posttranscriptional gene silencing techniques, such as antisense and gene knockout technology. It is easier to deliver, requires only small doses of siRNA to produce its silencing effect, and can inactivate a gene at almost any stage in development[14]. siRNA can be synthesized *in vitro*, but a specific gene silencing induced by synthetic siRNA might not be maintained long enough to achieve a phenotypic change[15]. In order to solve this problem some investigators have developed several vector-based expression systems to produce endogenous, functional siRNA molecules *in vivo*[16,17].

In this study, we present data showing that RNAi technology can be used to down-regulate Bcl-$X_L$ expression, resulting in suppression of cell growth and induction of apoptosis in esophageal cancer cells Eca109. It can be concluded that Bcl-$X_L$ is an alternative target in developing new therapeutic strategies for the treatment of esophageal cancers.

## MATERIALS AND METHODS

### Preparation of siRNA-expressing vector

siRNA-expressing vector was constructed from pTZ-U6+1 vector (Provided by Rossi JJ, Division of Molecular Biology, Beckman Research Institute of the City of Hope, Duarte, California 91010, USA) according to the instructions of the manufacturer. Briefly, a pair of oligonucleotides were synthesized *in vitro*. Each oligonucleotide contained a 21 nucleotide target sequence followed by a short loop sequence, the reverse complement of the target sequence, and five thymidines as a RNA polymerase Ⅲ transcriptional stop signal. The oligonucleotides were annealed in a buffer (potassium acetate 100 mmol/L, 30 mmol/L HEPES-KOH pH 7.4, and magnesium acetate 2 mmol/L) and the mixture was incubated at 95℃ for 5 min and then at 37℃ for 1 h. The double stranded oligos were cloned into the *Sal*Ⅰ and *Xba*Ⅰ sites of the pTZ-U6+1 vector where short hairpin RNAs were expressed under the control of the U6 promoter. The target sequences, corresponding oligonucleotides and resulting siRNA-expressing vectors in this study are shown in Table 1.

### Cell culture and transfection

The human esophageal cancer cell line Eca-109 was obtained from Shanghai Institute of Cell Biology, Chinese Academy of Sciences. Eca-109 cells were cultured in medium RPMI-1640 (Invitrogen) supplemented with 10% fetal bovine serum (FBS) and penicillin (100 kU/L) and streptomycin (100 mg/L) at 37℃ in a humidified incubator with 5% $CO_2$. For cell transfection, lipofectamine 2000 (Invitrogen) was used for transfecting the siRNA-expressing plasmids (or the control plasmids) following the manufacturer's instructions. The transfected cells were cultured for 5 h and then transferred to fresh medium with 10% FBS.

### Semiquantitative RT-PCR analysis for Bcl-$X_L$ gene expression

Cells were harvested 48 h after transfection. Total RNA was purified using the Total RNA Isolation System (Qiagen). Reverse transcription-PCR was performed with the isolated total RNA (100 ng) using the Omniscript RT kit and HotStarTaq PCR kit (Qiagen) according to the manufacturer's instructions. The primers were bcl-xl: 5'-GGCCTGAAGCCGGTGCAC -3', 5' -CACGGCGATACC GCTGGA -3'; β-actin: 5'-CTGG ATGCGATTCCAAGCAC-3', 5'-GAAGGACTTGGG ATCGTCCGG-3'. When RT-PCR was finished, 1 μL from the reaction mixture was withdrawn and analyzed by agarose gel electrophoresis followed by ethidium bromide staining. The 500 bp bcl-xl bands were cut from the gel and extracted using a DNA gel extaction kit (Qiagen). DNA concentration was determined using the GeneQuant *pro* RNA/DNA Calculator (Biochrom Ltd). Bcl-$X_L$ gene expression was calculated by dividing the concentration of the RT-PCR product of the treated cells by the concentration of the RT-PCR product of the untreated cells (taken as 100%). Each point represents the average of triplicate tests.

### Western blotting analysis

Seventy-two hours after the transfection, cells were washed twice in PBS and total protein was extracted in 150 mmol/L NaCl, 50 mmol/L Tris·HCl (pH 7.5), 1% sodium deoxycholate, 0.1% SDS, 1% Triton X-100, 5 mmol/L EDTA, 10 mg/mL leupeptin, 1% aprotinin and 2 mmol/L PMSF. Ten micrograms of protein sample was loaded onto a 10% SDS-PAGE and electroblotted onto a PVDF nylon membrane (Millipore, Bedford). Membranes were blocked in 0.05% Tween 20 (v/v) PBS containing 5% skim milk， and then incubated with rabbit polyclonal Bcl-$X_L$ antibodies and rabbit polyclonal β-Actin antibodies (Santa Cruz Biotechnology). Membranes were then incubated with a HRP-linked goat anti-rabbit IgG secondary antibody (Santa Cruz Biotechnology). Finally, the membrane was reacted with DAB reagent and washed with PBS once protein bands had appeared.

### Cell growth assay

Untreated cells or the transfected cells were harvested and reseeded at $1 \times 10^4$ cells/well in a 12-well, flat-bottomed plate. Cells were cultivated with RPMI 1640 medium in the $CO_2$ incubator at 37℃. The total cell number was determined every two days with a hematocytometer and under an inverted microscope (Olympus). Cell viability was determined by trypan blue staining. Each value represents the average of triplicate wells.

### Analysis of apoptosis

Cell apoptosis was assessed under an inverted fluorescence microscope (Olympus).The green fluorescent DNA intercalant dye YO-PRO-1 was purchased from Invitrogen Company. Dual staining with YO-PRO and PI made it possible to detect early apoptotic cells that have undergone initial changes in permeability to small molecules. At 72 h post cell transfection, YO-PRO-1 dyes were added to the culture medium at 0.1 μmol/L for 30 min. Cell apoptosis was quantified by determining the percentage of cells that were YO-PRO-1-positive in every 200 cells. Each value represents the average of triplicate wells.

**Table 1 Target sequences, corresponding oligonucleotides and resulting siRNA-expressing vectors in this study**

| Target sequences and corresponding oligonucleotides | siRNA-expressing vector |
|---|---|
| Target 1: 5′-ggaagagaacaggactgaggc-3′ (Target site: 90-110) | siRNA- expressing vector No.1 |
| Oligo 1 F: 5′-tcgaggaagagaacaggactgaggcttcaagagagcctcagtcctgttctcttcctttttt-3′ | |
| Oligo 1 R: 5′-ctagaaaaaggaagagaacaggactgaggctctcttgaagcctcagtcctgttctcttcc-3′ | |
| Target 2 :5′- gaacaggtagtgaatgaactc-3′(Target site: 370-390) | siRNA- expressing vector No.2 |
| Oligo 2 F: 5′-tcgagaacaggtagtgaatgaactcttcaagagagagttcattcactacctgttctttttt-3′ | |
| Oligo 2 R: 5′-ctagaaaaagaacaggtagtgaatgaactctctcttgaagagttcattcactacctgttc-3′ | |
| Target 3: 5′- gaacgcttcaaccgctggttc-3′(Target site: 622-642) | siRNA-expressing vector No.3 |
| Oligo3 F: 5′-tcgagaacgcttcaaccgctggttcttcaagagagaaccagcggttgaagcgttctttttt-3′ | |
| Oligo 3 R: 5′-ctagaaaaagaacgcttcaaccgctggttctctcttgaagaaccagcggttgaagcgttc-3′ | |
| Control: 5′-gaggaccgttactagatcata-3′ | Control siRNA- expressing vector |
| Oligo F: 5′-tcgagaggaccgttactagatcatattcaagagatatgatctagtaacggtcctctttttt-3′ | |
| Oligo R: 5′-ctagaaaaagaggaccgttactagatcatatctcttgaatatgatctagtaacggtcctc-3′ | |

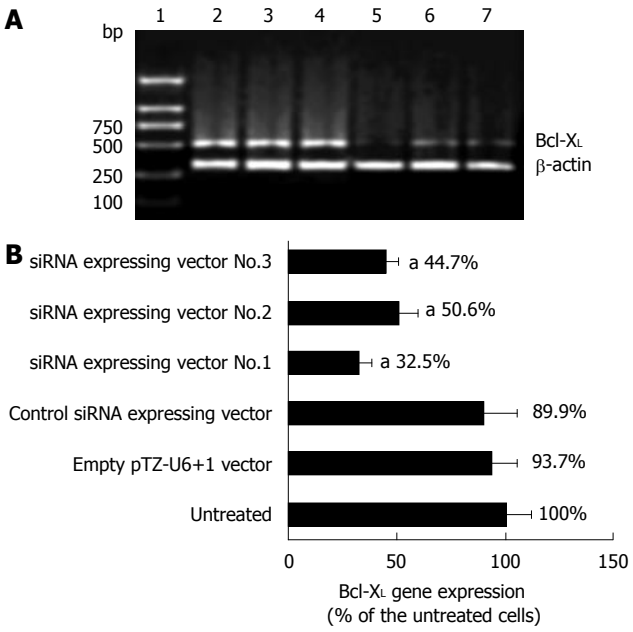


**Figure 1** RT-PCR analysis of the effect of siRNA-expressing vector on Bcl-XL gene expression. **A:** Argarose gel electrophoresis of the RT-PCR products. L1: DNA marker; L2: RT-PCR product of the untreated cells; L3: RT-PCR product of the empty pTZ-U6+1 vector treated cells; L4: RT-PCR product of the control siRNA-expressing vector treated cells; L5: RT-PCR product of the siRNA-expressing vector No.1 treated cells; L6: RT-PCR product of the siRNA-expressing vector No.2 treated cells; L7: RT-PCR product of the siRNA-expressing vector No.3 treated cells. **B:** Quantification of the RT-PCR products (mean ± SD, $n = 3$, $^aP < 0.05$ *vs* untreated esophageal cancer cells).

### *Statistical analysis*

Statistical analysis was performed by the Student's $t$ test or $\chi^2$ test. $P < 0.05$ was considered statistically significant. Data are expressed as mean ± SD. All statistical calculations were performed using SPSS10.0 statistical software package.

## RESULTS

### *siRNA-expressing vector inhibiting Bcl-XL mRNA expression*

We examined three siRNA-expressing vectors which target human Bcl-XL as shown in Figure 1A. Of the three siRNA-expressing vectors, siRNA-expressing vector No.1 potently suppressed the synthesis of Bcl-XL mRNA in human esophageal cancer cells. RT-PCR product quantification showed that siRNA expressing vector No.1 suppressed Bcl-XL mRNA production to 32.5% of that in the control as shown in Figure 1B. On the basis of these results, we selected siRNA-expressing vector No.1 as the most highly functional siRNA-expressing vector in further studies.

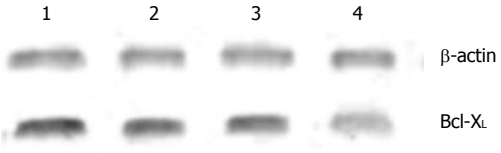


**Figure 2** Western blotting analysis and effect of siRNA-expressing vectors. L1: Untreated esophageal cancer cells; L2: Esophageal cancer cells were transfected with empty pTZ-U6+1 vector; L3: Esophageal cancer cells were transfected with control siRNA expressing vector; L4: Esophageal cancer cells were transfected with siRNA-expressing vector No.1.

### *Effect of siRNA-expressing vector on Bcl-XL protein expression*

We evaluated the effect of siRNA-expressing vector No.1 on target protein Bcl-XL by Western blotting analysis. Figure 2 shows that siRNA-expressing vector No.1 markedly down-regulated the expression of Bcl-XL in human esophageal cancer cells as compared with untreated cells. However, treatment with control siRNA-expressing vector and empty pTZ-U6+1 vector did not change the expression of Bcl-XL in human esophageal cancer cells as compared with untreated cells.

### *Effect of siRNA-expressing vector on cell proliferation*

We have analyzed siRNA-expressing vector No.1 on the cell proliferation of Eca-109 esophageal cancer cells. Figure 3 shows that siRNA-expressing vector No.1 target Bcl-XL significantly inhibited the cell growth in esophageal cancer cells as compared with the untreated cells ($P < 0.05$). Cells treated with control siRNA expressing vector and empty pTZ-U6+1 vector showed only slight cell growth inhibition and had no difference as compared with the untreated cells.

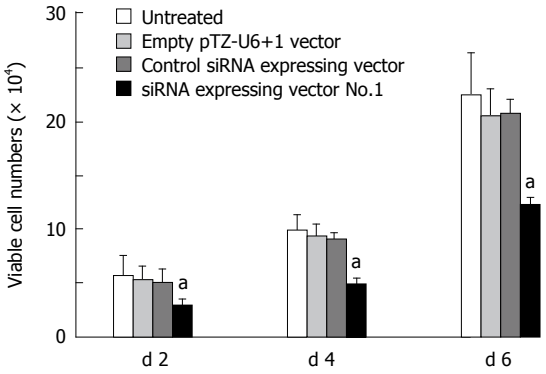


**Figure 3** Effect of siRNA-expressing vector on cell growth post transfection (mean ± SD, $n = 3$, $^aP < 0.05$ *vs* untreated esophageal cancer cells).

### *Bcl-X$_L$ siRNA-expressing vector potentiating apoptosis of esophageal cancer cells*

YO-PRO-1 is a membrane-impermeable DNA-binding dye and is generally excluded from variable cells, whereas early-stage apoptotic cells are YO-PRO-1-positive. Typical photographs of YO-PRO-1 staining are shown in Figure 4A. The nucleus of early-stage apoptotic cell was stained green under the inverted fluorescence microscope. Figure 4B shows that the apoptotic cells significantly increased in siRNA-expressing vector No.1 treated cells compared to untreated cells ($P < 0.05$). However, treatment with control siRNA expressing vector and empty pTZ-U6+1 vector did not significantly affect the apoptosis of esophageal cancer cells.

## DISCUSSION

Members of the Bcl-2 family of proteins play important roles in regulating cell survival and apoptosis. The Bcl-2 family includes pro-apoptotic members and antiapoptotic proteins such as Bcl-2 and Bcl-X$_L$ that inhibit apoptosis by blocking the release of cytochrome C. Bcl-X$_L$ is over-expressed in numerous types of cancer including myelomas, lymphomas, hepatomas, gastric carcinomas and ovarian cancers[6,7,9,18,19]. This over-expression of Bcl-X$_L$ is associated with decreased apoptosis in tumors, resistance to chemotherapeutic drugs and a poor clinical outcome. In esophageal cancer, some investigators observed that Bcl-X$_L$ expression correlated well with depth of tumor invasion, lymphatic invasion, and lymph node metastasis in superficial squamous cell carcinoma of the esophagus. Patients with high Bcl-X$_L$ expression showed significantly shorter survival than those with low Bcl-X$_L$ expression[20,21]. However, the precise role of Bcl-X$_L$ in the development of esophageal cancer remains to be elucidated. Thus, the performance to specifically reduce Bcl-X$_L$ level by genetic means in established esophageal cancer cell lines should be helpful for a better understanding of its role in maintaining the malignant phenotype. Several approaches have been developed to inhibit the function of Bcl-X$_L$ including antisense, peptide nucleic acid, small organic compounds[22-24]. Most of these antagonists of Bcl-X$_L$ were reported to elicit spontaneous apoptosis of cancer cells *in vivo* as well as *in vitro* and to enhance the sensitivity to chemotherapy in cancer cells. Bcl-X$_L$ has been successfully

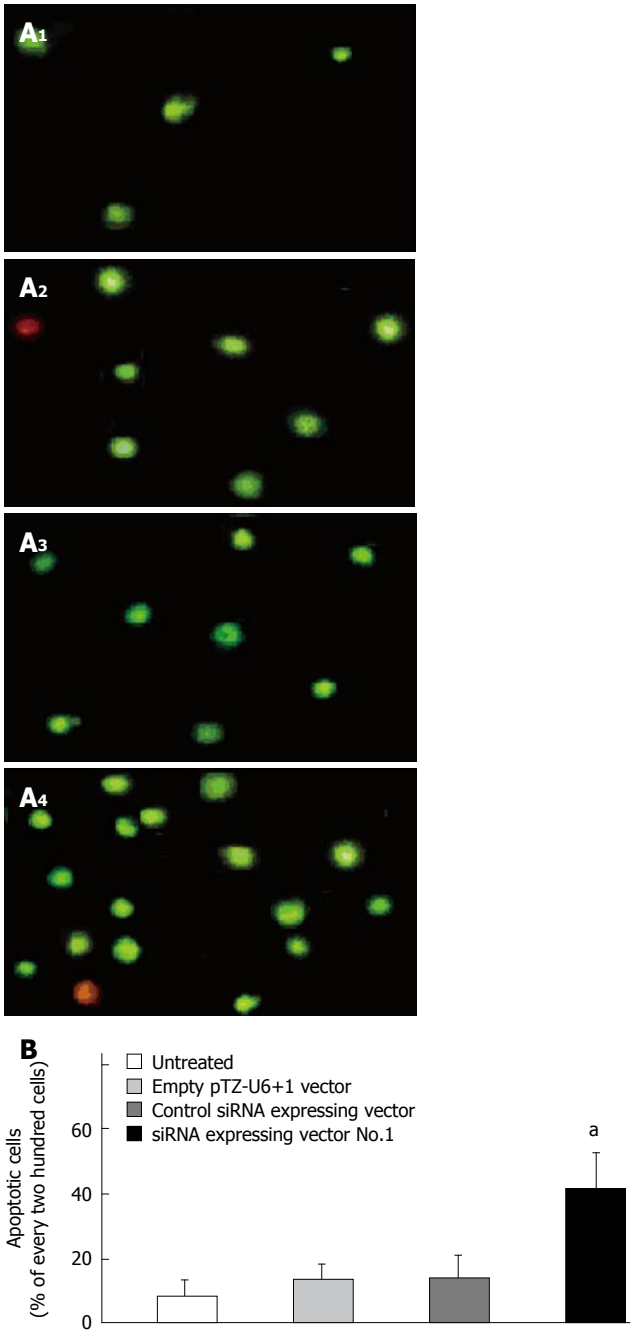


**Figure 4** Effect of siRNA-expressing vector targeting Bcl-X$_L$ on apoptosis of esophageal cancer cells. **A**: Photographs of YO-PRO-1 staining under the inverted fluorescence microscope at 72 h after transfection. 1: Untreated esophageal cancer cells; 2: Esophageal cancer cells were transfected with empty pTZ-U6+1 vector; 3: Esophageal cancer cells were transfected with control siRNA-expressing vector; 4: Esophageal cancer cells were transfected with siRNA-expressing vector No.1. **B**: Percentage of apoptotic cells in every 200 cells (mean ± SD, $n = 3$, $^aP < 0.05$ *vs* untreated cells).

down-regulated by RNAi in some previous studies, but the down-regulation of Bcl-X$_L$ expression by siRNA-expressing vector or other approaches in esophageal squamous cell carcinoma has not been performed until now.

In this study, we constructed three siRNA expressing vectors targetting human Bcl-X$_L$. Down-regulation of Bcl-X$_L$ gene expression was observed in the esophageal carcinoma cell line Eca109 transfected with these three siRNA-expressing vectors. Of the three siRNA-expressing

vectors, siRNA-expressing vector No.1 potently suppressed the synthesis of Bcl-$X_L$ mRNA in semiquantitative RT-PCR assay. RT-PCR product quantification showed that siRNA-expressing vector No.1 suppressed Bcl-$X_L$ mRNA production to 32.5% of that in the untreated esophageal cancer cells. Western blotting analysis showed that siRNA-expressing vector No.1 decreased the synthesis of Bcl-$X_L$ protein in esophageal cancer cells. To further investigate the role of Bcl-$X_L$ in the pathogenesis of esophageal cancer, cell growth and apoptosis were analyzed to determine the functional consequence of the siRNA-expressing vectors mediated decrease of Bcl-$X_L$ in established esophageal cancer cells. Our data showed that knockdown of Bcl-$X_L$ by siRNA-expressing vector suppressed cell growth and potentiated apopotosis in established esophageal cancer cells in a stable manner. These results were consistent with previous reports, Zhu *et al*[25] found that knockdown of Bcl-$X_L$ protein expression by small interfering RNA inhibited the proliferation of 5-FU-resistant human colon cancer cells. Lei X *et al*[26] demonstrated that siRNA targeting Bcl-$X_L$ genes specifically suppressed Bcl-$X_L$ expression and increased spontaneous apoptosis in the human gastric cancer cell line MGC-803. All these investigations suggest that Bcl-$X_L$ may serve as a potential target in cancer therapy. In summary, our study indicates that down-regulation of Bcl-$X_L$ by siRNA-expressing vectors can suppress cell growth and induce apoptosis in human esophageal cancer cells and siRNA technique may provide a novel therapeutic approach in the treatment of human esophageal cancer.

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*GASTRIC CANCER*

# Phosphorylated vasodilator-stimulated phosphoprotein is localized on mitotic spindles of the gastric cancer cell line SGC-7901

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## Abstract

**AIM:** To elucidate the localization of vasodilator stimulated phosphoprotein (VASP), a cytoskeletal organizing protein and a substrate of protein kinases A and G in mitotic gastric cancer cells.

**METHODS:** Immunofluorescence microscopy was used to observe the localization of α-tubulin, VASP and Ser157 phosphorylated VASP (p-VASP) in interphase of mitotic gastric cancer of the cell line SGC-7901.

**RESULTS:** Immunofluorescence staining showed that p-VASP but not VASP was co-localized with α-tubulin on spindle poles and fibers in prophase, metaphase and anaphase of the mitotic process of the gastric cancer cell line SGC-7901. H89, an inhibitor of protein kinases A and G, had no effect on the localization of p-VASP on the spindles.

**CONCLUSION:** VASP may play a role in assembling and stabilizing the mitotic spindle of cells, and phosphorylation of the protein is the precondition for it to exert this function.



**Key words**: Phosphorylated vasodilator-stimulated phosphoprotein; Localization; Mitotic spindle; Gastric cancer cell

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## INTRODUCTION

The spindle is a vital apparatus of mitotic cells. The mechanism of its formation has attracted great research attention. In centrosome-containing cells, centrosomes are thought to be instrumental for organization of the spindle poles and determination of both the microtubule polarity and the spindle axis[1]. The spindle poles often contain widely different amounts of known spindle-pole components, such as γ-tubulin. At the beginning of mitosis, γ-tubulin is recruited to the poles and forms a γ-tubulin ring complex (γTuRC), which can collect both α and β tubulins to form spindle fibers[2,3]. At the same time, accessory proteins also join the process. One of these types is microtubule-associated proteins (MAPs). Three major neural MAPs (MAP1, MAP2 and tau) and one non-neural MAP (MAP4) have been identified and well characterized at the biochemical level[4,5]. These MAPs possess the ability to stimulate tubulin polymerization and to bind to microtubules *in vitro* and are believed to play an important part in the regulation of microtubule formation and stabilization *in vivo*[6].

Vasodilator-stimulated phosphoprotein (VASP) is a proline-rich founding member of the Ena (drosophila enabled)/VASP family, a group of multifunctional proteins associated with regulation of actin. VASP was first found in platelets and is known to be a substrate for cAMP dependent protein kinase (PKA) and cGMP dependent protein kinase (PKG) in a variety of cells, such as vascular smooth muscle cells and endothelial cells[7,8]. It is also known to be actin cytoskeletal regulatory proteins. In a variety of cells like vascular endothelial cells, platelets, smooth muscle cells, and fibroblasts, VASP has been found to be highly expressed where it can be found in focal adhesions, along stress fibers, and in the areas with highly dynamic membrane activity, such as extending lamellipodia and filopodia[9-12]. *In vivo* evidence indicates that VASP is a crucial factor in the formation of actin filaments and the integration of signals transmitted between

cytoskeleton, cytoskeleton-membrane interface and the two cyclic nucleotide signal transduction pathways[9]. Even though increasing data address the importance of VASP, the biological role of this protein still remains largely unknown. We have recently found that phosphorylated VASP (p-VASP) is co-located with α-tubulin on the spindles of mitotic cells, indicating that this cytoskeletal organizing protein may also have a role in assembling and stabilizing the mitotic spindles.

## MATERIALS AND METHODS

### Cell line

Human gastric epithelial cell line SGC-7901 was provided by Institute of Cell Biology of China (Shanghai, China).

### Reagents

Dulbeccos's modified Eagle's medium (DMEM) was from GiBco (Grand Island, NY). Newborn calf serum (NBCS) was from Minhai Bio-engineering Co., LTD (Lanzhou, China). Antibodies against VASP (Cat. No. sc-46668) and phosphorylated VASP (Cat. No. sc-23506-R) were from Santa Cruz Biotechnology (Santa Cruz, CA). Antibody against α-tubulin (BM1452) was from Wuhan Boster Biological Technology Ltd (Wuhan, China). FITC- and TRITC- conjugated secondary antibodies were from Jackson ImmunoResearch Laboratories (West Grove, PA). Protein kinase A inhibitor H89 (Cat. No. 371963) was from Calbiochem (San Diego, CA). Nuclear fluorochrome Hoechst 33342 was from Sigma (St. Louis, Missouri).

### Immunofluorescent microscopy

The cells grown on cover slips were incubated to reveal nuclei with 0.2 μmol/L Hoechst 33 342 for 10 min. Then, the cells were fixed with freshly prepared 40 g/L paraformaldehyde in PBS at 4℃ overnight (o/n). After being penetrated with 30 mL/L Triton × 100 and blocked with 30 g/L BSA, the cells were incubated with primary antibodies at 4℃ o/n, and then with FITC- or TRITC- conjugated secondary antibodies for 1 h at room temperature (RT), and washed three times after each time of incubation. For immunofluorescence co-staining of α-tubulin and p-VASP, the cells on cover slips were subsequently incubated with mouse monoclonal antibody against α-tubulin at 4℃ o/n, rabbit polyclonal antibody against p-VASP for 2 h at RT, goat anti-mouse IgG conjugated with FITC for 1 h at RT, and finally TRITC-conjugated goat anti-rabbit IgG for 1 h at RT, and washed three times after each time of incubation. The cover slips were mounted in 900 mL/L glycerol and 100 mL/L Tris-HCl (25 mmol/L, pH 7.5). The fluorescence of FITC (green), TRITC (red), and Hoechst 33 342 (blue) was observed under a fluorescence microscope with a CCD camera (Leica).

## RESULTS

### Distribution of p-VASP and α-tubulin in interphase of SGC-7901 cells

Immunofluorescence staining showed that both p-VASP and α-tubulin were mainly distributed in the cytoplasm of the cells in interphase (Figure 1A and B)

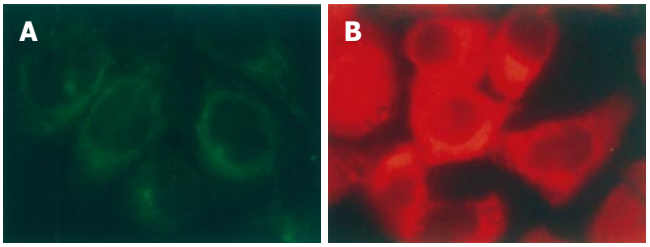


**Figure 1** Distribution of α-tubulin (**A**) and p-VASP (**B**) in interphase of SGC-7901 cells (immunofluorescent staining × 200). α-tubulin was stained with monoclonal antibody against the protein. p-VASP was stained with polyclonal antibody specifically against VASP phosphorylated at Ser157. Both α-tubulin and p-VASP were located in cytoplasm of the cells.

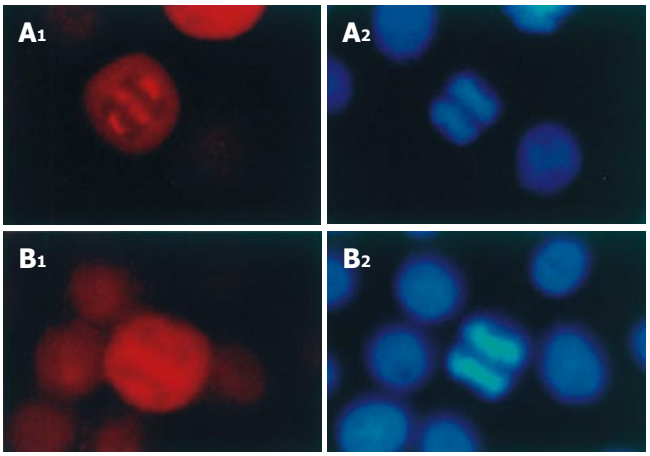


**Figure 2** Distribution of p-VASP (**A**) and VASP (**B**) in mitotic SGC-7901 cells (× 200). p-VASP and VASP in mitotic SGC-7901 cells were immunofluorescently stained with polyclonal and monoclonal antibodies respectively. The chromosomes were stained with Hoechst 33342.

### p-VASP but not VASP was located with α-tubulin on the spindle of mitotic cells

Immunofluorescence staining showed that p-VASP but not VASP was localized both on the spindle poles and on the spindle fibers of mitotic cells (Figure 2A and B). In prophase, the chromosomes became condensed with formation of p-VASP-containing spindle poles (Figure 3A). In metaphase, the chromosomes converged at the metaphase plate (Figure 3B) and the p-VASP-containing spindle poles were located at the opposite sides of chromosomes and the p-VASP-containing spindle fibers irradiated to the chromosomes (Figure 3B). In anaphase, daughter chromosomes were separated and moved toward the opposite p-VASP-containing spindle poles (Figure 3C). Both p-VASP-containing spindle poles and fibers could be seen in this phase (Figure 3C). In telophase, p-VASP-containing spindle disappeared and new daughter nuclei formed (Figure 3D). However, p-VASP could be detected at the cleavage furrow between two separating cells (Figure 3D). Immunofluorescence co-staining showed that α-tubulin was co-localized with p-VASP throughout all phases of mitosis (Figure 3A-D).

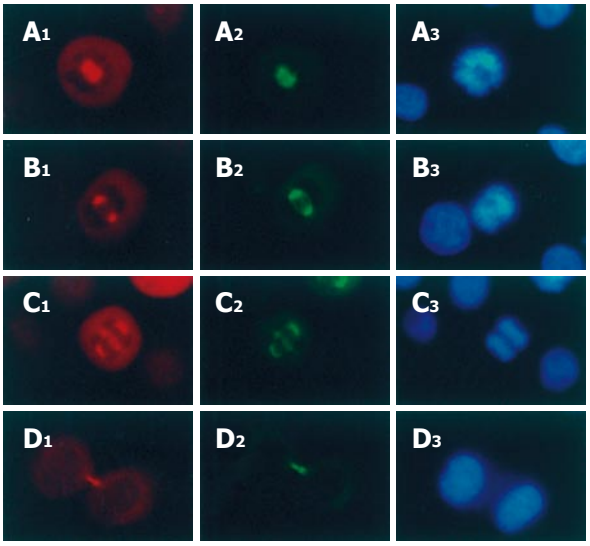


**Figure 3** p-VASP co-localization with α-tubulin and chromosomes on spindles of mitotic SGC-7901 cells (× 200). p-VASP and α-tubulin were stained with polyclonal and monoclonal antibodies respectively. **A1-D1**: p-VASP localization in prophase, metaphase, anaphase and telophase respectively; **A2-D2**: α-tubulin localization in the same phases; **A3-D3**: chromosomes of the cells were stained with Hoechst 33342.

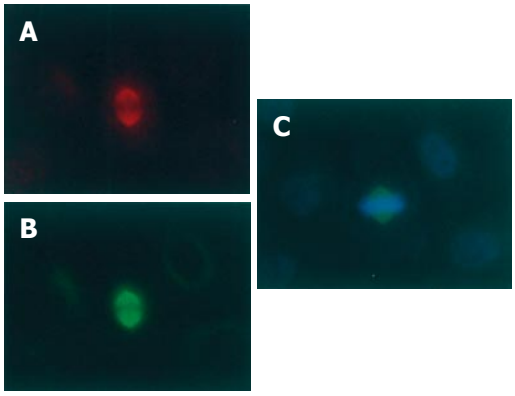


**Figure 4** No effect of PKA and PKG inhibitor H89 on location of p-VASP on spindles of mitotic SGC-7901 cells (× 200). SGC-7901 cells were treated with H89 (10 μmol/L) for 6 h and immunofluorescently stained for p-VASP (**A**) and α-tubulin (**B**). The chromosomes were stained with Hoechst 33342 (**C**).

### *PKA and PKG inhibitor had no effect on the distribution of p-VASP in mitotic spindle*

H89, an inhibitor of PKA and PKG, had no obvious effect on the localization of p-VASP on the mitotic spindle. Immunofluorescent staining of p-VASP could still be seen on the spindles of mitotic cells treated with H89 (10 μmol/L) for different periods of time (Figure 4).

## DISCUSSION

Mitosis is an important event of the cell cycle. During this process, duplicated DNA genetic material is condensed to chromosomes moving into two daughter cells with high fidelity. In eukaryotic cells, the accurate segregation of chromosomes during cell division occurs on the spindle. The formation of the spindle is a complicated process that requires the participation of several types of proteins such as tubulins, spindle-microtubule motor proteins, and microtubule-associated proteins (MAPs)[13,14]. Even though accumulating data address the dynamic process of spindle formation, the exact mechanism and process still need to be further clarified.

In this experiment, we showed for the first time that p-VASP was localized both on spindle poles and on spindle fibers of the mitotic cells. The localization of p-VASP exerted a dynamic change associated with stages of the cell cycle. In interphase, p-VASP was detected in the cytoplasm. In mitotic cells, p-VASP was localized on the mitotic spindle from prophase to anaphase. In prophase, p-VASP was localized on the poles of the spindle. p-VASP could be detected on both the poles and fibers of the spindles from metaphase to anaphase. The localization of p-VASP in mitotic cells was exactly coincident with the localization of alpha tubulin, suggesting that p-VASP might play a role in the formation of mitotic spindles, which is associated with tubulin.

Microtubule-associated proteins (MAPs) play an important role in the formation of spindles, and can bind to tubulin and assist the assembling and stabilizing of spindles. The microtubule-binding domains of MAPs are divided into three sub-domains: a proline-rich region, an AP sequence region and a short hydrophobic tail[4]. The importance of the proline-rich region in *in vitro* MAP-induced microtubule assembly has been widely recognized[15-18]. Tokuraku *et al*[6] showed that this proline-rich region plays a new role as the protofilament linker in *in vitro* MAP-induced microtubule assembly. Since microtubules consist of tubulin as its major component, the proline-rich region of MAPs may be one of the structural features that permit the protein be able to bind to tubulin. Interestingly, VASP also has a proline-rich region in its structure. This protein is organized in three domains, an Ena-VASP-homology 1 (EVH1) $NH_2$-terminal domain, a proline-rich central domain, and an EVH2 COOH-terminal domain. The proline-rich region comprises a $(GP_5)_3$ sequence presenting as a single or repeated motif in different proteins. It has been confirmed that profilin binds to VASP *in vitro* and cell extracts probably *via* the $(GP_5)_3$ repeats[19]. Considering this structural feature of VASP, it is reasonable to speculate that p-VASP may be able to bind to tubulin and the possible function of the p-VASP-tubulin binding is to stimulate the polymerization of tubulin on spindle fibers and increase the stability of microtubules of the spindles, a role similar to that of MAPs.

Research data indicate that phosphorylation is necessary for the accessory proteins to exert their role in forming and stabilizing the spindles. For example, mutations that cause loss of function of the protein kinase aurora prevent centrosome separation in Drosophila[20], while phosphorylation of Eg5 has been shown to be necessary for its localization on spindle poles[21]. Since our results showed that p-VASP was a form of this protein detected on spindles, it is possible that phosphorylation is the precondition for VASP to exert its role in spindle formation and stabilization. VASP has a peptide chain containing 380 amino acid residues. Three of them are known as phosphorylation sites, of which two

serine residues are Ser157 and Ser239 respectively and the threonine residue is Thr278. Phosphorylation of VASP at these sites is directly regulated by PKA and PKG, and VASP has also been shown to be directly phosphorylated on Ser157 by protein kinase C (PKC)[22]. It has been confirmed that VASP phosphorylation is a modulatory event for VASP-dependent regulation of the actin cytoskeleton. The phosphorylation can affect the cytoskeleton structure and may induce morphological changes of cells[23-25]. Research data also indicate that phosphorylation of VASP on different amino acid residues has different functions. For example, serum-induced VASP phosphorylation at serine157 by both PKA and PKC promotes smooth muscle cell (SMC) proliferation, whereas VASP phosphorylation at serine239 induced by nitric oxide (NO)/PKG is required to induce the inhibitory effects of NO on SMC proliferation[26]. One interesting phenomenon observed in our experiment was that PKA (also PKG) inhibitor H89 could not prevent the localization of p-VASP on the mitotic spindles, suggesting that VASP on the spindle is not the substrate of PKA and PKG.

In conclusion, p-VASP might play a role in mitotic spindle formation and stabilization, which implies a potential target for controlling the division and proliferation of gastric cancer cells. The exact mechanism and which protein kinase is responsible for the phosphorylation of VASP on the mitotic spindle need to be further investigated.

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*COLORECTAL CANCER*

# Effects of gastrin 17 on β-catenin/Tcf-4 pathway in Colo320WT colon cancer cells

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## Abstract

**AIM:** To explore the effect of gastrin 17 (G17) on β-catenin/T cell factor-4 (Tcf-4) signaling in colonic cancer cell line Colo320WT.

**METHODS:** The pCR3.1/GR plasmid, which expresses gastrin receptor, cholecystokinin-2 receptor (CCK-2R), was transfected into a colonic cancer cell line Colo320 by Lipofectamine ™2000 and the stably expressing CCK-2R clones were screened by G418. The expression levels of gastrin receptor in the Colo320 and the transfected Colo320WT cell line were assayed by RT-PCR. Colo320WT cells were treated with G17 in a time-dependent manner (0, 1, 6, 12, 24 and 48 h), then with L365,260 ($Gastrin_{17}$ receptor blocker) for 30 min, and with G17 again for 12 h or L365,260 for 12 h. Expression levels of β-catenin in a TX-100 soluble fraction and TX-100 insoluble fraction of Colo320WT cells treated with G17 were detected by co-immuniprecipation and Western blot. Immunocytochemistry was used to examine the distribution of β-catenin in CoLoWT320 cells. Expression levels of c-myc and cyclin D1 in Colo320WT cells treated with G17 were assayed by Western blot.

**RESULTS:** Expression levels of β-catenin in the TX-100 solution fraction decreased apparently in a time-dependent fashion and reached the highest level after G17 treatment for 12 h, while expression levels of β-catenin in the TX-100 insoluble fraction were just on the contrary. Immunocytochemistry showed that β-catenin was translocated from the cell membranes into the cytoplasm and nucleus under G17 treatment. Expression levels of c-myc and cyclin D1 in the G17-treated Colo320WT cells were markedly higher compared to the untreated Colo320WT cells. In addition, the aforementioned G17-stimulated responses were blocked by L365,260.

**CONCLUSION:** Gastrin17 activates β-catenin/Tcf-4 signaling in Colo320WT cells, thereby leading to over-expression of c-myc and cyclin D1.



**Key words:** Gastrin17; Cholecystokinin-2 receptor; Colorectal carcinoma; β-catenin/Tcf-4 pathway

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## INTRODUCTION

Gastrin is a hormone produced by G-cells in the normal gastric antrum. As a peptide hormone and trophic factor, in addition to regulating gastric acid secretion, gastrin exerts a growth-promoting action on gastrointestinal malignancy[1,2]. And there is substantial evidence that gastrin can stimulate the growth and proliferation of some colorectal cancers *in vivo* and *in vitro*[3-6]. Colorectal carcinoma cells may also aberrantly produce gastrin. Thus, gastrin may act as an autocrine/paracrine or endocrine factor in initiation and progression of colorectal carcinoma[1]. As yet, some research has shown that gastrin exerts its effect in promoting proliferation and growth by binding its receptor CCK-2[7-11]. We have demonstrated that G17 can cause tyrosine phosphorylation of focal adhesion kinase (FAK), paxillin, and $p130^{Cas}$ in human colon cancer cells. We have also confirmed that G17 may promote colon cancer cell invasion and metastasis by phosphorylating FAKTyr397 and activating FAK pathway[12,13].

The β-catenin/Tcf-4 signaling pathway plays a critical role in gastrointestinal malignancy. As an effector to transmit a receptor-mediated signal from cytosol to the nucleus in the β-catenin/Tcf-4 pathway, β-catenin interacts with and activates the Tcf/Lef transcription factor in the nucleus. The activation of β-catenin/Tcf-4 transcriptional complex can result in expression of multiple target genes,

such as c-myc, cyclin D1 and matrilysin, which induce tumor cells invasion and metastasis[14-18]. However, till now, little research has been carried out on whether gastrin exerts its effects on β-catenin/Tcf-4 pathway in colorectal carcinoma cells. We, therefore, aimed to explore the effects of gastrin on β-catenin/Tcf-4 pathway in human colorectal carcinoma cells.

## MATERIALS AND METHODS

### Materials

Gastrin-17 amide was purchased from Sigma. The gastrin receptor antagonist L-365,260 and eukaryotic expression vector pCR3.1/GR were kindly provided by St. Josef-Hospital, Ruhr-University Bochum, Germany. Mouse monoclonal antibodies for β-catenin (E-5), E-cadherin (G10), c-myc (9E10), cyclin D1 (A-12) and enhanced chemoluminescence (ECL) reagents were purchased from Santa Cruz Biotechnology. Mouse polyclonal antibodies for Tcf-4 (6H5-3) were obtained from Upstate Biotechnology. Protein G Sepharose 4 Fast Flow and nitrocellulose membranes were obtained from Amersham Pharmacia. Horseradish peroxidase-conjugated anti-mouse secondary antibody was obtained from PIERCR.

### Cell culture

Culture of the human colon cancer cell lines Colo320 (ACTCC) was maintained at 37℃ in RPMI-1640 medium, supplemented with 100 mL/L fetal bovine serum (FBS) in a humidified atmosphere containing 50 mL/L $CO_2$ and 950 mL/L air. For experimental purposes, cells were plated in 35-mm dishes at a density of $1 \times 10^5$ cells per dish and grown in RPMI 1640 medium containing 100 mL/L FBS for 5-7 d.

### Stable transfection

Colo320 cells were stably transfected with wild-type CCK2 receptor cDNA cloned into the eukaryotic expression vector pCR3.1/GR using the Lipofectamine$^{TM}$2000 according to the manufacturer's instructions. Following transfection, cells were seeded at very low density to obtain a single cell in an individual well of 96-well plates and further expanded in the presence of 500 mg/mL G418. G418-resistant clones were screened for CCK2 receptor expression by RT-PCR.

### RNA extraction and reverse transcription polymerase chain reaction

Total RNA was extracted from Colo320 and Colo320WT cells by using Trizol reagent. The first strand cDNA was synthesized from 1 μg of total RNA using murine moloney leukemia virus (MuMLV) reverse transcriptase and the first strand cDNA synthesis kit (MBI Fermants) in a total volume of 20 μL. One microliter of each product was subjected to PCR for 30 cycles, each amplification cycle consisting of denaturation at 95℃ for 30 s, primers annealing at 60℃ for 45 s, and extension at 72℃ for 60 s. The primers used were as follows: CCK2, 5'-GTGACAG CGACAGCCAAAGCAG-3' (sense) and 5'-CGAGGCGT AGCTCAGCAAGTGA-3' (antisense); β-actin, 5'-CGAC GGGAAATCGTGCGTGACATTAAGGAGA-3' (sense) and 5'-CGTCATACTCCTGCTTGCTGATCCACATCT GC-3' (antisense). The PCR products were visualized on ethidium bromide-stained 7 g/L agarose gels.

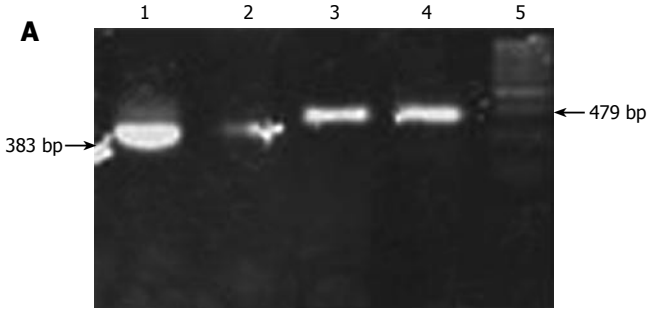
### Cell fractionation and co-immunoprecipitation

The following procedures were carried out at 4℃ as previously described[19-21]. Cells were extracted in the tissue-culture dish with 1 mL of cytoskeleton extraction buffer [CSK: 300 mmol/L, sucrose, 10 mmol/L pipes (pH 6.8), 50 mmol/L NaCl, 3 mmol/L $MgCl_2$, 5 mL/L Triton X-100, 1.2 mmol/L PMSF, 0.1 mg/mL DNase, 0.1 mg/mL RNase]. Cells were harvested with a rubber policeman from the tissue-culture dish, centrifuged at 14 000 *g* for 10 min and the TX-100-soluble fraction separated from the pellet. The TX-100-insoluble pellet was resuspended in 100 μL of SDS immunoprecipitation buffer [10 g/L SDS, 10 mmol/L Tris-HCl (pH 7.5), 2 mmol/L EDTA, 0.5 mmol/L DTT, 0.5 mmol/L PMSF] and boiled for 10 min. SDS concentration was reduced to 0.1% by the addition of 900 μL of CSK buffer prior to immunoprecipitation. For total cell extract immunoprecipitation, cultures were extracted with 1 mL of lysis buffer [10 mmol/L Tris-HCl (pH 7.5), 1 mmol/L EDTA, 1 mmol/L EGTA, 2 mmol/L PMSF and 5 mL/L Triton X-100] for 10 min and harvested as described above.

The soluble and insoluble fractions were precleared for 1 h by incubation with 1 μg of purified mouse serum IgG and 5 μL of 50% protein G Sepharose at 4℃. Following centrifugation at 3000 r/min for 15 min, the TX-100-soluble fraction was processed for immunoprecipitation with 1 μg of E-cadherin antibodies and 20 μL of 50% protein G Sepharose overnight at 4℃. TX-100-insoluble fractions were processed for immunoprecipitation with 1 μg of β-catenin antibodies and 20 μL of 50% protein G Sepharose overnight, because TX-100-insoluble fractions have to be solubilized prior to immunoprecipitation and the solubilization conditions (10 g/L SDS at 100℃) caused dissociation of the complex. Purified mouse serum Ig was used as a negative control. The beads were washed sequentially with high stringency buffer [15 mmol/L Tris-HCL (pH 7.5), 5 mmol/L EDTA, 2.5 mmol/L EGTA, 10 mL/L TX-100, 10 g/L sodium deoxycholate, 1 g/L SDS, 120 mmol/L NaCl and 25 mmol/L KCl], high-salt buffer [15 mmol/L Tris-HCl (pH 7.5), 5 mmol/L EDTA, 2.5 mmol/L EGTA, 10 mL/L TX-100, 10 g/L sodium deoxycholate, 1 g/L SDS and 1 mol/L NaCl] and low-salt buffer [15 mmol/L Tris-HCl (pH 7.5), and 5 mmol/L EDTA]. The beads were then resuspended in an equal volume of sample buffer, boiled for 5 min and centrifuged at 14 000 *g* for 5 min, and the supernatant was dissolved by SDS-PAGE on an 80 g/L polyacrylamide gel and transferred by electroblotting onto nitrocellulose membrane and probed with anti-β-catenin antibodies. For β-catenin/Tcf-4 complex coimmunoprecipitation, the total cell extract was precipitated with anti-Tcf-4 antibody and the immunoprecipitated proteins were subjected to probe with anti-β-catenin or anti-Tcf-4, respectively.

### Immunocytochemistry

Cells were grown to approximately 80% confluence on tissue-culture multispot glass microscope slides at 37℃




Lane 1: CCKR-2 from Colo320WT; Lane 2: CCKR-2 from Colo320; Lane 3: β-actin from Colo320WT; Lane 4: β-actin from Colo320; Lane 5: DNA marker.

Lane 1: Colo320 cells; Lane 2: Colo320WT cells.

**Figure 1** Expression and receptor binding characteristics of CCK2 receptor in Colo320 and Colo320 WT cells. **A**: RT-PCR using RNA extracted from Colo320 and Colo320WT cells; **B**: Immunoblotting using protein extracted from Colo320 and Colo320WT cells. Blotting result of β-actin was used to show the equal loading.

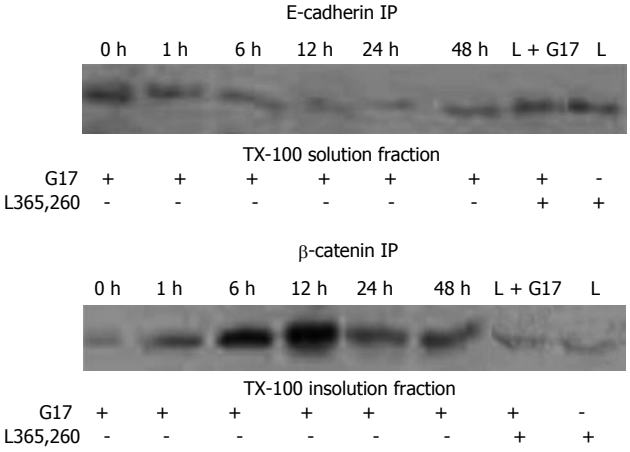



**Figure 2** Immunoprecipitation of β-catenin in TX-100 solution fraction and the insoluble fraction of Colo320WT cells treated with G17 ($10^{-8}$ mol/L). TX-100 solution fraction was immunoprecipitated with anti-E-cadherin antibody, while TX-100 insoluble fraction was immunoprecipitated with anti-β-catenin antibody. E-cadherin immunoprecipitate and β-catenin immunoprecipite were separated on 100 g/L SDS/PAGE gel and probed with β-catenin antibody. Stimulation with G17 induced an increase in the level of β-catenin protein extracted from TX-100 -insoluble fraction and reached the highest at 12 h, while a reduction in the level of β-catenin protein extracted from TX-100 solution fraction. But the responses stimulated by G17 were blocked by L365,260 (Right lane: L).

in an incubator containing 50 mL/L $CO_2$ in absence of serum. The next day, cells were treated with $10^{-8}$ mol/L G17 or with $10^{-6}$ mol/L CCK2 receptor antagonist L365,260 for 30 min, followed by treatment with G17 for 12 h. The cells were then fixed in acetone for 10 min at 4℃. Endogenous peroxidase was blocked by incubating the slides in 3 mL/L hydrogen peroxide ($H_2O_2$) in phosphate-buffered saline (PBS) for 15 min. The slides were incubated in normal goat serum for 20 min to block the non-specific binding. Primary antibody was added to the cells (β-catenin, 1:50), and the slides were incubated overnight at 4℃. The cells were washed in PBS and then appropriate biotinylated secondary antibody was added to each well. The slides were incubated for 30 min at room temperature, washed in PBS, and then incubated with streptavidin-HRP for 30 min at room temperature. The slides were examined by a conventional light microscope, and cellular distributions of the proteins between the membrane, cytoplasm and nucleus were assessed.

### *Western blot*

Cells were grown to approximately 80% confluence and then serum-starved for 24 h. Then the cells were treated with $10^{-8}$ mol/L G17 or with $10^{-6}$ mol/L CCK2 receptor antagonist L365,260 as described above. The stimulation was terminated on ice by aspirating the medium and solubilizing the cells in 1 mL of ice-cold RIPA buffer (10 mL/L NP-40, 1% DOC, 1 g/L SDS, 150 mmol/L NaCL, 10 mmol/L Tris-HCl, 1 μmol/L PMSF, 1 μg/mL leupeptin, 1 μg/mL Aprotinin, 1 μg/mL Pepstatin). Cell lysates were centrifuged at 14 000 *g* for 5 min. The supernatants were transferred into new ice-cold micro-centrifuge tubes. Following SDS-PAGE, proteins were transferred onto nitrocellulose membranes. For detection of proteins, membranes were blocked using 50 g/L non-fat dried milk in Tris buffer containing 1 g/L Tween (TBST) and then incubated overnight at 4℃ with specific antibodies diluted in TBS-T containing 50 g/L non-fat milk. Bound primary antibodies to immunoreactive bands were visualized by ECL detection with horseradish peroxidase-conjugated anti-rabbit or anti-mouse antibodies.

## RESULTS

### *Expression of CCK2 receptor in Colo320WT cells*

The transfection efficiencies in Colo320 and Colo320 WT cells were evaluated by RT-PCR and immunoblotting. Although no method is capable of discriminating between endogenous and transfected CCK2 receptor expressions, our results of RT-PCR and immunoblotting showed that Colo320 cells expressed low levels of CCK2 receptor mRNA and protein, and that stable transfection with CCK2 receptor cDNA led to a 4-fold over-expression of the CCK2 receptor at protein and mRNA levels (Figure 1A and B).

### *Immunoprecipitation of β-catenin*

β-catenin in the TX-100 solution fraction was precipitated with anti-E-cadherin antibody. Expression of β-catenin in the TX-100 solution fraction increased in a time-dependent fashion under G17 treatment, and the expression level reached the highest when treated for 12 h. While the expression of β-catenin in the TX-100 insoluble fraction was obviously decreased, and β-catenin in free pool increased (Figure 2).

### *Coimmunoprecipitation of β-catenin/Tcf-4*

β-catenin interacts with Tcf-4 and forms a complex in the nuclei. We used anti-Tcf-4 antibody to precipitate the complex in the total cell extract, and then go on to probe by anti-β-catenin or anti-Tcf-4 antibody, respectively. As a result, we found that the expression levels of β-catenin

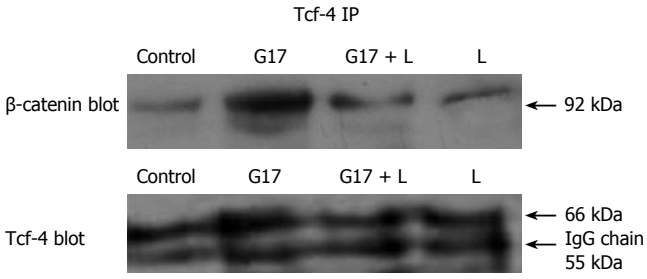



**Figure 3** Immunoprecipitation of β-catenin/Tcf-4 transcription complex in cell protein fraction treated with G17 ($10^{-5}$ mol/L). Cells were stimulated with G17 ($10^{-8}$ mol/L) for the times indicated and lysed. Whole cell lysates were immunoprecipitated with anti-Tcf-4 antibody and Tcf-4 immunoprecipitate was probed separately with anti-β-catenin or anti-Tcf-4 antibody. The results showed that the level of Tcf-4 and β-catenin from immunoprecipitation with Tcf-4 increased under G17 stimulation.

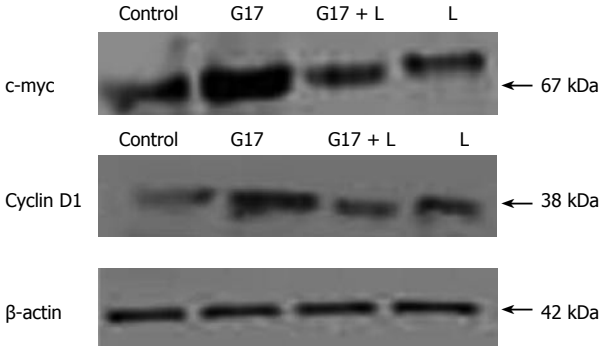



**Figure 5** Over-expression of c-myc and cyclin D1 in Colo320WT cells stimulated by G17. Expression levels of c-myc and cyclin D1 were determined by immunoblotting. The results showed that expression levels of both proteins increased markedly under G17 stimulation for 12 h. But L365,260 could abrogate the responses stimulated by G17.

and Tcf-4 proteins in the complex increased greatly after G17 stimulation, which confirmed that more β-catenin translocated into the nucleus where it bound and interacted with Tcf-4. The active β-catenin/Tcf-4 transcriptional complex further resulted in the expression of the target genes, such as c-myc and cyclin D1 (Figure 3).

### *Immnodetection of β-catenin*

Before treatment with gastrin, scattered Colo320WT cells showed cytoplasmic and little nuclear immunoreactivity for β-catenin in the absence of serum. When confluent, Colo320WT cell membrane localization of β-catenin was at sites of cell-cell contact, but free borders of the cells showed little membranous staining. After G17 stimulation, distribution of β-catenin in the cells changed greatly and β-catenin was translocated from the membrane to the cytoplasm and nucleus, especially to scattered cells. In cohesive cells, staining decreased at sites of cell-cell contact. But after application of L365,260, straining of β-catenin in the cells was the same as that of the cells untreated by G17 (Figure 4).

### *Immumoblot of c-myc and cyclin D1 protein*

After stimulating Colo320WT cells by G17, expression levels of c-myc and cyclin D1 protein were greatly

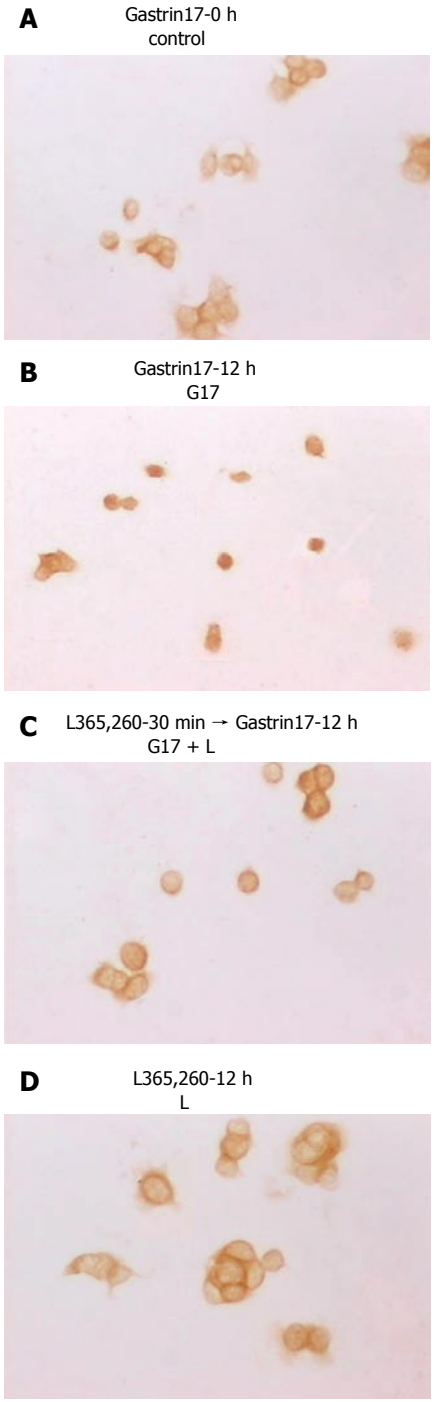



**Figure 4** Immuno-cytochemical staining of β-catenin in Colo320WT cells treated with G17. **A**: Expression of β-catenin in Colo320WT cells stimulated for 0 h by G17 ($10^{-8}$ mol/L); **B**: Expression of β-catenin in Colo320WT cells stimulated for 12 h by G17 ($10^{-8}$ mol/L); **C:** Expression of β-catenin in Colo320WT cells stimulated for 12 h by L365,260 ($10^{-6}$ mol/L); **D**: Expression of β-catenin in Colo320WT cells stimulated for 30 min by $10^{-6}$ mol/L L365,260 and then for 12 h with G17 ($10^{-8}$ mol/L).

increased, showing that gastrin can translocate β-catenin into the nucleus where it interacts with Tcf-4, resulting in over-expression of downstream molecules c-myc and cyclin D1, which may cause tumor cell invasion and metastasis (Figure 5).

## DISCUSSION

The gastrointestinal (GI) peptide hormone gastrin has

been shown to regulate multiple cellular functions, including growth, apoptosis and secretion[22]. Many studies have also shown that gastrin can stimulate the growth and proliferation of colorectal cancer cells from animals and human[3-6] and gastrin exerts its effects in promoting proliferation and growth by binding its high affinity receptor CCK-2, which belongs to the family of G protein-coupled receptors[7-11]. The gastrin receptor antagonist can abrogate responses stimulated by gastrin[23,24]. We have confirmed that G17 may increase invasion of human colorecatal cancer cell by activating the FAK pathway[12,13]. Recently, we have also found that G17 can lead to phosphorylated FAKTyr397 to accumulate at lamellipodia and to form the FAK-Src-p130Cas-Dock180 signaling complex in human colorectal cancer cells (unpublished data).

β-catenin is a member of the catenin family as a proto-oncogene. Adenomatous polyposis coli (APC) can compete with E-cadherin to bind and interact with β-catenin. β-catenin binds the intracellular domain of E-cadherin and forms a complex, which keeps low levels of β-catenin in the cytoplasm. Alpha-catenin mediates the anchorage of the E-cadherin/β-catenin complex to actin filaments of the cytoskeleton and participates in cytoskeleton remodeling[25-28]. Cytosolic β-catenin is degraded by the ubiquitin-proteasome pathway, requiring active glycogen synthase kinase-3β (GSK-3β), APC and axin[25,29-31].

Our study showed that G17 could stimulate redistribution of β-catenin in human colon cancer cells Colo320WT, which is different from the results achieved by Song *et al*[32] that gastrin can lead only to high expression of β-catenin, but not redistribution of β-catenin in mouse colorectal cells. The different results probably resulting from different genus cells (mouse and human) are to be studied in our following research. Our coimmunoprecipation results showed that the expression level of β-catenin in the TX-100 insoluble fraction markedly increased in a time-dependent manner, and the expression level of β-catenin reached the highest when G17 was exogenously applied for 12 h. But the expression level of β-catenin in the TX-100 soluble fraction was the other way around. It indicates that free cytosolic β-catenin increases greatly under G17 treatment, because G17 may inhibit activation of APC, axin, PTEN (the phosphatase and tensin homologue) and GSK-3β, and further inhibit β-catenin phosphorylation. As a result, free β-catenin in the cytoplasmic pool increases on gastrin stimulation. When we used L365,260 (gastrin receptor antagonist) to block effects caused by G17, expression level of β-catenin changed a little in the TX-100-insoluble fraction and the TX-100-soluble fraction, which confirmed that G17 exerts its effects on Colo320WT cells by binding and interacting with its high-affinity receptor CCK-2R.

Immunocytochemistry demonstrated a link between cell-cell contact and the distribution of β-catenin. Without G17 stimulation, scattered Colo320WT cells showed cytoplasmic and little nuclear immunorectivity for β-catenin, and the confluent Colo320WT cells membrane localization of β-catenin was at sites of cell-cell contact, but free borders of the cells showed little membranous staining. But after G17 stimulation, distribution of β-catenin in the cells changed greatly and β-catenin was translocated from the membrane to the cytoplasm and nucleus, especially to scattered cells. In cohesive cells, staining decreased at sites of cell-cell contact. Taken together, G17 can affect redistribution of β-catenin in Colo320WT cells and decrease cell-cell cohesion, resulting in cell invasion and metastasis.

G17 leading to redistribution of β-catenin in Colo320WT cells resulted in an increase of the cytoplasmic pool of β-catenin. The increased free β-catenin was translocated into the nucleus, where it bound and interacted with Tcf-4 transcription factor. Thus, activated β-catenin/Tcf-4 pathway led to the up-regulation of downstream target genes c-myc and cyclin D1.

In conclusion, G17 can cause redistribution of β-catenin and activate β-catenin/Tcf-4 pathway which leads to high expression of c-myc and cyclin D1, thereby promoting invasion and metastasis of Colo320WT cells.

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## COMMENTS

### Background

Colorectal cancer is one of human malignant tumor, and tumor metastasis affects therapeutic effect and prognosis, playing a key role in death to a patient with cancer. But the biologic mechnism of tumor cells' motility, invasion and metastasisis to be studied as yet.

### Research frontiers

Gastrin may mediate intracellular signal transduction in human colon cancer cells, and can increase invasion and metastasis of tumor cells by binding to its receptor CCK-2.

### Innovations and breakthroughs

Our study shows that Gastrin17 may induce redistribution of β-catenin in human colon cancer cells Colo320WT, which is different from the results achieved by Song DH *et al* that gastrin can lead only to high expression of β-catenin, but not redistribution of β-catenin in mouse colorectal cells.

### Applications

The research explores the mechanism of colorectal cancer invasion and metastasis in order to provide a new thinking of preventing and curing tumor invasion and metastasis.

### Terminology

Beta -catenin in the TX-100 solution fraction is cytoplasmic beta -catenin, while beta -catenin in TX-100 insoluble fraction is cytoskeleton bound beta -catenin.

### Peer review

Gastrin is a peptidic hormone essentially secreted by gastric antrum and proximal duodenum, which belongs to the same family as cholecystokinin (CCK). More recent findings suggest that gastrin can mediate proliferative effects in digestive tract neoplasia by the CCK2 receptor. And some clinical evidence and animal experiments have shown that gastrin may promote tumor's invasivness and metastasis, regrettably, its mechanism is still to be explored as yet.

The experiment aims to explore the mechanism by which gastrin increases tumor's invasion and metastasis by plasmid transfection, RT-PCR, coimmunoprecipitation and Western blot methods. The results showed that G17 induced redistribution of β-catenin and increased free cytoplasmic β-catenin which translocated into the nucleus, where it was bound and interacted with Tcf-4 transcription factor. G17 activated the β-catenin/Tcf-4 pathway, and further upregulated downstream target genes c-myc and cyclinD1.

This article is clear-cut and easy to understand. The paper provides new thoughts on how gastrin affects gastrointestinal tract tumorigenesis, development, invasion and metatasis.

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*VIRAL HEPATITIS*

# N-terminal and C-terminal cytosine deaminase domain of APOBEC3G inhibit hepatitis B virus replication

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## Abstract

**AIM:** To investigate the effect of human apolipoprotein B mRNA-editing enzyme catalytic-polypeptide 3G (APOBEC3G) and its N-terminal or C-terminal cytosine deaminase domain-mediated antiviral activity against hepatitis B virus (HBV) *in vitro* and *in vivo*.

**METHODS:** The mammalian hepatoma cells HepG2 and HuH7 were cotransfected with APOBEC3G and its N-terminal or C-terminal cytosine deaminase domain expression vector and 1.3-fold-overlength HBV DNA as well as the linear monomeric HBV of genotype B and C. For *in vivo* study, an HBV vector-based mouse model was used in which APOBEC3G and its N-terminal or C-terminal cytosine deaminase domain expression vectors were co-delivered with 1.3-fold-overlength HBV DNA *via* high-volume tail vein injection. Levels of hepatitis B virus surface antigen (HBsAg) and hepatitis B virus e antigen (HBeAg) in the media of the transfected cells and in the sera of mice were determined by ELISA. The expression of hepatitis B virus core antigen (HBcAg) in the transfected cells was determined by Western blot analysis. Core-associated HBV DNA was examined by Southern blot analysis. Levels of HBV DNA in the sera of mice as well as HBV core-associated RNA in the liver of mice were determined by quantitative PCR and quantitative RT-PCR analysis, respectively.

**RESULTS:** Human APOBEC3G exerted an anti-HBV activity in a dose-dependent manner in HepG2 cells, and comparable suppressive effects were observed on genotype B and C as that of genotype A. Interestingly, the N-terminal or C-terminal cytosine deaminase domain alone could also inhibit HBV replication in HepG2 cells as well as Huh7 cells. Consistent with *in vitro* results, the levels of HBsAg in the sera of mice were dramatically decreased, with more than 50 times decrease in the levels of serum HBV DNA and core-associated RNA in the liver of mice treated with APOBEC3G and its N-terminal or C-terminal cytosine deaminase domain as compared to the controls.

**CONCLUSION:** Our findings provide probably the first evidence showing that APOBEC3G and its N-terminal or C-terminal cytosine deaminase domain could suppress HBV replication *in vitro* and *in vivo*.



**Key words**: Cytosine deaminase domain; Apolipoprotein B mRNA-editing enzyme catalytic-polypeptide 3G; Hepatitis B virus; Antiviral therapy

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## INTRODUCTION

Hepatitis B virus (HBV) infects more than 350 million people worldwide and is a leading cause of end-stage liver disease and of hepatocellular carcinoma[1]. HBV is non-cytopathic for hepatocytes; however, most newly HBV-

infected adult patients develop acute hepatitis because of a strong immune response that clears HBV from the liver, whereas approximately 5% of newly HBV-infected adult patients generate insufficient immunity and become chronically infected[1,2]. HBV and other hepadnaviruses replicate their partially double-stranded DNA genome within cytoplasmic core particles by reverse transcription of encapsidated pregenomic RNA and thus are related to retroviruses[3].

The cytidine deaminase APOBEC3G (A3G), which is encoded within a cluster of seven related editing enzymes (APOBEC3A-G) on chromosome 22, provides a broad innate immunity against exogenous and endogenous retroelements[4-10]. It has been shown that the propagation of HIV-1 strains lacking the accessory protein virion infectivity factor (VIF) is suppressed in non-permissive cells due to expression of the cytidine deaminase APOBEC3G[5,11]. Encapsidated into the retroviral particle, APOBEC3G induces massive C→U deamination of single-stranded retroviral DNA, resulting in DNA degradation or lethal G→A hypermutation[6-8]. HIV overcomes this innate defense barrier in T-cells with the help of the HIV-encoded VIF protein that specifically targets A3G to proteosomal degradation[12-14]. In addition, APOBEC3F (another cytidine deaminase of APOBEC family) is a potent retroviral restrictor, but its activity, unlike that of APOBEC3G, is partially resistant to HIV-1 VIF and results in retroviral hypermutation. Moreover, APOBEC3F and APOBEC3G appear to be coordinately expressed in a wide range of human tissues and are independently able to inhibit retroviral infection. Thus, APOBEC3F and APOBEC3G are likely to function alongside one another in the provision of an innate immune defense, with APOBEC3F functioning as the major contributor to HIV-1 hypermutation *in vivo*[15]. Recent evidence suggests that some of the HIV restriction exerted by A3G may be independent of its cytidine deaminase activity[16-18].

Interestingly, APOBEC3G can also interfere with the HBV life-cycle in co-transfected cells[19,20]. However, reduced levels of encapsidated viral pgRNA rather than extensive editing was found to be the major contributing factor[19], APOBEC3G-mediated editing did occur but was only detected in a minority of clones produced in the transfected HepG2 hepatoma cells[20]. In addition, the C-terminal cytosine deaminase domain catalytically inactive APOBEC3G derivatives no longer inhibited VIF-defective HIV-1 but did conserve wild-type levels of activity on HBV[19]. As a result, the mechanisms of APOBEC3G blocking HBV production are unclear.

A previous study shows that APOBEC family members contain a domain structure characteristic of cytidine deaminases[21]. A short a-helical domain is followed by a catalytic domain (CD), a short linker peptide and a pseudocatalytic domain (PCD). In APOBEC3G, the entire unit is duplicated to form the domain structure helix1- CD1-linker1-PCD1-helix2-CD2-linker2-PCD2. Each catalytic domain contains the conserved motif H-X-E-(X)27-28-P-C-X-X-C in which the His and Cys residues coordinate $Zn^{2+}$ and the Glu serves as a proton shuttle in the deamination reaction[22]. Based on the molecular structure of APOBEC3G and recent research results, we speculated that the N-terminal or C-terminal cytosine deaminase domain of APOBEC3G could also display HBV inhibition activity.

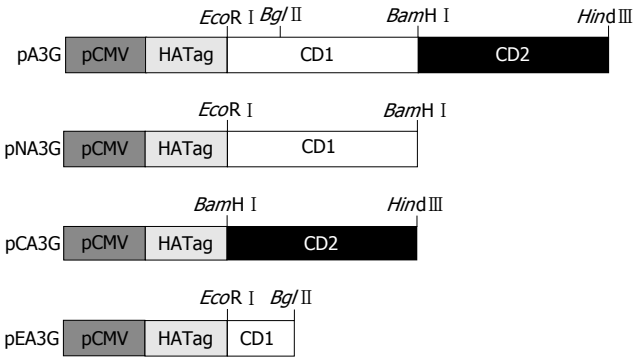



**Figure 1** Construction of APOBEC3G and its N-terminal and C-terminal cytosine deaminase domain expression plasmids. Full-length APOBEC3G sequence was cloned into *Eco*R Ⅰ/*Hind*Ⅲ restriction sites of the CMV-driven expression vector fused with a hemagglutinin fusion epitope tag at its N- terminal end (pXF3H) to construct APOBEC3G expression plasmid (pA3G). pA3G was digested with *Eco*R Ⅰ/*Bam*H Ⅰ and *Bam*H Ⅰ/*Hind*Ⅲ, these fragments were inserted into pXF3H to construct the N-terminal and C-terminal cytosine deaminase domain expression plasmids (pXFNA3G and pXFCA3G), respectively. pA3G was digested with *Eco*R Ⅰ/*Bgl*Ⅱ, and used for the construction of the N-terminal region of APOBEC3G which did not contain any cytosine deaminase domain plasmid (pXFEA3G). pCMV: CMV promoter; CD1: N-terminal cytosine deaminase domain; CD2: C-terminal cytosine deaminase domain.

In the present study, we performed a detailed analysis of the inhibitory effect of APOBEC3G and its N-terminal or C-terminal cytosine deaminase domain on hepatitis B virus replication in cell culture and in an HBV vector-based mouse model.

## MATERIALS AND METHODS

### Plasmid constructs

For construct expression vectors coding for human APOBEC3G (pXFA3G), total RNA was extracted from the peripheral blood mononuclear cells (PBMCs). RT-PCR amplification of human APOBEC3G sequence was carried out using forward primer 5'-CGGAATTCAAGCCTCACTTCAGAAACAC-3' and reverse primer 5'-CGAAGCTTTCTGCCTTCCTTAGAGACTG-3'. The PCR product was cloned into *Eco*R Ⅰ/*Hind*Ⅲ restriction sites of the CMV-driven expression vector fused with a hemagglutinin fusion epitope tag at its carboxy terminal end (pXF3H). For construction of the N-terminal and C-terminal cytosine deaminase domain expression plasmids, the unique restriction enzyme site *Bam*H Ⅰ in the linker area of APOBEC3G, two cytosine deaminase domains were used. pXFA3G was digested with *Eco*R Ⅰ/*Bam*H Ⅰ and *Bam*H Ⅰ/*Hind*Ⅲ, these fragments were inserted into pXF3H to construct the N-terminal and C-terminal cytosine deaminase domain expression plasmids (pXFNA3G and pXFCA3G), respectively. Next, pXFA3G was digested with *Eco*R Ⅰ/*Bgl*Ⅱ, and used for construction of the N-terminal region of APOBEC3G which did not contain any cytosine deaminase domain plasmid (pEA3G) (Figure 1). Replication competent of wild-type HBV 1.3-fold-overlength plasmid (subtype, ayw) and linear monomeric HBV genomes of

genotype B and genotype C with *Sap* I ends have been constructed previously in our laboratory.

### Cell culture, transfection and harvesting

HepG2 and Huh7 cells were grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 100 mL/L fetal calf serum (Invitrogen, CA, USA) at 37℃ in a humidified atmosphere containing 50 mL/L $CO_2$. The cells were plated at a density of $4.5 \times 10^5$ cells per well in 6-well plates 18 h prior to transfection. Transfecion of cells was performed with lipofectamine 2000 (Invitrogen, USA) following the manufacturer's guidelines. On d 3 after transfection, the cells were removed from the culture dish *via* treatment with trypsin-EDTA, resuspended in culture medium, washed with phosphate-buffered saline (PBS), pelleted, and resuspended in 1 mL chilled lysis buffer (140 mmol/L NaCl, 1.5 mmol/L $MgCl_2$, 50 mmol/L Tris-HCl [pH 8.0]) containing 5 mL/L NP-40. Nuclei were removed *via* centrifugation for 5 min at 2000 r/min in an eppendorf centrifuge, and the supernatant was cleared of cell debris by centrifugation for another 5 min at 14 000 *g*.

### HBV vector-based mouse model

For the *in vivo* experiments, 6 to 8-wk-old female BALB/c mice were used. A total of 30 mice were randomly divided into 5 groups (6 mice/group). Replication-competent pHBV1.3 (10 μg) and APOBEC3G and its N-terminal or C-terminal cytosine deaminase domain expression vectors (10 μg) or pXF3H (10 μg) control plasmid DNA were co-injected into the tail vein of mice in a volume of Ringer's injection equivalent to 9% of the mouse body weight[23,24], the total volume was delivered within 5 s. The mice sera were collected at an indicated time after hydrodynamic injection, and secreted hepatitis B virus surface antigen (HBsAg) levels and HBV DNA content were measured. All mouse experiments were carried out according to the guidelines established by the Institutional Animal Care and Use Committee at the Tongji Medical College, Huazhong University of Science and Technology.

### HBsAg and HBeAg assays and Western blot analysis

Levels of HBsAg and HBeAg in the media of the transfected cells, and in the sera (1:100 dilution) of the treated mice were determined using an ELISA kit (Shanghai Shiye Kehua Company, China). For Western blot analysis, cytoplasmic lysates were incubated with 1 vol 2 × loading buffer containing 100 mL/L beta-mercaptoethanol for 10 min at 95℃ before loading on a 125 g/L SDS-PAGE. Proteins were transferred onto a nitrocellulose membrance via electroblotting. The membranes were incubated with HBV core-specific rabbit antiserum (Santa Cluz, USA) or with anti-hemagglutinin fusion epitope monoclonal antibody (Santa Cluz, USA), followed by horseradish peroxidase-conjugated mouse anti-rabbit antibody. Proteins were visualized by Enhanced Chemiluminescene (Roche, Germany).

### HBV DNA purification and analysis

The method for purification of cytoplasmic core-associated HBV DNA was adapted from Pugh *et al*[25]. Briefly, HepG2 cells were disrupted in lysis buffer (100 mmol/L Tris-HCl, pH 8.0, 2 mL/L NP-40). The cell lysate was clarified by centrifugation at 13 000 *g* for 1 min to pellet nuclei and insoluble material. The supernatant was adjusted to 6 mmol/L $MgOAC_2$ and incubated for 2 h at 37℃ with 200 μg/mL DNase I and 100 μg/mL RNase A. Following digestion, the lysate was centrifuged for 1 min at 13 000 *g*. The supernatant was incubated for 1 h at 55℃ after addition of 10 mmol/L EDTA, 10 g/L SDS, 100 mmol/L NaCl and 200 μg/mL proteinase K. Finally, the sample was extracted with phenol:chloroform. The DNA was ethanol precipitated, resuspended in TE at pH 8 (10 mmol/L Tris-HCl, pH 7.5; 1 mmol/L EDTA) and digested with 100 μg/mL RNase A for 30 min at 37℃. The purified DNA was subjected to Southern blot analysis. DNA samples were loaded onto 13 g/L agarose gels, blotted onto nylon membranes, and probed with a Dig-labeled full-length HBV genome in EasyHyb hybridization solution (Roche, Germany).

For quantitative PCR, 100 μL of mouse serum was adjusted to 6 mmol/L $MgOAC_2$ and incubated for 2 h at 37℃ with 200 μg/mL DNase I and 100 μg/mL RNase A. Following proteinase K digestion, the sample was extracted with phenol:chloroform. HBV DNA levels were analyzed with the Light Cycle real-time PCR system (Roche, Germany), using the primer sequences as follows: 5'-TCACAATACCGCAGAGTC-3' (nt231-248, forward) and 5'-AGCAACAGGAGGGATACA-3' (nt569-552, reverse). The pHBV1.0 vector containing the full-length HBV genome was used as a standard curve to calculate HBV copies per milliliter of serum.

### RNA isolation and analysis

For quantitative RT-PCR, approximately 20 mg of liver tissue was obtained from each mouse for total RNA extraction with RNeasy Total RNA kit (QIAGEN, Germany), according to the manufacture's protocol. cDNA was synthesized from 2 μg of total RNA with oligo$(dT)_{18}$ primer in a total volume of 20 μL. Quantitative RT-PCR was performed with the Light Cycler real-time PCR system (Roche, Germany). The PCR primers were as follows: GAPDH, 5'-GTTGTCTCCTGCGACTTCA-3' (forward) and 5'-GGTGGTCCAGGGTTTCTTA-3' (reverse); HBV, 5'-TCACAATACCGCAGAGTC-3' (nt231-248, forward) and 5'- AGCAACAGGAGGGATACA-3' (nt569-552, reverse). A standard curve was constructed by the simultaneous amplification of serial dilutions of the expression plasmid encoding HBV used as templates. Target cDNAs were normalized to the endogenous RNA levels of the DAPDH. Quantitative amplification was carried out using the SYBR Green kit (Invitrogen, USA). Gene expression was determined using the relative quantification: $\Delta\Delta C_T$ = $(C_{THBV}\text{-}C_{T\ GAPDH})$Test -$(C_{T\ HBV}\text{-}C_{T\ GAPDH})$ Control. $C_T$ is the fractional cycle number that reaches a fixed threshold, $C_{Test}$ is the test of interest, and $C_{Control}$ is the reference control (RNA from control group). ΔCT is the difference between gene expression in the treated cells and the reference control cells. The fold increase was calculated using $2^{\Delta\Delta CT}$[26].

## RESULTS

### Inhibitory effect of APOBEC3G on HBV DNA replication in cell culture

Various amounts of a CMV-driven expression vector

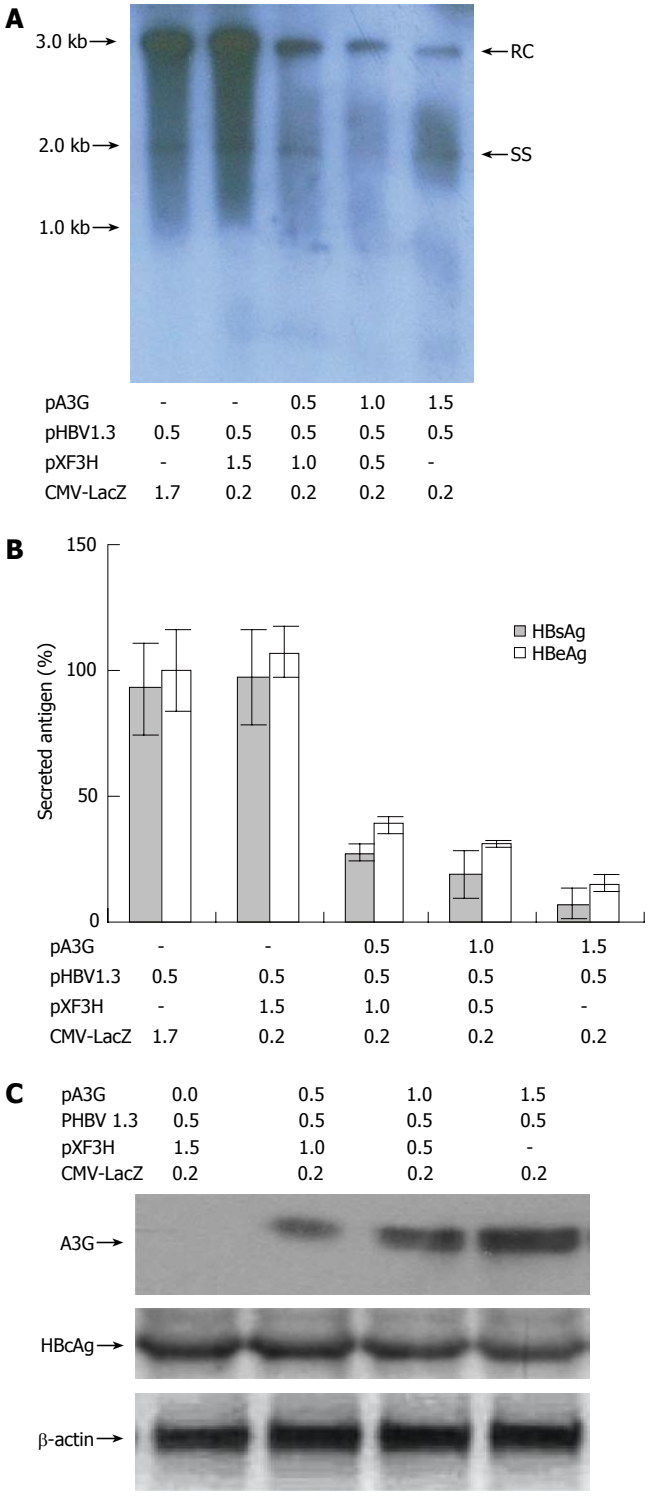



**Figure 2** Effect of APOBEC3G on HBV replication in co-transfected HepG2 cells. (**A**) Human hepatoma HepG2 cells were transiently co-transfected with pHBV1.3 and various amounts of a CMV-driven expression vector encoding A3G or with empty vector pXF3H and pCMV-LacZ using Lipofectamine 2000 reagents. The cells were harvested 3 d after transfection. HBV core-associated viral DNA was prepared from nuclease-treated cytoplasmic lysates. Viral replicative DNA intermediates were analyzed by Southern blotting using a Dig-labeled full-length HBV DNA probe. (**B**) HBsAg and HBeAg levels were determined in the media of co-transfected HepG2 cells by ELISA, normalized to the activity of co-tansfected β-galactosidase in the cell lysates. The mean ± SE of six independent experiments is shown (error bar indicates standard error). (**C**) Western blot analysis of cytoplasmic extracts from HepG2 cells co-transfected with the indicated plasmids. Numbers at the end or top of the lines indicate the amount of transfected plasmid DNA in micrograms. HBV: hepatitis B virus; pA3G: APOBEC3G expression plasmids; RC: relaxed circular DNA; SS: single-stranded DNA; HBcAg: hepatitis B virus core antigen; HBsAg: hepatitis B virus surface antigen; HBeAg: hepatitis B virus e antigen.

encoding A3G (pA3G) and empty vector (pXF3H) were co-transfected into the human hepatoma cells HepG2 together with replication-competent 1.3-fold-overlength HBV and with pCMV-LacZ to normalize for the transfection efficiency. Three days after transfection, core-associated viral DNA was prepared from nuclease-treated cytoplasmic lysates and analyzed with Southern blotting. HBsAg and HBeAg levels in the media of co-transfected cells were determined by ELISA. As shown in our previous study[27], A3G reduced the level of HBV-replicative intermediates in a dose-dependent manner (Figure 2A). In a series of six independent experiments, the expression of A3G resulted in a dose-dependent decrease in the levels of extracellular production of HBsAg and HBeAg even after correction for transfection efficiency by determination of the co-transfected β-galactosidase (Figure 2B). However, the expression of core protein did not change (Figure 2C).

### *Suppression of the replication of HBV clinical isolates of different genotypes by APOBEC3G*

Based on sequence divergence, HBV can be classified into at least 8 genotypes[28]. These results, in agreement with our and other's results[19,27], showed that APOBEC3G inhibited the replication of a laboratory clone of genotype A (subtype ayw). Further testing concerned whether or not APOBEC3G could also inhibit the replication of HBV with different genotypes from clinical isolates. As genotypes B and C are the most prevalent genotypes in China, this study mainly focused on these 2 genotypes. A previous study has demonstrated that linear monomeric HBV genomes with *Sap* I ends can initiate a full replication cycle, leading to viral replication and antigen expression[29].

In order to examine the effect of APOBEC3G on different genotypes of clinical isolates, a full-length HBV genome of genotype B or C was amplified from the patient's serum by PCR and cloned into pUC19 vector. After digestion with *Sap* I , the linear monomeric HBV genomes were transfected into HepG2 cells with APOBEC3G expression plasmid (pA3G) or the control plasmid (pXF3H). Three days after transfection, HBV core-associated DNA was analyzed by Southern blot. As shown in Figure 3, pA3G could also inhibit the replication of HBV genotypes B and C from clinical isolates to a similar extent as on genotype A (pHBV1.3).

### *Inhibitory effect of N-terminal and C-terminal cytosine deaminase domain of APOBEC3G on HBV DNA replication in cell culture*

APOBEC family members contain a domain structure characteristic of cytidine deaminases[21]. A short a-helical domain is followed by a catalytic domain (CD), a short linker peptide and a pseudocatalytic domain (PCD). In APOBEC3G, the entire unit is duplicated to form the domain structure helix1-CD1-linker1-PCD1-helix 2-CD2-linker2-PCD2. Each catalytic domain contains the conserved motif H-X-E-(X)27-28-P-C-X-X-C in which the His and Cys residues coordinate $Zn^{2+}$ and the Glu serves as a proton shuttle in the deamination reaction[22]. A recent study has shown that APOBEC3G can interfere with the HBV life-cycle, but APOBEC3G-mediated

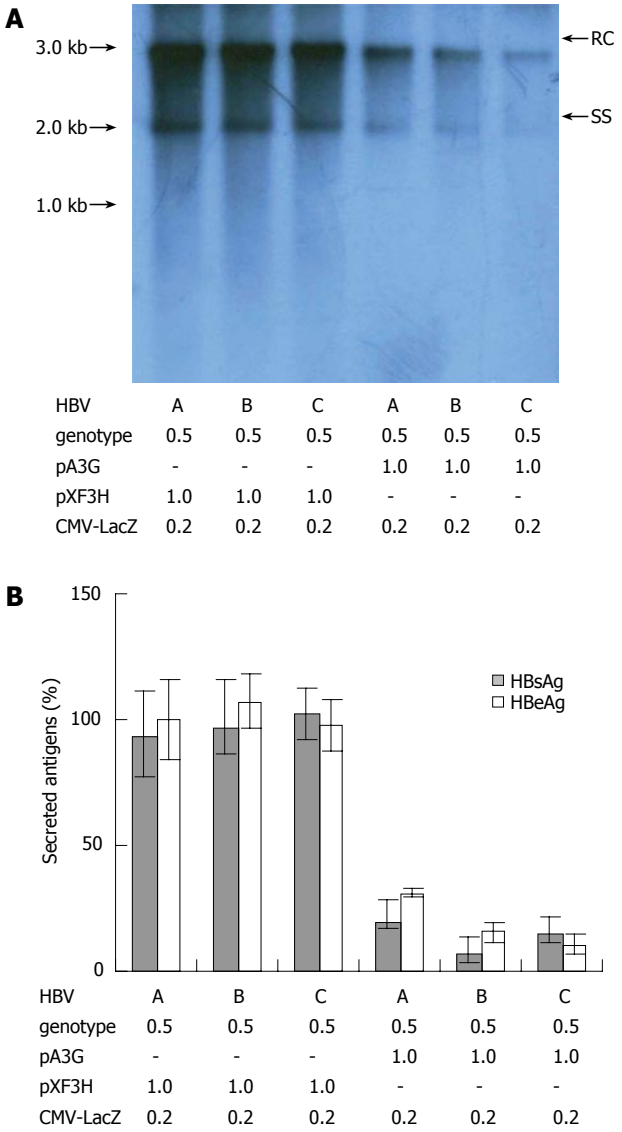



**Figure 3** Suppression of replication of HBV clinical isolates of different genotypes by APOBEC3G. (**A**) pHBV1.3 or the linear monomeric HBV genomes of genotype B and C were transiently co-transfected into HepG2 cells with the CMV-driven expression vector encoding A3G or empty vector pXF3H and pCMV-LacZ using Lipofectamine 2000 reagents. The cells were harvested 3 d after transfection. HBV core-associated viral DNA was prepared from nuclease-treated cytoplasmic lysates. Viral replicative DNA intermediates were analyzed by Southern blotting using a Dig-labeled full-length HBV DNA probe. (**B**) HBsAg and HBeAg levels were determined in the media of co-transfected HepG2 cells by ELISA, normalized to the activity of co-tansfected β-galactosidase in the cell lysates. The mean ± SEM of six independent experiments is shown (error bar indicates standard error). pA3G: APOBEC3G expression plasmids; RC: relaxed circular DNA; SS: single-stranded DNA.

editing of nucleic acids does not seem to represent the major contributing factor[10]. We speculated that the N-terminal or C-terminal cytosine deaminase domain of APOBEC3G could also display HBV inhibitory activity.

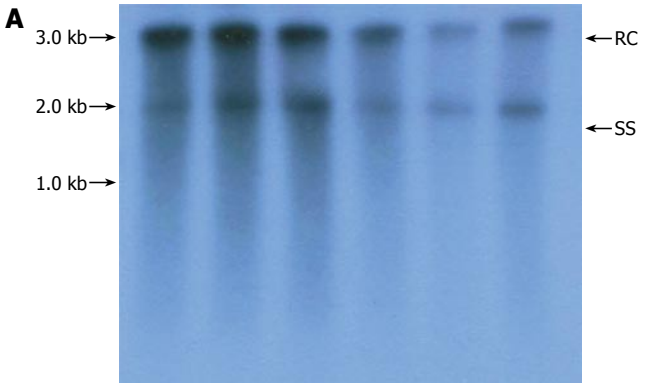
To verify this hypothesis, APOBEC3G and its N-terminal or C-terminal cytosine deaminase domain expression plasmids were co-transfected with replication-competent 1.3-fold-overlength HBV DNA into HepG2 and HuH7 cells, and with pCMV-LacZ to normalize for the transfection efficiency. HBV core-associated viral DNA was prepared 3 d after transfection and analyzed by Southern blotting. HBsAg and HBeAg levels were determined by ELISA. As expected, N-terminal or C-terminal cytosine deaminase domain of APOBEC3G expression plasmid (pNA3g and pCA3g) could also reduce the level of replicative HBV intermediates and HBsAg and HBeAg levels in the media of co-transfected HepG2 cells, while the N-terminal region of APOBEC3G, which did not contain any cytosine deaminase domain (pEA3G), had no inhibitory effect on HBV production, and the same results were observed in another human hepatoma cell Huh7 (Figures 4A and 4B). In a series of six independent experiments, the expression of A3G and its N-terminal or C-terminal cytosine deaminase domain, but not of pEA3G, resulted in a consistent decrease of secreted HBsAg and HBeAg even after correction for transfection efficiency by determination of the co-transfected galactosidase (Figure 4C). Compared with the empty vector control and normalized to the amount of secreted HBsAg, the N-terminal or C-terminal cytosine deaminase domain decreased the formation of intracellular HBV-replicative intermediates in this series to approximately 60% and thus achieved a similar level of inhibition as A3G (Figure 4D). In all of these experiments, pEA3G showed no apparent inhibition of HBV replication (Figure 4D). However, Western blot analysis, using the polyclonal anti-HBV core antibody, indicated that the levels of core protein were unaffected by APOBEC3G and its N-terminal or C-terminal cytosine deaminase domain (Figure 4E).

### *Inhibition of HBV replication in mice by N-terminal or C-terminal cytosine deaminase domain of APOBEC3G*

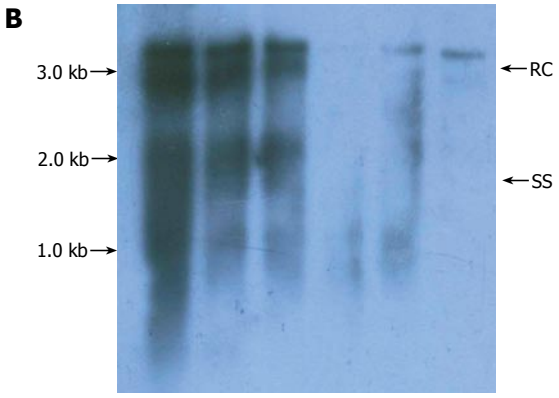
To examine whether the N-terminal or the C-terminal cytosine deaminase domain of APOBEC3G could also display HBV inhibition *in vivo*, replication-competent HBV plasmid (pHBV1.3) was co-transfected with the expression plasmids of N-terminal or C-terminal cytosine deaminase domain of APOBEC3G or the empty control plasmid (pXF3H) to mouse liver by hydrodynamic injection. As previously shown[30], hydrodynamic injection of pHBV1.3 into mouse resulted in HBV replication in the liver and secretion of viral antigens to the serum. Consistent with *in vitro* experiments, serum HBsAg and viral DNA in mice that received pHBV1.3 were suppressed dramatically at different time points by the N-terminal or the C-terminal cytosine deaminase domain of APOBEC3G compared to the mice treated with control plasmid (Figures 5A and 5B). Real-time PCR quantification of core-associated HBV RNA in the liver of mice treated with APOBEC3G and its N-terminal or C-terminal cytosine deaminase domain of APOBEC3G expression plasmid showed approximately 50 times decrease compared to the controls (Figure 5C).

## DISCUSSION

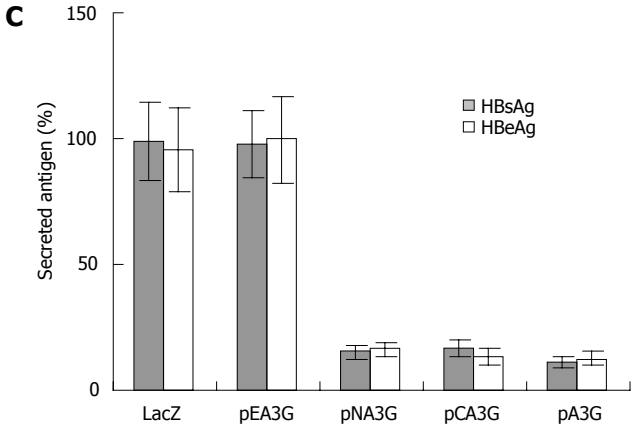
Our study describes the inhibitory effects of human APOBEC3G and its N-terminal or C-terminal cytosine deaminase domain on HBV DNA production in mammalian hepatoma cells as well as in HBV vector-based mouse model. Our data confirm and extend the recent results by Turelli *et al*[19] who reported A3G-mediated inhibition of HBV DNA production in human

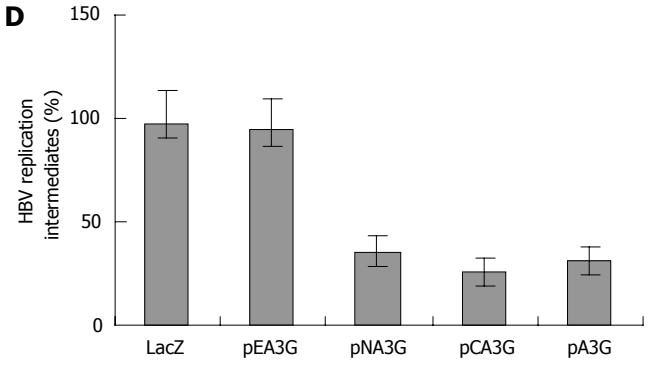


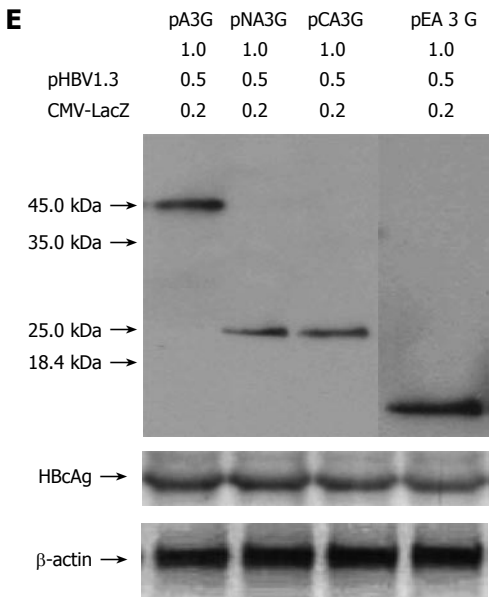

| | - | pXF3H | pEA3G | pNA3G | pCA3G | pA3G |
|---|---|---|---|---|---|---|
| | | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 |
| pHBV1.3 | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 |
| CMV-LacZ | 1.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 |




| | - | pXF3H | pEA3G | pNA3G | pCA3G | pA3G |
|---|---|---|---|---|---|---|
| | | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 |
| pHBV1.3 | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 |
| CMV-LacZ | 1.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 |




| | pA3G | pNA3G | pCA3G | pEA 3 G |
|---|---|---|---|---|
| | 1.0 | 1.0 | 1.0 | 1.0 |
| pHBV1.3 | 0.5 | 0.5 | 0.5 | 0.5 |
| CMV-LacZ | 0.2 | 0.2 | 0.2 | 0.2 |

**Figure 4** Inhibitory effect of the N-terminal and C-terminal cytosine deaminase domain on HBV production in the co-transfected cells. (**A** and **B**) Human hepatoma HepG2 and HuH7cells were transiently co-transfected with the pHBV1.3 and CMV-driven expression vector encoding A3G and its N-terminal or C-terminal cytosine deaminase domain or with the empty vector pXF3H and pCMV-LacZ using Lipofectamine 2000 reagents. The cells were harvested 3 d after transfection. HBV core-associated viral DNA was prepared from nuclease-treated cytoplasmic lysates. Viral replicative DNA intermediates were analyzed by Southern blotting using a Dig-labeled full-length HBV DNA probe. (**C**) HBsAg and HBeAg levels were determined in the media of co-transfected HepG2 cells by ELISA, normalized to the activity of co-tansfected β-galactosidase in the cell lysates. The mean ± SE of six independent experiments is shown (error bar indicates standard error). (**D**) The hybridized HBV DNA from the replicative intermediates was quantified by radiophosphorimaging, normalized to the amount of secreted HBsAg (normalized for co-transfected β-galactosidase activity) and calculated relative to the empty vector control (pXF3H). The mean ± SE of six independent experiments is shown. (**E**) Anti-HA monoclonal antibody for Western blot analysis of cytoplasmic extracts from HepG2 cells co-transfected with the indicated plasmids. Numbers at the end or top of the lines indicate the amount of transfected plasmid DNA in micrograms. pXF3H: empty vector; pA3G: APOBEC3G expression plasmids; pNA3G: N-terminal cytosine deaminase domain of APOBEC3G expression plasmids; pCA3G: C-terminal cytosine deaminase domain of APOBEC3G expression plasmids; pEA3G: N-terminal region of APOBEC3G which does not contain any cytosine deaminase domain plasmid; HBV: hepatitis B virus; RC: relaxed circular DNA; SS: single-stranded DNA; HBcAg: hepatitis B virus core antigen; HBsAg: hepatitis B virus surface antigen; HBeAg: hepatitis B virus e antigen.

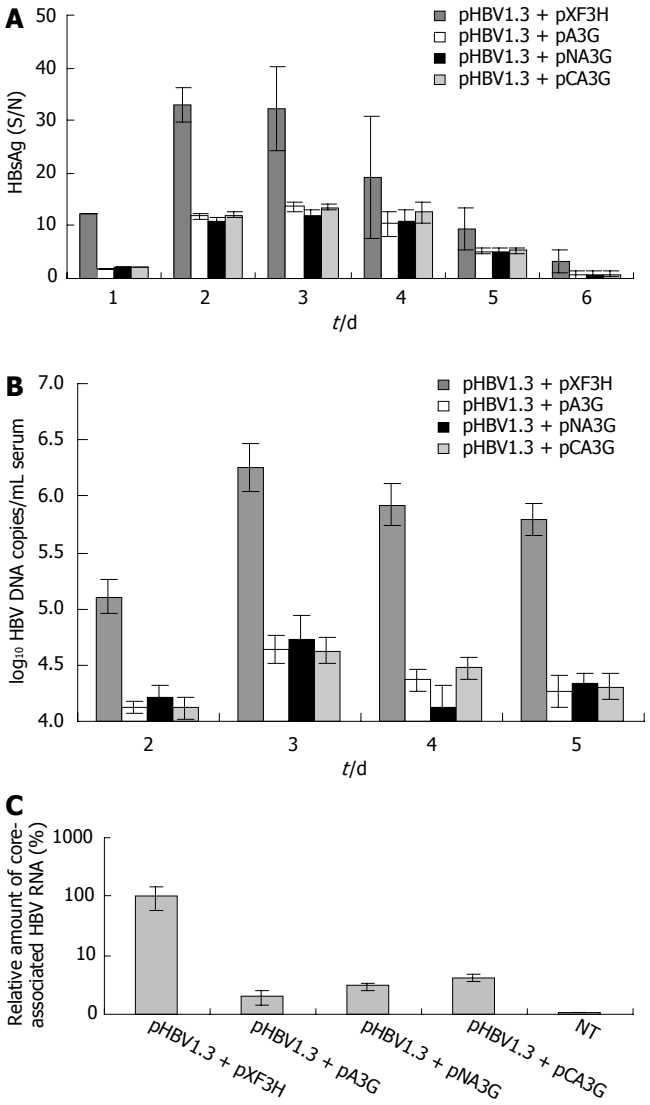



**Figure 5** The N-terminal or C-terminal cytosine deaminase domain of APOBEC3G inhibits HBV replication and gene expression in mice. (**A**) Serum HBsAg levels in the mice treated with APOBEC3G and its N-terminal and C-terminal cytosine deaminase domain expression vector. HBsAg levels (S/N, S: absorbance of the sample, N: absorbance of the negative control) in the sera of BALB/c mice were significantly reduced after treatment with APOBEC3G and its N-terminal and C-terminal cytosine deaminase domain expression plasmids (error bar indicates standard error) in the indicated time points. (**B**) Real-time PCR quantification of HBV DNA in the sera of mice treated with APOBEC3G and its N-terminal and C-terminal cytosine deaminase domain expression plasmid. BALB/c mice were co-injected with pHBV1.3 vector (10 μg) and the indicated expression plasmids (10 μg). HBV DNA levels of sera were determined by quantitative PCR ($n$ = 6 per treatment group). (**C**) Real-time PCR quantification of core-associated HBV RNA in the liver of mice treated with APOBEC3G and its N-terminal or C-terminal cytosine deaminase domain expression plasmids. BALB/c mice were co-injected with pHBV1.3 vector (10 μg) and the N-terminal or C-terminal cytosine deaminase domain expression vector of APOBEC3G (10 μg) 3 d after injection, and the levels of the liver HBV RNA were determined by quantitative RT-PCR, normalized to GAPDH mRNA and reported as a ratio of HBV mRNA/GAPDH mRNA (± SD) ($n$ = 6 per treatment group). NT: non-treated group; pXF3H: empty vector; pA3G: APOBEC3G expression plasmids; pNA3G: N-terminal cytosine deaminase domain of APOBEC3G expression plasmids; pCA3G: C-terminal cytosine deaminase domain of APOBEC3G expression plasmids; HBsAg: hepatitis B virus surface antigen.

HuH-7 hepatoma cells. Previous studies suggested that G-to-A hypermutation could influence HBV pathogenesis. Specific G-to-A changes yielded HBeAg-negative HBV variants, often isolated from patients with acute fulminant hepatitis, as well as HBV vaccine escape mutants[31,32]. However, there is no evidence so far that APOBEC3G-induced lethal hypermutation represents an important innate defense mechanism down-regulating hepadnavirus production.

APOBEC3G site-directed mutation experiments showed that both the N- and the C-terminal cytosine deaminase domains were required for inhibiting the infectivity of HIV-based retroviruses[6,7], and the C-terminal cytosine deaminase domain of APOBEC3G governed the catalytic activity and the retroviral hypermutation specificity, while the N-terminal deaminase domain was to perform other aspects of retroviral restriction[33]. Another study suggested that the N-terminal deaminase domain of APOBEC3G mediates encapsidation and RNA binding, while the C-terminal cytosine deaminase domain mediates cytidine deaminase activity[17].

Although APOBEC3G could inhibit HBV replication, extensive editing was not found to be the major contributing factor[19,20]. Each catalytic domain contains the conserved motif in which the His and Cys residues coordinate $Zn^{2+}$ and the Glu serves as a proton shuttle in the deamination reaction[22]. In this study, we found the N-terminal and C-terminal cytosine deaminase domain of APOBEC3G could inhibit HBV DNA replication and gene expression in cell culture and the HBV vector-based mouse model. Together with the fact that catalytically inactive APOBEC3G derivatives no longer inhibited VIF-defective HIV-1 but did conserve wild-type levels of activity on HBV, confirming the hypothesis that APOBEC3G might act on HBV and retroviruses through different mechanisms. As a result, we speculated that the RNA binding activity might play a role in these processes, for example, APOBEC3G might bind to the pregenome RNA or interfere with the binding of the ε subunit and RT domain of HBV DNA polymerase, but this needs further investigation.

Given the high heterogeneity of HBV sequences, HBV can be classified into 8 genotypes according to the sequence divergence[28]. Our results and other group showed that APOBEC3G inhibited the replication of genotype A. Whether APOBEC3G could also inhibit the replication of HBV with different genotypes (genotypes B and C) from clinical isolates is a great concern. As we expected, pXFA3G could also inhibit the replication of the most prevalent genotypes B and C from clinical isolates to a similar extent as on pHBV1.3, thereby indicating the inhibitory effect of APOBEC3G is different than that of the siRNAs which is sequence-specific.

The hydrodynamic delivery of nucleic acids in the mouse was described by Liu *et al*[23], who showed that the vast majority of the injected nucleic acid was delivered to the liver by this technique. Yang *et al*[30] first demonstrated that hydrodynamic injection of a replication-competent HBV vector resulted in high levels of HBV replication in the livers of the injected mice. In the vector-based model, HBV replicates in the liver of immunocompetent mice for 7-10 d, resulting in detectable levels of HBV RNA and antigens in the liver and of HBV DNA and antigens in the serum. Several reports have documented the use of the HBV vector model to examine the *in vivo* activity

of co-HDI-administered HBV-targeted unmodified siRNAs[34,35] or vector-expressed short hairpin RNAs[36] in silencing HBV gene expression. Most notably, we have demonstrated *in vivo* activity of APOBEC3G and its N-terminal or C-terminal cytosine deaminase domain expression vector via standard intravenous administration. This is probably the first demonstration of APOBEC3G *in vivo* activity in a hepatitis animal model with a clinically viable route of administration. Although we observed a more than 50 times reduction in serum HBV levels after treatment with APOBEC3G and its N-terminal or C-terminal cytosine deaminase domain in an HBV mouse model, the potential contribution of toxicity from the APOBEC3G has not been ruled out and will need to be investigated further.

During the course of an acute infection, HBV DNA clearance apparently occurs through noncytopathic mechanisms in which interferon γ (IFN-γ) and tumor necrosis factor α (TNF-α) play an important role[37,38]. APOBEC3G might participate in this type of antiviral response. Although APOBEC3G is not normally expressed in the liver, but a recent study has shown that APOBEC3G is induced by IFN-α stimulation in human hepatocytes[39]. It is possible that it is induced by IFN in the course of HBV infection. Several studies have shown that IFNs inhibit HBV replication *in vitro* and *in vivo* in human hepatoma cells and HBV transgenic mice[40-42]. Currently, IFN-α is an approved treatment for chronic hepatitis B. We speculate that APOBEC3G might be responsible for the anti-HBV action of IFNs under hepatic inflammation. Further analyses will be necessary to determine whether APOBEC3G plays roles in the human innate defense against hepatitis viruses *in vivo*.

Our study demonstrated that APOBEC3G and its N-terminal and C-terminal cytosine deaminase domain effectively inhibited HBV replication in the culture cells and mammalian liver, showing that such an approach could be useful in the treatment of HBV infection. However, whether suppression of viral replication by APOBEC3G plays a role during natural HBV infection seems speculative at present, because expression of APOBEC3G in human liver tissue has not yet been shown. Nevertheless, a better understanding of the mechanisms of A3G action may help identify new therapeutic strategies against chronic hepatitis B.

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*BASIC RESEARCH*

# Toxicological study of the hepatotherapeutic herbal formula, Chunggan extract, in beagle dogs

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## Abstract

**AIM:** To evaluate the pharmaceutical safety of a Chinese herbal formula, Chunggan extract (CGX), traditionally prescribed as a hepatotherapeutic drug *via* systemic acute and subacute toxicological study.

**METHODS:** Twenty male dogs and 20 female dogs were fed doses 50 times and 4 times greater than the clinically-recommended drug dosages in an acute and a subacute toxicological study, respectively. Adverse effects were examined by comparing the differences between normal and drug-administered groups using clinical signs, necropsies, histopathologic findings, haematology, urinalysis, and biochemical analysis.

**RESULTS:** In the acute study no change in the body weight, diarrhoea, apetite, mortality rate and histopathology of major organs was observed in male or female dogs with a single administration of CGX at 5 g/kg. No drug-induced abnormalities at analysis of histopathology, haematology, urinalysis, and biochemistry were found with any dose of this drug.

**CONCLUSION:** CGX is supposed to be very safe when used in a clinical application with a wide therapeutic index.



**Key words:** Hepatotherapeutics; Herbal remedy; Chunggan extract; Safety; Toxicological study

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## INTRODUCTION

Worldwide use of complementary and alternative medicines, including herbal products for various health benefits, has recently increased[1,2]. Botanical compounds and medical plants have also attracted attention among drug investigators and the pharmaceutical industry. Many herbal formulae have been traditionally prescribed for patients with hepatic disorders, and researchers have recently studied many compounds from natural resources as potential hepatotherapeutic candidates[3-5].

However, many issues related to a lack of scientific evidence about the efficacy and safety of herbal remedies remain unresolved[6,7]. Many reports and warnings have been published, particularly about the potential hepatotoxicity of herbal products, because the liver is a prime target for the toxic effects of general drugs[8-10].

Chunggan extract (CGX) is a potentially hepatotherapeutic drug derived from natural herbs. It has hepatoprotective effects on alcohol-, dimethylnitrosamine (DMN)-, and D-galactosamine-induced liver injury, a therapeutic effect on chronic liver disease, and an inhibitory effect on intestinal absorption and storage of cholesterol[11-14]. However, no animal-based high-dose toxicological study has been performed in conjunction with tests to confirm the drug's safety and efficacy in the appropriate dosages.

This study applied acute and subacute toxicological tests using beagle dogs to evaluate the wide-range tolerance and safety of Chunggan extract. This report aims to provide vital information about the efficacy and safety of multi-herbal plant-derived traditional Chinese medicine.

## MATERIALS AND METHODS

### *Components of CGX and fingerprinting analysis*

The ingredients of CGX include 5 g each of Artemisia capillaris Herba, Carapax Trionycis, Semen Raphani; 3 g each

of Rhizoma Atractylodis Macrocephalae, Poria, Alismatis Rhizoma, Atractylodis Rhizoma, Salviae Miltiorrhizae Radix; 2 g each of Polyporus, Amomi Fructus, Aurantii Fructus, and 1 g of Glycyrrhizae Radix or Helenii Radix. The 10.71% (w/w) lyophilized water-extract was obtained from the initial dried mixture according to the Korean standard over-the-counter (OTC) monographs, and its high-performance thin layer chromatography (HPTLC)-based fingerprint was produced by the CAMAG application system (Muttenz, Switzerland) (Figure 1A-D). For the HPTLC analysis, water extracts of CGX, Artemisia capillaris Herba, Aurantii Fructus, Glycyrrhizae Radix, Poria cocos and their standard components, 6,7-dimethoxycoumarin (Sigma Chemical Co., St. Louis, MO, USA), Poncirin (Fluka, St. Louis, MO, USA), Glycyrrhizic acid ammonium salt (Sigma Chemical Co., St. Louis, MO, USA) and Pachyman (Megazyme Co., Wicklow, Ireland) were dissolved in HPLC-grade methanol and applied to pre-washed silica gel 60 F254 HPTLC plates (size 20 cm × 10 cm, thickness of the silica gel 0.2 mm; Merck, Darmstadt, Germany) with an automated applicator (Linomat IV; CAMAG). The samples were then separated (migration distance 80 mm) using HPLC-grade ethyl acetate/formic acid/acetic acid/water (15:1:1:2) except for Poria cocos (migration distance 70 mm, n-butanol/methanol/water as 50:25:20). The migrated components were visualized under UV radiation at 366 nm or 254 nm using Reprostar 3 with a digital camera (CAMAG). Only Poria coco was visualized under white light after derivatization with aniline-diphenylamine-phosphoric acid solution.

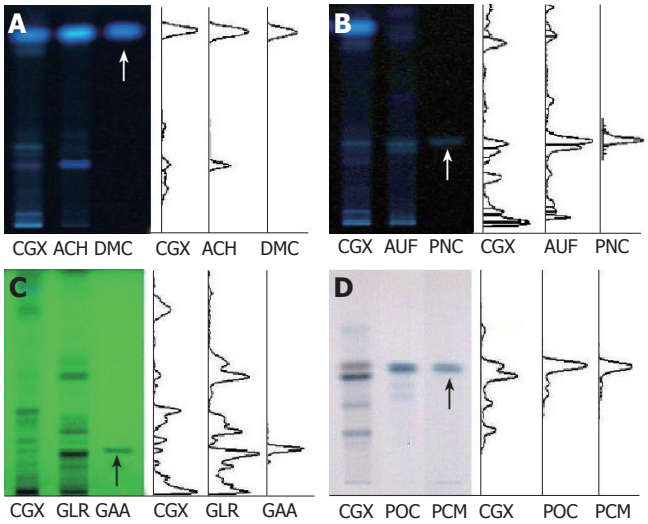


**Figure 1** High-performance thin layer chromatography method-based fingerprint for Chunggan extract. HPTLC analysis was performed to characterize CGX and its three major components (extracts from Artemisia capillaris Herba, Aurantii Fructus, Glycyrrhizae Radix and Poria cocos) with reference components, 6,7-dimethoxycoumarin, Poncirin, Glycyrrhizic acid ammonium salt and Pachyman (indicated by arrows). **A**: 2 μL of CGX (200 mg/mL) and 1 μL of the extract from Artemisia capillaris Herba (50 mg/mL) were subjected to HPTLC with 1 μL of 6,7-dimethoxycoumarin (0.1 mg/mL), then visualized under UV radiation at 366 nm; **B**: 2 μL of CGX (200 mg/mL) and 1 μL of the extract from Aurantii Fructus (50 mg/mL) were subjected to HPTLC with 1 μL of Poncirin (5 mg/mL), then visualized under UV radiation at 366 nm; **C**: 4 μL of CGX (200 mg/mL) and 4 μL of the extract from Glycyrrhizae Radix (50 mg/mL) were subjected to HPTLC with 4 μL of Glycyrrhizic acid ammonium salt (1 mg/mL), then visualized under UV radiation at 254 nm; **D**: 2 μL of CGX (40 mg/mL) and 1 μL of the extract from Poria cocos (10 mg/mL) were subjected to HPTLC with 10 μL of Pachyman (100 mg/mL), then visualized under white light after derivatization.

### *Animals*

In total, 40 beagle dogs (20 males and 20 females) were obtained from Jung-Ang Lab Inc. (Seoul, Korea). Each dog was acclimatized to conditions in kennel units (1.08 $m^2$) and was subjected to routine examination and acceptance procedures for 6 wk. Animal housing was maintained at 22 ± 3℃ with a 12-h light/dark cycle, and air was exchanged approximately 13-18 times/h. Each dog was fed 400 g/d of a standard dry diet (Gold Pet; Agribrands Purina Korea Inc., Seoul, Korea) and had free access to automatically filtered tap water that was checked using routine chemical monitoring (Korea Chemicals Inspection & Testing Institute). At the beginning of treatment, the dogs averaged approximately 5 mo of age and 8.5-11.5 kg in weight.

Prior to the experiment, a veterinarian performed an animal health review. This study complied with the Testing Guidelines of the Korea Food and Drug Administration (KFDA; notification no. 1999-61).

### *Acute toxicity*

After 12 h of fasting, two males and two females in each of four groups received oral doses (5 000, 2 500, 1 250, and zero mg/kg respectively) of CGX dissolved in distilled water through a catheter. Clinical observations were performed hourly for 6 h, after which mortality or clinical signs of toxicity were monitored daily for the following 2 wk. Body weight was measured weekly, and necropsy-gross findings were recorded on the final day.

### *Subacute toxicity*

Over 4 wk, three male and three female dogs received orally administered CGX in one of four dosages: 400, 200, 100, and zero mg/kg as a control. They were subsequently checked and measured carefully for mortality and clinical signs of toxicity (daily), body weight and food intake (weekly), ocular fundus examination, and slit lamp examination (before drug administration and 2 d before the final day). On the final day, all dogs were deprived of food, but not water, for 16 h. Urinalysis, hematologic, and various biochemical parameters of all dogs were measured using blood samples after collection via the jugular vein (before and on the final day of the experiment). After necropsy, organs (brain, hypophysis, heart, liver, spleen, kidneys, adrenal glands, prostate, testes, and ovaries) were weighed. Microscopic examination of the following organs was also done: lungs, trachea, heart, thymus, liver, kidneys, pancreas, spleen, thyroid, adrenal glands, testes, epididymes, ovaries, uterus, esophagus, stomach, duodenum, jejunum, ileum, cecum, colon, urinary bladder, aorta, brain, pituitary, prostate gland, and tongue. A histopathologist performed a complete examination of the tissue samples after they were stained with haematoxylin-eosin (H&E) following a 10% formalin fixation and embedded in paraffin wax.

### *Statistical analysis*

Distributions of body and organ weight, food intake, and

**Table 1 Summary for clinical observation and histological examination**

| CGX (mg/kg per day) | 0 | 100 | 200 | 400 |
|---|---|---|---|---|
| Mortality | - | - | - | - |
| Clinical signs | NAD | NAD | NAD | NAD |
| Anorexia | - | - | - | - |
| Soft feces | - | - | - | - |
| Food intake | NAD | NAD | NAD | NAD |
| Ophthalmologic findings | NAD | NAD | NAD | NAD |
| Relative organ weight | NS | NS | NS | NS |
| Necropsy findings | NAD | NAD | NAD | NAD |
| Histopathological findings | Rarely in some samples | Rarely in some samples | | |
| Liver | Focal necrosis | Subcutaneous necrosis | | |
| Lung | Yellow pigmentation | Yellow pigmentation | | |
| Kidney | Glomerular hyperemia | Glomerular hyperemia | | |
| Spleen | White pulp atrophy | White pulp atrophy | | |
| Stomach | *Helicobacter* | *Helicobacter* | | |

Three male and three female beagle dogs in each group were fed CGX, and the clinical symptoms including mortality were monitored for 4 wk. At the end of the experiment, ophthalmologic, necropsy-based, and histopathological examinations were performed. NAD: No abnormality detected; NS: Not significant; -: Absent.

haematological and biochemical parameters were analysed using Levene's test of equality of variance. When variance was homogenous, a one-way ANOVA was applied. Statistical differences in the means among groups were analysed using Dunnett's multiple comparison test[15,16]. When variance was not homogenous, Dunnett's *t*-test was performed. Statistical analyses were conducted using SPSS Base 10.0 (SPSS, Inc., Chicago, IL, USA).

## RESULTS

### *Acute toxicity*

After administration of CGX and distilled water (DW) as a control, all dogs exhibited decreased activity for 1 h. No clinical symptoms including diarrhoea, anorexia, or death were observed in any of the dogs during the remaining 2 wk of the experiment. Each group exhibited a normal range in weight gain (Figure 2A), and no abnormal findings were seen at necropsy in the major organs of males or female dogs treated with CGX (data not shown).

### *Subacute toxicity*

**Food consumption and body weight:** Food consumption did not appear to markedly differ in any CGX-treated group compared to the control over 4 wk (Figure 2B), although females in the CGX-treated groups consumed less food than the control group. This result may have been due to a lower body weight among females at the starting point, leading to reduced food consumption. Similarly, no groups exhibited significant gains or losses in body weight during the 4-wk experimental period (Figure 2C and D).

**Clinical signs, necropsy, and histopathological findings:** No clinical signs including anorexia or soft faeces were observed in any dogs during the 4 wk of the experiment. No tested groups exhibited any changes in ophthalmologic signs (summarised in Table 1). Macroscopic autopsy

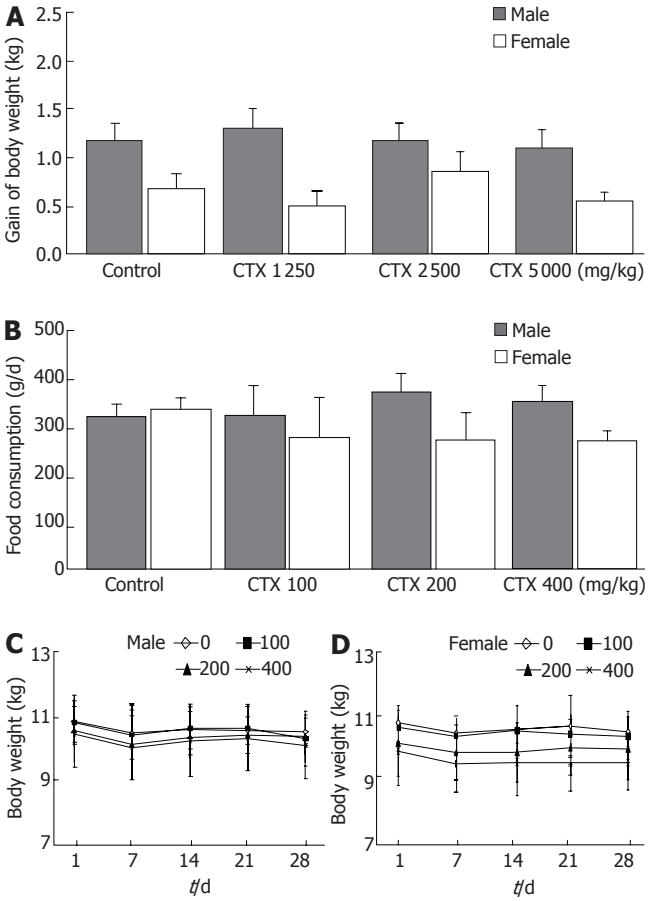


**Figure 2** Body weight and food consumption. **A**: Final gains in body weight for each group administered with indicated Chunggan extract (CGX) after 2 wk of the acute toxicity experiment ($n$ = 2 males and 2 females); **B**: Average daily food consumption for each group ($n$ = 3 males and 3 females) during the subacute toxicity experiment; **C**: Change in body weight during 4 wk of the subacute toxicological experiment, shown separately for males ($n$ = 3) and females ($n$ = 3). Values represent the mean ± SD.

examination did not reveal any pathological findings in any organs, and no significant changes to body weight ratio were found in any of the 13 tested organs of either treated or control groups: liver, kidneys, spleen, adrenals, ovaries, brain, pituitary gland, lungs, heart, thymus, thyroid, uterus, and urinary bladder (data not shown). However, microscopic examination detected rare pathological findings, such as focal necrosis with macrophage, lymphocyte, plasma cells, hyperaemia, and subcutaneous edema in livers, bronchopneumonia in lungs, glomerular hyperaemia in kidneys, white pulp atrophy in spleens, and *Helicobacter* in stomachs; similar findings were exhibited by groups treated with CGX and the control group. However, no systematic histopathological differences were observed between groups. Figure 3 presents the only observed focally pathological features in kidneys and livers between the control group and the group treated with the highest levels of CGX (400 mg/kg per day).

**Haematology and urinalysis:** Before the experiment began, tests indicated that all dogs (12 males and 12 females) had normal haematological values (data not shown). Haematology was measured again on the final day of the experiment, and results revealed a significant decrease in mean corpuscular haemoglobin concentration (MCHC)

**Table 2 Haematological analysis after administration of Chunggan extract for 4 wk**

| Number of dogs | Male (12) | | | | Female (12) | | | |
|---|---|---|---|---|---|---|---|---|
| CGX (mg/kg·d) | 0 | 100 | 200 | 400 | 0 | 100 | 200 | 400 |
| Erythrocyte (M/UL) | 6.4 ± 0.6 | 6.80 ± 0.4 | 6.56 ± 0.4 | 6.70 ± 0.1 | 6.22 ± 0.5 | 6.70 ± 0.5 | 6.77 ± 0.5 | 6.59 ± 0.2 |
| Hemoglobin (g/dL) | 18.6 ± 2.1 | 17.6 ± 0.4 | 18.2 ± 1.0 | 18.1 ± 1.3 | 16.5 ± 1.7 | 17.5 ± 1.8 | 17.8 ± 0.4 | 17.7 ± 0.1 |
| Hematocrit (%) | 41.3 ± 4.1 | 42.8 ± 1.8 | 42.8 ± 2.2 | 42.2 ± 1.0 | 39.2 ± 2.8 | 40.1 ± 2.1 | 43.3 ± 3.9 | 41.6 ± 0.9 |
| MCV (fL) | 64.1 ± 0.3 | 63.1 ± 2.4 | 65.4 ± 2.8 | 62.9 ± 1.0 | 63.1 ± 0.1 | 60.1 ± 4.1 | 63.9 ± 1.4 | 63.1 ± 1.9 |
| MCH (pg) | 28.9 ± 1.1 | 26.0 ± 1.4 | 27.8 ± 2.3 | 27.0 ± 1.7 | 26.5 ± 1.2 | 26.1 ± 2.7 | 26.4 ± 2.4 | 26.8 ± 0.7 |
| MCHC (g/dL) | 45.2 ± 1.5 | 41.7 ± 0.6[a] | 42.4 ± 2.2 | 42.9 ± 2.2 | 42.1 ± 2.2 | 43.5 ± 2.5 | 41.3 ± 4.6 | 42.6 ± 0.7 |
| RDW (%) | 15.1 ± 0.1 | 15.9 ± 0.3 | 15.3 ± 0.4 | 15.9 ± 0.3 | 15.7 ± 0.1 | 15.9 ± 0.6 | 15.7 ± 0.5 | 15.1 ± 0.1 |
| Reticulocytes (%) | 0.30 ± 0.1 | 0.23 ± 0.1 | 0.37 ± 0.2 | 0.43 ± 0.2 | 0.77 ± 0.2 | 0.50 ± 0.4 | 0.53 ± 0.6 | 0.50 ± 0.2 |
| Platelets (k/UL) | 349 ± 13 | 320 ± 125 | 334 ± 50 | 344 ± 46 | 347 ± 94 | 333 ± 90 | 239 ± 87 | 245 ± 18 |
| MPV (fL) | 12.8 ± 0.3 | 15.1 ± 1.2 | 14.6 ± 2.2 | 14.0 ± 1.3 | 13.4 ± 1.6 | 12.8 ± 1.6 | 16.4 ± 4.2 | 15.0 ± 4.7 |
| PT (s) | 6.73 ± 0.2 | 7.20 ± 0.6 | 6.63 ± 0.3 | 6.77 ± 0.2 | 6.73 ± 0.3 | 7.00 ± 0.4 | 6.77 ± 0.1 | 6.93 ± 0.5 |
| aPTT (s) | 14.2 ± 0.5 | 19.3 ± 5.2 | 15.4 ± 0.7 | 15.2 ± 0.9 | 15.7 ± 0.2 | 15.4 ± 0.9 | 16.1 ± 2.6 | 16.5 ± 0.7 |
| Leukocytes (k/UL) | 9.86 ± 1.2 | 9.51 ± 1.3 | 8.56 ± 1.3 | 11.4 ± 1.4 | 9.64 ± 1.3 | 10.6 ± 0.2 | 11.8 ± 3.8 | 8.9 ± 1.6 |
| Neutrophil (%) | 61.0 ± 10 | 59.8 ± 15 | 65.7 ± 14 | 66.9 ± 10 | 57.0 ± 13 | 60.9 ± 14 | 49.2 ± 2.3 | 62.8 ± 13 |
| Lymphocyte (%) | 19.5 ± 4.1 | 23.7 ± 4.2 | 21.4 ± 4.7 | 21.8 ± 2.6 | 28.2 ± 5.2 | 26.7 ± 8.5 | 35.5 ± 13 | 27.1 ± 7.9 |
| Monocytes (%) | 7.5 ± 2.0 | 8.0 ± 3.2 | 6.9 ± 1.2 | 5.3 ± 0.9 | 7.2 ± 1.0 | 7.6 ± 5.7 | 10.4 ± 5.1 | 7.8 ± 2.2 |
| Eosinophil (%) | 11.6 ± 9.1 | 8.41 ± 4.2 | 5.96 ± 5.8 | 5.70 ± 1.7 | 7.47 ± 11 | 4.25 ± 1.9 | 4.15 ± 1.7 | 2.70 ± 2.4 |
| Basophil (%) | 0.30 ± 0.3 | 0.11 ± 0.1 | 0.12 ± 0.3 | 0.26 ± 0.2 | 0.31 ± 0.6 | 0.13 ± 0.1 | 0.34 ± 0.5 | 0.11 ± 0.1 |

After administration of Chunggan extract (CGX) for 4 wk, haematology was analysed to compare CGX-treated groups to the control group. MCV: Mean corpuscular volume; MCH: Mean corpuscular haemoglobin; MCHC: Mean corpuscular haemoglobin concentration; RDW: Red cell distribution width; MPV: Mean platelet volume; PT: Prothrombin time; APPT: Activated partial thromboplastin time. aindicates a significant difference at a level of $P < 0.05$.

only in male dogs treated with 100 mg/kg per day CGX compared to control dogs; 200 or 400 mg/kg per day male and all female dogs produced normal values (Table 2). No other haematological indicators revealed any significant change.

No other unusual results were revealed by urinalysis before and after administration of CGX (data not shown).

**Serum biochemistry:** The serum chemistry in all dogs (12 males and 12 females) was checked before beginning CGX administration. Another chemistry analysis was conducted on the final day. Results did not reveal any significant changes to biochemistry values between the control and CGX groups, except the BUN levels in female dogs who were administered 400 mg/kg per day CGX when compared to the control group (Table 3).

## DISCUSSION

Among the variety of complementary and alternative medicines, Asian doctors have prescribed herbal mixtures to treat various diseases according to traditional Oriental pharmacology. Since this traditional treatment is based on extensive knowledge gathered from applications of natural resources to humans, people have usually assumed that the treatment is safe. However, as Oriental remedies have been rapidly growing in popularity worldwide, researchers are paying as much attention to safety issues as to their therapeutic efficacy[17-21]. Many studies have therefore been conducted to evaluate the safety of Oriental herbal medicines or the associated risk of adverse effects[22-25].

This study applied a systemic acute and subacute toxicological examination using beagle dogs to investigate the possibility of adverse effects associated with Chunggan extract, a modified-traditional hepatotherapeutic herbal drug.

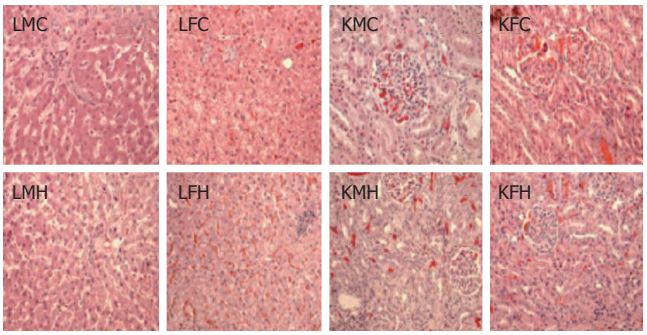


**Figure 3** Histopathological findings. On the final day of the subacute toxicological experiment, 13 main organs (indicated in the text) were removed from each dog, and microscopic examinations were carefully performed after a general H&E staining process. Livers, lungs, kidneys, spleens, and stomachs from the control and the group fed CGX revealed uniformly rare focal pathological findings. This figure presents the only pathological findings among livers and kidneys from the control and the group treated with the highest level of CGX, such as focal necrosis and hyperemia in livers, and glomerular hyperemia in kidneys (HE stain, X 200). LMC: liver from male control; LFC: liver from female control; LMH: liver from male high dose (400 mg/kg); LFH: liver from female high dose (400 mg/kg); KMC: kidney from male control; KFC: kidney from female control; KMH: kidney from male high dose (400 mg/kg); KFH: kidney from female high dose (400 mg/kg).

In clinical application, the therapeutic dosage of CGX is 100 mg/kg per day, usually divided into three daily doses. We treated dogs with 50 times the clinically recommended dosage of CGX, but observed no clinical signs of adverse effects except slightly decreased activity for 1 h in every group including the control. The decrease in activity might have resulted from the forced administration of a high volume of CGX (or water in the control group) via a catheter. Typical adverse signs of any drug include changes in appetite or faeces; even a 50-fold increase in CGX did

**Table 3 Serum chemistry analysis after administration of Chunggan extract for 4 wk**

| Number of dogs | Male (12) | | | | Female (12) | | | |
|---|---|---|---|---|---|---|---|---|
| CGX (mg/kg per day) | 0 | 100 | 200 | 400 | 0 | 100 | 200 | 400 |
| AST (IU/L) | 37.0 ± 3.4 | 36.3 ± 2.0 | 40.3 ± 14.2 | 41.3 ± 8.3 | 40.6 ± 8.5 | 34.3 ± 5.7 | 37.0 ± 4.3 | 40.0 ± 9.5 |
| ALT (IU/L) | 57.6 ± 7.3 | 48.6 ± 8.1 | 49.3 ± 29.7 | 65.6 ± 24.0 | 38.0 ± 7.0 | 46.6 ± 16.2 | 44.0 ± 1.7 | 33.6 ± 9.0 |
| ALP (IU/L) | 328 ± 46 | 299 ± 102 | 390 ± 221 | 238 ± 74 | 271 ± 104 | 391 ± 93 | 377 ± 159 | 174 ± 43 |
| BUN (mg/dL) | 18.2 ± 1.3 | 20.1 ± 3.1 | 22.8 ± 3.6 | 19.3 ± 3.5 | 13.4 ± 1.9 | 17.6 ± 2.6 | 13.9 ± 3.9 | 24.4 ± 4.8[a] |
| Creatinine (mg/dL) | 1.05 ± 0.1 | 1.20 ± 0.1 | 1.23 ± 0.1 | 1.18 ± 0.1 | 1.03 ± 0.2 | 1.15 ± 0.1 | 1.09 ± 0.1 | 1.11 ± 0.1 |
| Glucose (mg/dL) | 99.0 ± 15.5 | 115.6 ± 5.1 | 112.3 ± 11.9 | 110.7 ± 4.2 | 114 ± 14 | 121 ± 10.2 | 117 ± 5.6 | 116 ± 26.1 |
| Total chol. (mg/dL) | 252 ± 30 | 223 ± 34 | 260 ± 25 | 217 ± 51 | 299 ± 73 | 296 ± 34 | 263 ± 24 | 261 ± 58 |
| Total bilirubin (mg/dL) | 0.23 ± 0.1 | 0.27 ± 0.1 | 0.23 ± 0.1 | 0.30 ± 0.1 | 0.30 ± 0.1 | 0.30 ± 0.0 | 0.30 ± 0.1 | 0.33 ± 0.1 |
| Total protein (g/dL) | 7.83 ± 0.2 | 7.37 ± 0.2 | 7.33 ± 0.2 | 7.37 ± 0.3 | 7.60 ± 0.3 | 7.47 ± 0.6 | 7.40 ± 0.4 | 7.53 ± 0.2 |
| Albumin (g/dL) | 4.73 ± 0.3 | 4.33 ± 0.2 | 4.47 ± 0.1 | 4.60 ± 0.4 | 4.77 ± 0.3 | 4.50 ± 0.3 | 4.63 ± 0.3 | 4.57 ± 0.2 |
| AG ratio | 1.53 ± 0.2 | 1.43 ± 0.2 | 1.60 ± 0.2 | 1.67 ± 0.3 | 1.70 ± 0.1 | 1.50 ± 0.1 | 1.70 ± 0.1 | 1.57 ± 0.1 |
| C.phosphokinase (IU/L) | 133 ± 15 | 130 ± 39 | 171 ± 89 | 157 ± 48 | 199 ± 30 | 116 ± 24 | 155 ± 37 | 144 ± 23 |
| Triglyceride (mg/dL) | 25.0 ± 3.0 | 35.6 ± 24.8 | 30.0 ± 3.6 | 287 ± 7.2 | 36.0 ± 16.6 | 28.4 ± 9.5 | 36.0 ± 5.2 | 47.3 ± 17.1 |
| Calcium (mg/dL) | 14.8 ± 0.5 | 14.5 ± 0.5 | 14.6 ± 0.3 | 14.4 ± 0.2 | 14.5 ± 0.5 | 14.7 ± 0.9 | 14.1 ± 0.4 | 14.6 ± 0.7 |
| Inorganic pho. (mg/dL) | 3.33 ± 1.1 | 4.43 ± 0.3 | 4.23 ± 0.3 | 4.43 ± 0.6 | 3.80 ± 0.5 | 3.90 ± 0.6 | 4.03 ± 0.8 | 3.63 ± 0.3 |
| LDH (IU/L) | 105 ± 45.7 | 132 ± 73.5 | 91.7 ± 32.8 | 106 ± 60.3 | 80.0 ± 11.5 | 68.3 ± 9.7 | 146 ± 60.7 | 146 ± 126.1 |
| GGT (IU/L) | 4.63 ± 0.3 | 3.93 ± 1.3 | 4.03 ± 0.4 | 4.37 ± 1.3 | 4.93 ± 1.0 | 5.13 ± 0.4 | 4.57 ± 1.0 | 5.53 ± 0.5 |
| Sodium (mmol/dL) | 148 ± 2.0 | 146 ± 1.1 | 144 ± 2.1 | 144 ± 0.6 | 146 ± 1.2 | 148 ± 1.00 | 147 ± 2.0 | 147 ± 0.4 |
| Potassium (mmol/dL) | 4.21 ± 0.1 | 4.32 ± 0.1 | 4.31 ± 0.2 | 4.18 ± 0.2 | 4.08 ± 0.23 | 3.95 ± 0.2 | 4.24 ± 0.3 | 4.42 ± 0.4 |
| Chloride (mmol/dL) | 116 ± 1.1 | 115 ± 2.5 | 115 ± 2.3 | 116 ± 0.6 | 117 ± 1.5 | 114 ± 1.5 | 114 ± 3.2 | 114 ± 1.5 |

After administration of Chunggan extract (CGX) for 4 wk, serum chemistry was analysed to compare CGX-treated groups to the control group. AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; ALP: Alkaline phosphatase; BUN: Blood urea nitrogen; AG ratio: Albumin globulin ratio; LDH: Lactate dehydrogenase; GGT: Gamma glutamyl transferase. [a]$P < 0.05$ among BUN in female grops treated with 0 mg, 100 mg, 200 mg and 400 mg CGX.

not affect appetite or faeces in any group, nor did it affect body weight changes in any group (Figure 2A). A similar pattern was exhibited over repeated CGX administration for 4 wk with dosages ranging from 100 to 400 mg/kg (Table 1). However, no dog in any group (male or female) gained body weight during the 4 wk, which may have resulted from the daily repeated stress of drug or water administration. The variations in body weight on the final day corresponded to the differences in initial body weight at the starting point (Figure 2C and D).

During the subacute toxicological study, no groups exhibited changes in clinical signs. Ophthalmologic examination, analysis of organ weights, and autopsy findings on the final day revealed dogs were free of pathologic states. Because the microscopic examination revealed that every group including the control exhibited focally pathological findings in livers, kidneys, lungs, stomachs, and spleens (Table 1, Figure 3), they were not caused by CGX administration. Furthermore, the positive results for *H pylori* in all groups might be related to statistics showing that this disease has infected 78% of dogs in Korea[26].

Haematology and chemistry analysis revealed that groups treated with CGX showed no significantly changed values except MCHC in males treated with 100 mg (41.7 ± 0.6) and BUN in females treated with 400 mg (24.4 ± 2.8), shown in Tables 2 and 3 respectively. However, the significance of these changes is doubtful since the values were within the normal range for dogs and the results did not exhibit a dose-dependent or histopathological correlation. It is important to examine potential hepatotoxicity in herbal products because therapeutic and toxic properties apply similar biological processes, especially in the liver[27]. Serum values of aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), and bilirubin are among the most sensitive markers of liver damage[28]. As expected, treatment with CGX during this study resulted in normal values identical to the control group.

In conclusion, this study verifies that CGX could be safely used in a clinical application with a very large therapeutic index.

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*BASIC RESEARCH*

# Comparison between bioartificial and artificial liver for the treatment of acute liver failure in pigs

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## Abstract

**AIM:** To characterize and evaluate the therapeutic efficacy of bioartificial liver (BAL) as compared to that of continuous hemodiafiltration (CHDF) with plasma exchange (PE), which is the current standard therapy for fulminant hepatic failure (FHF) in Japan.

**METHODS:** Pigs with hepatic devascularization were divided into three groups: (1) a non-treatment group (NT; $n$ = 4); (2) a BAL treatment group (BAL; $n$ = 4), (3) a PE + CHDF treatment group using 1.5 L of normal porcine plasma with CHDF (PE + CHDF, $n$ = 4). Our BAL system consisted of a hollow fiber module with 0.2 μm pores and 1 x $10^{10}$ of microcarrier-attached hepatocytes inoculated into the extra-fiber space. Each treatment was initiated 4 h after hepatic devascularization.

**RESULTS:** The pigs in the BAL and the PE + CHDF groups survived longer than those in the NT group. The elimination capacity of blood ammonia by both BAL and PE + CHDF was significantly higher than that in NT. Aromatic amino acids (AAA) were selectively eliminated by BAL, whereas both AAA and branched chain amino acids, which are beneficial for life, were eliminated by PE + CHDF. Electrolytes maintenance and acid-base balance were better in the CPE + CHDF group than that in the BAL group.

**CONCLUSION:** Our results suggest that PE + CHDF eliminate all factors regardless of benefits, whereas BAL selectively metabolizes toxic factors such as AAA. However since PE + CHDF maintain electrolytes and acid-base balance, a combination therapy of BAL plus CPE + CHDF might be more effective for FHF.



## INTRODUCTION

Fulminant hepatic failure (FHF) has been one of the most challenging problems in clinical medicine. Although plasma exchange (PE) is still a main therapeutic modality in Japan, PE was found not to affect mortality in cases of liver failure[1]. Therefore, recently the combination of continuous PE and continuous hemodiafiltration (CHDF) has been proposed, and its initial clinical results have been encouraging[2-4]. In fact, however, no studies have compared CHDF with other therapeutic modalities.

We have developed a BAL system utilizing porcine hepatocytes cultured on collagen-coated beads and reported on its efficacy[5]. Although many studies have reported the efficacy of CPE + CHDF or BAL treatment, little is known about how these two modalities compare with each other.

The purpose of the present study is to compare the therapeutic efficacy of BAL support for ischemic FHF with that of CPE + CHDF treatment in pigs. In addition, a characterization of the benefit of each treatment was attempted.

## MATERIALS AND METHODS

All study protocols were reviewed and approved by the University of Nagasaki Research Animal Resources Animal Care Committee and met both institutional and national guidelines.

### *Hepatocyte isolation*

Hepatocytes were harvested from outbred female white pigs weighing 8 to 10 kg, according to the method described by Seglen with our modification[6]. Briefly, under isoflurane anesthesia, the portal vein was cannulated with a silicon tube and the liver was perfused *in situ* with 4 L of oxygenated 2 mmol/L ethylenediaminotetraacetic acid

(EDTA) solution at the rate of 400 mL/min at 37℃. Immediately after the perfusion, the liver was removed and placed in a sterile basin. Ten minutes later, the EDTA solution was replaced with a $Ca^{2+}$-enriched Leffert's buffer solution containing 0.05% type P collagenase (Sigma Chemical Co. St. Louis, MO). The solution was re-circulated through the liver in the basin at a rate of 400 mL/min for 20 min under a clean air-filtered hood. After the collagenase perfusion, the digested liver was manually disrupted in cold DMEM (Nissui Pharmaceutical, Tokyo, Japan) containing 10% heat-inactivated fetal calf serum (FCS, Pharmacia LKB Biotechnology, Piscataway, NJ) and 1% penicillin-streptomycin (Life Technologies, Grand Island, NY). The crude liver cells were filtered through a Mesh Filtration Unit (mesh space, 105 μm; Spectrum, Laguna Hills, CA), and the enriched fractions of the hepatocytes were separated by low-speed centrifugation (at 50 g for 2 min, at 4℃). Centrifugation was performed three times. The initial viability of the hepatocytes was determined using the trypan blue dye exclusion test.

### Preparation of the bioreactor

Isolated hepatocytes with a viability of better than 90% and collagen coated dextran microcarrier beads (Cytodex 3, Pharmacia LKB Biotechnology, Piscataway, NJ) were incubated with DMEM with 10% FCS in a cell culture bag (Si culture bag, Wako, Tokyo, Japan) for 4 h at 95% air and 5% $CO_2$ at 37℃. After washing with normal saline, ten billion microcarrier-induced hepatocytes were inoculated into the extra-fiber space (185 mL) of the hollow fiber module with 0.8 $m^2$ of total external fiber surface (Plasmaflo OP-08, Asahi Medical, Tokyo, Japan). The hollow fibers were made from polyethylene materials with an internal diameter of 340 mm, a pore size of 0.2 μm, and membrane thickness of 50 μm.

### Animal model of fulminant hepatic failure

Under general anesthesia with mechanical ventilation via an endotracheal tube, pigs (weighing 27 to 30 kg) underwent hepatic devascularization consisting of dissection of the biliary tract, hepatic arteries, and ligaments after creation of an end-to-side portocaval shunt according to the method described by Mazziotti *et al*[7]. This model was reported to reproduce the many pictures of FHF including, encephalopathy, coagulopathy and brain edema. A femoral artery was cannulated for sampling blood and monitoring blood pressure. A jugular vein was also cannulated with a double lumen catheter for blood access. The volume of the replacement fluid was adjusted over the range of 3-6 L, with 5% dextrose lactate Ringer solution depending on the response of the FHF pigs.

### Circulation system of BAL

The BAL system consisted of a hollow fiber module inoculated with microcarrier-attached porcine hepatocytes, a plasmapheresis unit (Plasauto 2500 and Plasmaflo OP-2, Asahi Medical, Tokyo, Japan), a roller pump, and a plasma reservoir (Figure 1). The system was oxygenated and warmed at 37℃ using a membrane-type oxygenator. Whole blood was removed at a rate of 35-45 mL/min from the jugular vein and was separated to plasma at a rate of 15 mL/min. The separated plasma was perfused through the bioreactor at a rate of 400 mL/min and was recirculated through the whole system with the transmission reservoir (Figure 1).

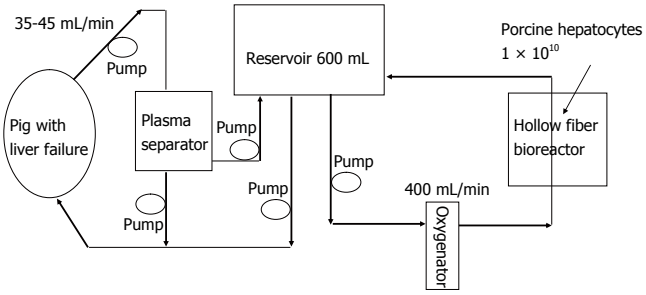


**Figure 1** Schematic drawing of our bioartificial liver (BAL) system. It consists of 1 × $10^{10}$ cultured allogeneic hepatocytes in the outer space of a hollow fiber.

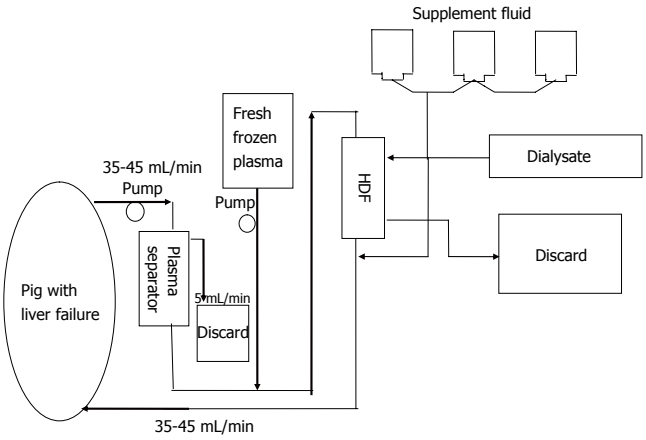


**Figure 2** Schematic drawing of continuous plasma exchange (CPE) with allogeneic plasma and continuous hemodiafiltration system (CHDF).

### Circulation system of PE + CHDF

The PE + CHDF system consisted of the plasmapheresis unit mentioned above, a high performance hemodiafiltration unit (Panflo, APF-03S, Asahi Medical,Tokyo, Japan) and three roller pumps (Figure 2). In this system, the apparatuses were connected in a series. PE + CHDF was performed for 6 h. An average of 1.5 L fresh porcine frozen plasma was used during PE treatment. Dialysis was performed concomitantly at a flow rate of 50 mL/min using a dialysis fluid containing bicarbonate buffer (Sublood B; Fuso Pharmaceutical, Tokyo, Japan).

### Experimental design

Animals undergoing hepatic devascularization were divided into three groups as follows: a non-treatment group (NT; only plasma separation, $n$ = 4), a BAL treatment group (BAL; $n$ = 4), and a CPE + CHDF treatment group (CPE + CHDF; $n$ = 4). The BAL or CPE + CHDF treatments were initiated at 4 h after completion of hepatic devascularization. The pigs in both the BAL and CPE + CHDF groups were treated for 6 h and were given 800 U/h of heparin as an anticoagulant. After 6 h BAL or CPE + CHDF was stopped and the pigs were observed until their death.

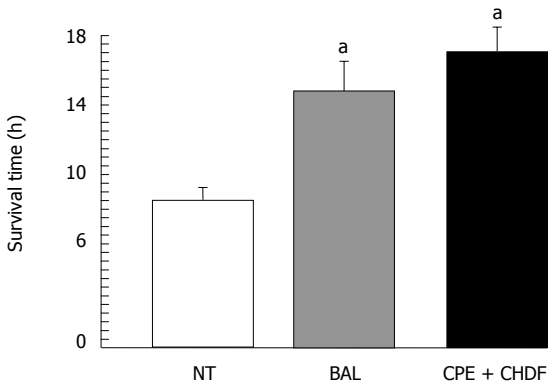


**Figure 3** Survival after induction of hepatic failure. $^{a}P < 0.05$ *vs* NT group.

### *Assessments*

Conventional liver function tests including aspartate aminotransferase (AST) and alanine aminotransferase (ALT), the plasma levels of branched chain amino acids (BCAA), aromatic amino acids (AAA), the ratios of the BCAA to the AAA (Fischer's ratio) after each treatment, and the changes in plasma ammonia were determined. Plasma levels of electrolytes, blood urea nitrogen (BUN), creatinine, and pH were measured. Survival time was also observed.

### *Statistical analysis*

Data were expressed as mean ± SD, and were analyzed statistically using a non-parametric test. $P < 0.05$ was considered to indicate a significant difference.

## RESULTS

### *Survival*

Although the pigs in the BAL and the CPE + CHDF groups survived significantly longer than those in the NT groups, there were no significant differences between the BAL group and the CPE + CHDF group (Figure 3; control: 10.3 ± 1.1 h; BAL: 18.0 ± 1.3 h; CPE + CHDF: 19.2 ± 0.7 h).

### *Liver functions*

Plasma levels of AST and ALT showed no significant differences among the three experimental groups. The Fischer's ratio was highest in the BAL group (NT: 0.8 ± 0.1; BAL: 1.5 ± 0.3; CPE + CHDF: 1.1 ± 0.2). In the NT group, plasma levels of both AAA and BCAA increased gradually during the treatments. In the BAL groups, whereas AAA levels in plasma decreased significantly, BCAA levels remained unchanged. In contrast, in the CPE + CHDF group, both AAA and BCAA levels in plasma decreased during the treatments (Figure 4).

The elimination capacity of ammonia in both the BAL and CPE + CHDF groups was significantly higher than that in the NT group, but there were no significant differences between the BAL and CPE + CHDF groups (Figure 5).

### *Electrolytes and pH*

As for electrolyte status, serum potassium level increased with time in both the NT and BAL group. In the CPE + CHDF group, the increase in potassium level was kept within the normal range (Figure 6). As for acid-base balance acidosis deteriorated with time in the NT and BAL group but was rather maintained in the CPE + CHDF group (Figure 6). Although the same pattern among the three groups was also seen, there were no statistical differences between the groups.

## DISCUSSION

Although most nonbiological or biological liver support systems rely on blood detoxification, the removal of medium-sized molecules, which cause hepatic coma, does not improve the survival rate of patients with severe encephalopathy[1]. This implies that the replacement of biotransformation and of liver synthetic functions is also needed in order to maintain life. At present, only intact hepatocytes seem to be able to achieve it[8-10].

In this experiment, the most striking findings for BAL were its effects on changes in plasma ammonia and on amino acids. The elimination capacity of plasma ammonia in BAL and CPE + CHDF was significantly higher than that in NT, although there were no significant differences between BAL and CPE + CHDF. These results indicated that hepatocytes in BAL functioned well and that its ammonia elimination capacity was not inferior to that of the CPE + CHDF treatment. The improvement of Fischer's ratio in BAL treatment was attributable to the decrease in AAA, which is one of the toxic substances in hepatic failure and which is metabolized by intact hepatocytes[11]. It is noteworthy that AAA was selectively eliminated in BAL, whereas both AAA and BCAA, which are beneficial amino acids, were eliminated in CPE + CHDF. This implies that CPE + CHDF eliminated all factors regardless of toxicity or necessity, whereas BAL selectively metabolized toxic factors such as AAA in the blood. To the best of our knowledge, the present results are the first to report the characteristics of two different liver support systems *in vivo*.

However, as for the maintenance of acid-base balance and electrolytes, CPE + CHDF was superior to BAL. That is, in the model of FHF in pigs, CPE + CHDF outperformed BAL in the maintenance of general status such as acid-base balance and electrolytes abnormalities. In addition, although there were no significant differences between these two groups, the pigs in the CPE + CHDF group survived slightly longer than those in the BAL group. We attributed this to better renal functions and maintenance of acid-base balance in the CPE + CHDF group.

Also, it was possible that CPE + CHDF removed all growth factors that might be needed for a diseased liver to regenerate. This kind of nonselective removal of the indispensable factors has been reported previously by researchers, including ourselves[12]. We showed that, in the rat model undergoing partial hepatectomy, blood exchange therapy reduced the serum level of hepatocyte growth factor significantly, resulting in better liver regeneration with a PCNA labeling index. In this sense, BAL might be valuable in light of its bio-transforming activity, which does not remove all factors needed.

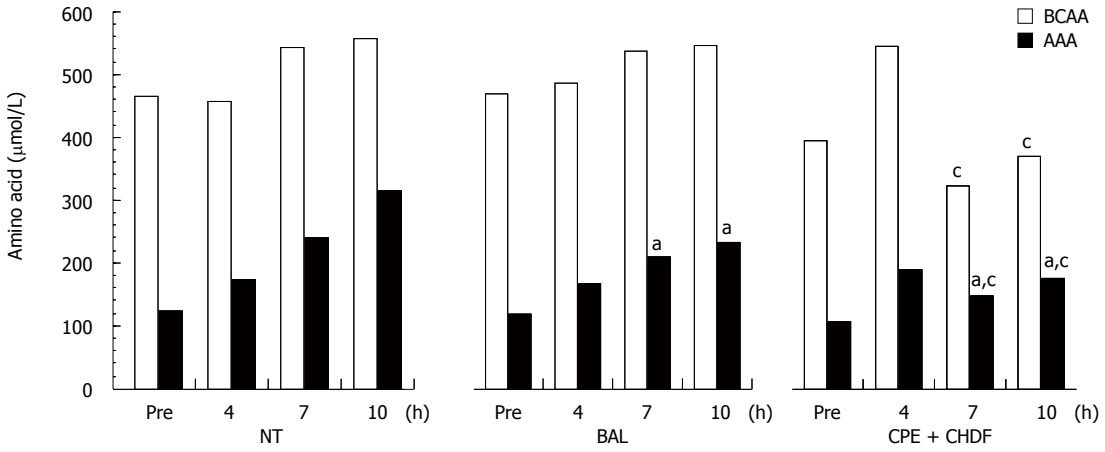


**Figure 4** Time transition of the levels of amino acid after induction of acute liver failure in (**A**) NT group, (**B**) BAL group and (**C**) CPE+CHDF group. $^{a}P < 0.05$ *vs* NT group, $^{c}P < 0.05$ *vs* NT group and BAL group.

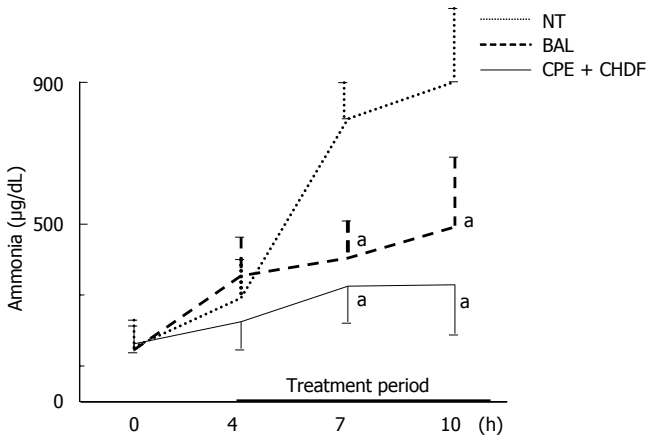


**Figure 5** Time transition of the levels of ammonia in the blood after induction of acute liver failure. $^{a}P < 0.05$ *vs* NT group.

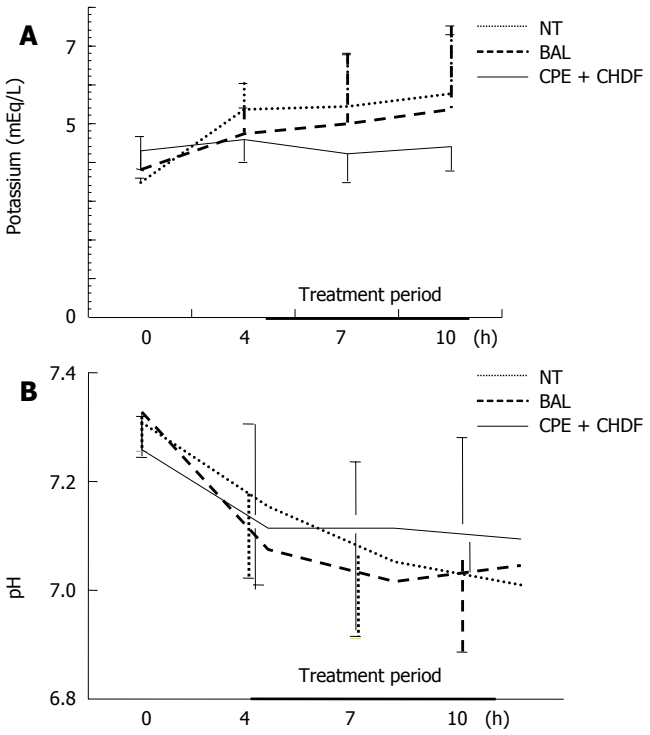


**Figure 6** Time transition of the serum levels of (**A**) potassium and (**B**) pH after induction of acute liver failure.

As to survival time, we did not find the difference between the BAL and CPE + CHDF, although both treatments prolonged the survival of pigs with ischemic liver. Therefore, it would be possible to use other models of liver failure such as the one recently published, since our model of liver failure was too aggressive with no chance of spontaneous liver regeneration[13].

In conclusion, we have compared the BAL and CPE + CHDF treatments, especially concerning their elimination capacity. We expect that the combination of BAL and CPE + CHDF would serve as an effective bridging therapy to liver transplantation or liver regeneration in patients with liver failure.

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*BASIC RESEARCH*

# Microbubble-enhanced ultrasound exposure improves gene transfer in vascular endothelial cells

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## Abstract

**AIM:** To explore the effects of ultrasound exposure combined with microbubble contrast agent (SonoVue) on the permeability of the cellular membrane and on the expression of plasmid DNA encoding enhanced green fluorescent protein (pEGFP) transfer into human umbilical vein endothelial cells (HUVECs).

**METHODS:** HUVECs with fluorescein isothiocyanate-dextran (FD500) and HUVECs with pEGFP were exposed to continuous wave (1.9 MHz, 80.0 mW/cm$^2$) for 5 min, with or without a SonoVue. The percentage of FD500 taken by the HUVECs and the transient expression rate of pEGFP in the HUVECs were examined by fluorescence microscopy and flow cytometry, respectively.

**RESULTS:** The percentage of FD500-positive HUVECs in the group of ultrasound exposure combined with SonoVue was significantly higher than that of the group of ultrasound exposure alone (24.0% ± 5.5% *vs* 66.6% ± 4.1%, $P < 0.001$). Compared with the group of ultrasound exposure alone, the transfection expression rate of pEGFP in HUVECs was markedly increased with the addition of SonoVue (16.1% ± 1.9% *vs* 1.5% ± 0.2%, $P < 0.001$). No statistical significant difference was observed in the HUVECs survival rates between the ultrasound group with and without the addition of SonoVue (94.1% ± 2.3% *vs* 91.1% ± 4.1%).

**CONCLUSION:** The cell membrane permeability of HUVECs and the transfection efficiency of pEGFP into HUVECs exposed to ultrasound are significantly increased after addition of an ultrasound contrast agent without obvious damage to the survival of HUVECs. This non-invasive gene transfer method may be a useful tool for clinical gene therapy of hepatic tumors.



**Key words:** Microbubble; Ultrasound; Gene transfer; Human umbilical vein endothelial cell; Enhanced green fluorescent protein

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## INTRODUCTION

Tumor angiogenesis plays a critical role in the development and progression of hepatocellular carcinoma (HCC)[1]. The emerging success of anti-angiogenic therapy for other cancers, such as colorectal cancer[2,3] and lung cancer[4], suggests that it may be useful for HCC because of its hyper-vascularity. To the best of our knowledge, the delivery method targeting the vascular endothelial cells of HCC has not been used in gene therapy as yet. As an effective strategy, gene therapy is largely dependent on the development of a vector or vehicle that can selectively and efficiently deliver a gene to cells or target tissues with minimal toxicity. There has been a great deal of attention paid to the development of different gene carriers to more effectively deliver DNA[5]. In addition to naked DNA, both viral (e.g., adenovirus) and non-viral (e.g., liposome) vectors have been used[6,7]. DNA may also be incorporated into nanospheres with biopolymers, such as chitosan and gelatin[8]. Ultrasound exposure has been proven to permeabilize plasma membranes[9] and reduce the thickness of the unstirred layer at the cell surface[10] because of the remarkable ability of ultrasound to produce cavitation activity[11]. Cavitation is the interaction between an ultrasonic field in a liquid and gaseous inclusion (i.e., microbubbles) within the insonated medium, which disrupts the structure of the carrier vesicle and releases the drug and also makes the cell membranes and capillaries more permeable to drugs. Cavitating gas bodies, such as microbubbles, are the mediators through which the energy of relatively non-interactive pressure waves is concentrated

to produce forces that permeabilize cell membranes and disrupt the vesicles that carry the gene[12,13]. SonoVue, as a microbubble contrast agent, associated with the gene on the surface, under ultrasound exposure, can lower the threshold for cavitation by acting as a cavitation nucleus and boosts the cavitation which alters cell membrane permeability without significantly affecting cell viability in biological tissue[10], which can be used to enhance gene transfer even further. In this study, we attempted to evaluate the effect of microbubble-enhanced ultrasound exposure in improving the transfection of enhanced pEGFP into HUVECs. The aims of the study were to elucidate the mechanism of microbubble-enhanced transfection and to construct a novel gene therapy that might be used to treat HCC.

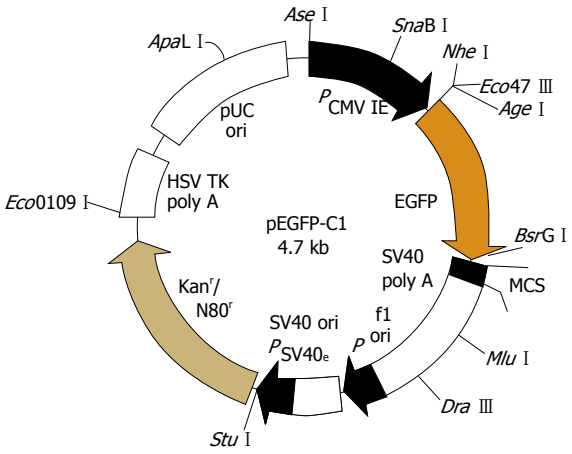


**Figure 1** Map of pEGFP-C1, showing the restriction sites.

## MATERIALS AND METHODS

### Cell culture

Primary human umbilical vein endothelial cells (HUVECs), separated with 0.2% collagenase (Sigma, St. Louis, MO, USA) from newborn aseptic umbilical cords after abdominal delivery from the healthy pregnant women who had normal routine urinalysis and blood tests (women with any complications of pregnancy or viral infections were excluded), were maintained in Human Endothelial-SFM (Gibco, California, USA) supplemented with 100 mL/L fetal bovine serum (Gibco), 2 ng/mL vascular endothelial cell growth factor (Sigma), 90 μg/mL heparin (Sigma), 100 U/mL penicillin and 50 μg/mL streptomycin. HUVECs were cultured in a 50 ml/L $CO_2$ incubator at 37℃[14], and the cellular viability was determined by counting the cells stained with trypan blue (Sangon Biological Engineering technology, Shanghai, China). The HUVECs were identified by detecting rabbit anti-human von Willebrand factor (Boshide Company, Wuhan, China), as well as by performing the acetylated low-density lipoprotein color reagent test (DiI-Ac-LDL) (Molecular Probes, Novato, Canada, Europe BV). Further experiments were performed only when the purity of the HUVECs was above 95%.

### Preparation of microbubble contrast agent and plasmid DNA

The microbubble-based ultrasound contrast agent SonoVue™ (Bracco SpA, Milan, Italy) was used in this study, which consists of 59 mg of sulfur hexafluoride gas (SF6) and 25 mg of a freeze-dried white powder. After adding 5 mL of normal saline into the vial and then shaking for several seconds, phospholipid-stabilized microbubbles filled with sulfur hexafluoride with a diameter of less than 8 μm (mean, 2.5 μm) were generated[15,16].

The EGFP plasmid DNA (donated by Doctor Zhangge, Laboratory of Molecular Medicine, Sun Yat-Sen University, China) was prepared with a special reagent (E.ZN.A Plasmid Miniprep Kit Ⅱ, Omega Bio-tek Company, Doraville, GA, USA). The purity of extract EGFP plasmid DNA was more than adequate given that the value of 260 was 1.8 as measured on ultraviolet spectrophotometry (DU800, BeckMan, Miami, USA). EGFP plasmid DNA was identified by enzyme digestive (*Sal* Ⅰ or *Xho* Ⅰ) and subsequent electrophoresis. The map of pEGFP was analyzed (Figure 1), two restriction sites *Sal* Ⅰ and *Xho* Ⅰ were included which proved that the obtained plasmid was EGFP.

### Analysis of cell membrane permeability

HUVECs were counted and resuspended in 24-well plates at a density of $1.5 \times 10^5$ cells/well. Fifty microliters of FD500 (25 mg/mL, Sigma, St. Louis, MO, USA) was added to the 24-well plates. The 24-well plates were randomly assigned into four groups, each consisting of six samples: group A (control group), sham-exposure to ultrasound without addition of SonoVue; group B, sham-exposure to ultrasound with the addition of SonoVue (600 μg/well); group C, exposure to ultrasound without addition of SonoVue; and group D, exposure to ultrasound with the addition of SonoVue (600 μg/well). Ultrasound was delivered using an HP-Viewpoint 2500 scanner (Hewlett-Packard Company, NY, USA). An E94K213 transcranial vector transducer (12 mm in diameter) with transmission gel (thickness, 10 mm) was placed on polyester film (thickness, 6 μm) that covered the 24-well plate, and the cells were exposed to 1.9 MHz continuous ultrasound at an 80.0 mW/cm$^2$ output intensity for 5 min (Figure 2). The cells were incubated for another 4 h. Subsequently, a flow cytometer (Elite, Beckman-Coulter, Miami, USA) was used to detect the percentage of FD500-positive cells in 5 wells from each group (10 000 cells in each well were detected). The other wells from each group were observed for fluorescent staining of HUVECs under the fluorescent microscope (Leica, Danaher Corporation, Wetzlar, Germany).

### Detection of gene transfection efficiency

HUVECs, at a density of $1.5 \times 10^5$ cells/well, were resuspended in 24-well plates, and 15.0 μg of pEGFP (280 μg/mL) was added to the 24-well plates. The total amount in each well was approximately 300 μL. The mixture of the HUVECs and pEGFP was incubated for 20 min at 37℃. The 24-well plates were divided into 4 groups, each consisting of 6 samples: group A, no ultrasound exposure and no SonoVue; group B, SonoVue (600 μg/well) only;

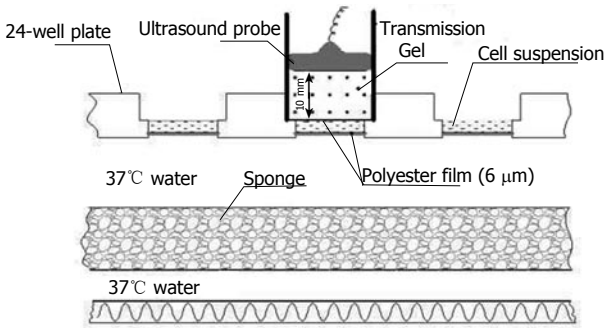


**Figure 2** Experimental setup for the ultrasound exposure. Ultrasound probe with transmission gel was placed on the cell suspension covered with polyester film, a 24-well plate covered by a 6-μm polyester film was kept in a water bath at 37℃, and also a sponge mat was placed in the water bath.

group C, ultrasound exposure only; and group D, both SonoVue (600 μg/well) and ultrasound exposure. The cells were exposed to 1.9-MHz of continuous waves at an 80.0 mW/cm$^2$ output intensity for 5 min. The cell viability was tested immediately with trypan blue after the exposure was completed. The cell suspensions were harvested from the wells, separated by centrifugation, and resuspended in medium. After 48-h culture, the pEGFP transfection efficiency was detected with fluorescent microscopy and flow cytometry.

### *Statistical analysis*

Data were expressed as mean ± SD. ANOVA was used to analyze the differences among the different groups. $P < 0.05$ was considered statistically significant.

## RESULTS

### *Examination of FD500-positive HUVECs*

Samples were evaluated with fluorescent microscopy and flow cytometry. Faint fluorescent staining in the cytoplasm was observed in few HUVECs in group A (control group) and group B (stimulated by SonoVue only). A few dispersed fluorescent-stained HUVECs were observed in group C (stimulated by ultrasound only), whereas an increased number and high concentration of fluorescent-stained HUVECs were detected in group D (stimulated by both ultrasound and SonoVue) (Figure 3). The percentages of FD500-positive HUVECs detected by flow cytometry are presented in Table 1.

### *Expression of EGFP in HUVECs*

In group D (stimulated by both SonoVue and ultrasound exposure), fluorescent microscopy revealed that the HUVECs transfected by the EGFP gene had an obviously green fluorescence in the cytoplasm, while the cells in other groups had almost no coloration (Figure 4). Flow cytometry showed that HUVECs with ultrasound exposure only barely expressed EGFP, whereas a little more than 16% of HUVECs with both ultrasound exposure and SonoVue expressed EGFP (Figure 5).

### *Cell survival rate*

The percentage of surviving cells was examined by trypan

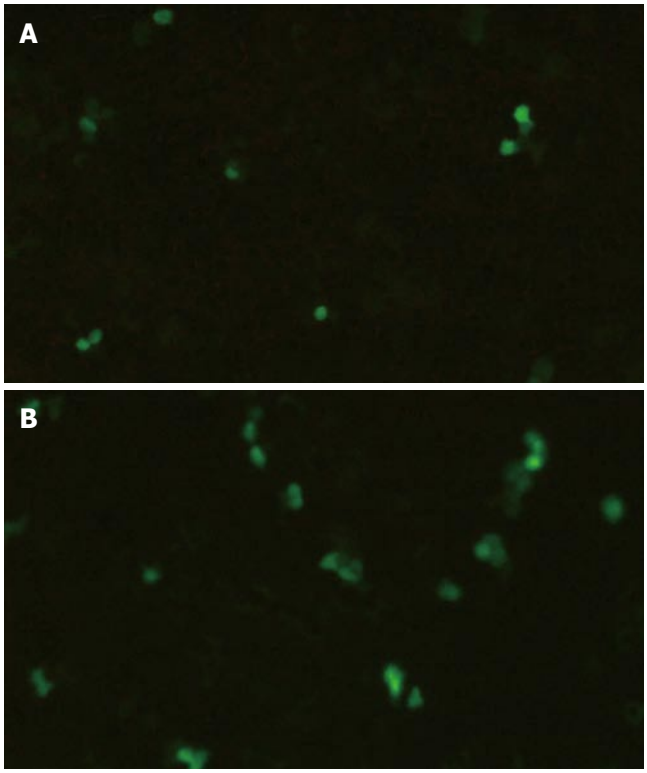


**Figure 3** Fluorescent microscopy showing FD500-positive HUVECs (200 ×). **(A)** A few FD500-positive HUVECs with ultrasound only; **(B)** significant increase in FD500-positive HUVECs after the addition of SonoVue.

**Table 1 The percentage of FD500-positive HUVECs**

| | Percentage of positive cells | Fluorescence intensity |
|---|---|---|
| Group A | 4.16 ± 1.63 | 2.07 ± 0.08 |
| Group B | 3.00 ± 0.90 | 2.15 ± 0.05 |
| Group C | 24.00 ± 5.50[a] | 1.89 ± 0.07 |
| Group D | 66.60 ± 4.10[b,d] | 2.04 ± 0.14 |

[a]$P < 0.05$ *vs* group A and B; [b]$P < 0.001$ *vs* group A and B; [d]$P < 0.001$ *vs* group C.

test after ultrasound exposure. The cell survival rates were 95.6% ± 1.4% in the control group, 96.0% ± 0.9% in group B, 94.1% ± 2.3% in group C, and 91.1 %± 4.1% in group D (Figure 6).

## DISCUSSION

Gene therapy has made significant progress in the therapy of liver neoplasms[17]. Hepatocellular carcinoma (HCC) is a typical malignancy characterized by neovascularization. Recent studies have shown that the angiogenic activity in HCC is correlated with metastasis and tumor recurrence after resection[18,19]. Angiogenesis provides a target for therapeutic approaches to HCC and may show clinical potential.

The success of gene therapy is largely dependent on the development of the gene delivery vector which is safe, easy to apply and give efficient transgene expression. DNA molecules are large, negatively charged molecules and have major difficulties in entering the cell or cell nucleus. DNA

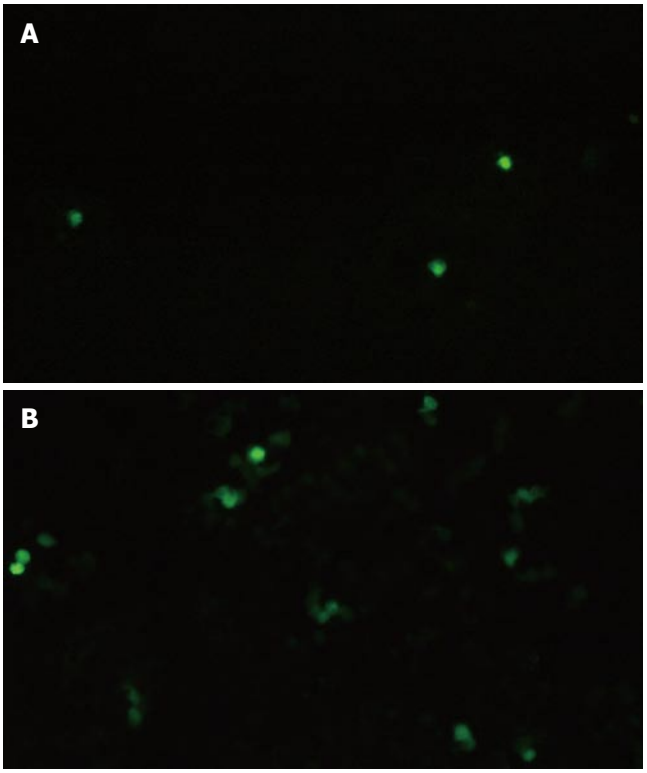


**Figure 4** Fluorescent microscopy after EGFP gene transfection (200 ×). (**A**) EGFP expression 48 h after ultrasound exposure only; (**B**) significant increase in EGFP expression in HUVECs 48 h after the addition of SonoVue.

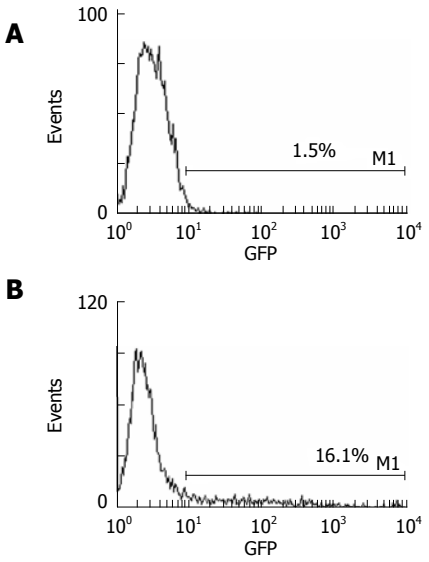


**Figure 5** Detection of EGFP gene transfection by flow cytometry. (**A**) Cells exposed to ultrasound only showed 1.5% of HUVECs within $M_1$ area; (**B**) cells exposed to both the ultrasound and SonoVue showed 16.1% of HUVECs within $M_1$ area.

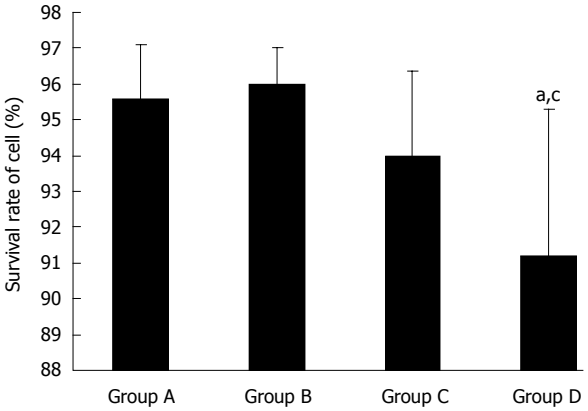


**Figure 6** HUVECs survival rate 48 h after gene transfection. $^aP < 0.05$ *vs* group A and B ; $^cP > 0.05$ *vs* group C.

becomes rapidly degraded by extra- and intra-cellular nucleases[20]. Therefore, suitable DNA delivery systems are under development. Viral vectors have been developed as highly efficient carriers for gene delivery to a variety of tissues; the efficiency of these vectors is due to the presence of viral proteins that interact with cell surface receptors. However, viral gene carriers have some major disadvantages: they often provoke an immune response and severe inflammation reactions. Additionally, the risk for insertional mutagenesis and the size

Limitations of the DNA that they can accommodate are other drawbacks of viral gene carriers[21]. Therefore, non-viral transfection systems based on cationic lipids or cationic polymers have gained more and more attention[22]. Although non-viral carriers may be safer and cheaper, they have, especially *in vivo*, a much lower transfection efficiency than viral gene carriers. To overcome the limitations of non-viral gene therapy, ultrasound energy, alone or in combination with gas-filled microbubbles, has recently been proposed to enhance the intracellular delivery of DNA, RNA, and proteins[23,24].

Microbubbles and ultrasound have been investigated with a view to improve the transfection efficiency of non-viral delivery systems. Ultrasound can create transient non-lethal perforations in cells and other membranes, which allow large molecules from the surrounding medium to enter into the cell, without remarkable damage so that the cell can reseal its membrane and survive[25,26]. Microbubbles, by acting as cavitation nuclei, applied in combination with the use of ultrasound, are thought to potentiate this effect, particularly effectively for gene delivery[27]. FD500 is the conjugate of fluorescein FITC and dextran with a molecular weight of 500 000, which makes it difficult for FD500 to penetrate the cellular membrane under normal conditions. In this study, HUVECs containing FD500 were treated with ultrasound alone and ultrasound with SonoVue, and the results showed that ultrasound exposure alone induced FD500 (24.0%) into HUVECs, as detected by flow cytometry, and the FD500-positive HUVECs increased to 66.6% after addition of SonoVue. Under ultrasound exposure, microbubbles suspended in liquid can be collapsed intentionally by insonation, and this collapse creates a mechanical force on the cell membrane and destroys the integrity of the adjoining cellular membrane.

Based on the aforementioned findings, we chose the pEGFP to examine the feasibility of gene transfer. GFP, as a report molecule, is used for detecting cytogene expression and protein location. EGFP is the optimized mutant of GFP; the fluorescence it emits is brighter and more sensitive than GFP[28]. The expression of plasmid

EGFP in HUVECs was just 1.5% in the group exposed to the ultrasound only, whereas the expression rate significantly increased to 16.1% ($P < 0.001$) when SonoVue was added, as detected by flow cytometry. The increase in transfection efficiency might be due to the transient holes that are produced in the cell membrane by the spreading of microbubbles. Several studies have reported that gas-body-based contrast agents enhance the non-thermal effects of ultrasound that induce cell membrane porosity[29]. SonoVue seemed to be rapidly destroyed by the sonication, which implies that most of the effects seen were not caused by any direct effect of SonoVue, but by ultrasonic-induced cavitation, which is used to mediate the transfection of the exogenous gene[30]. In the group with the addition of SonoVue only, there was no expression of plasmid EGFP in HUVECs. Compared to the cells treated with ultrasound alone, the addition of SonoVue did not significantly affect cell viability; more than 85% of the cells were viable.

In conclusion, our study demonstrates that ultrasound exposure combined with microbubble significantly improves the transfection efficiency of pEGFP in HUVECs, and the mechanism is based on the enhanced cellular membrane permeability caused by acoustic cavitation with no adverse effect on cellular viability. This method can be used as a new strategy for target gene therapy of HCC.

## ACKNOWLEDGMENTS

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## COMMENTS

### *Background*

It has been shown that ultrasonic waves may increase cell membrane permeability by inducing transient holes in the membrane and facilitate thereby the transfer of DNA into cells and ultrasound-mediated gene transfer is principally due to acoustic cavitation.

### *Research frontiers*

Ultrasound-mediated gene transfer can be further enhanced in the presence of microbubble contrast agents.

### *Innovations and breakthroughs*

Tumor angiogenesis plays a critical role in the development and progression of HCC, which is associated with a high propensity for vascular invasion. We attempted to evaluate the effect of microbubble-enhanced ultrasound exposure in improving the transfection of enhanced pEGFP into HUVECs.

### *Applications*

The gene therapy method of Microbubble¬-enhanced Ultrasound Exposure on targeting the vascular endothelial cells of HCC has not been used. The aims of the study was to elucidate the mechanism of microbubble-enhanced transfection and to construct a novel gene therapy that might be used to treat HCC.

### *Peer review*

HUVECs: Human umbilical vein endothelial cells; pEGFP: plasmid DNA encoding enhanced green fluorescent protein.

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*BASIC RESEARCH*

# Expression patterns and action analysis of genes associated with physiological responses during rat liver regeneration: Cellular immune response

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## Abstract

**AIM:** To study the cellular immune response during rat liver regeneration (LR) at a transcriptional level.

**METHODS:** Genes associated with the cellular immune response were obtained by collecting the data from databases and retrieving articles. Gene expression changes during LR were detected by rat genome 230 2.0 array.

**RESULTS:** A total of 127 genes were found to be associated with LR. The number of initially and totally expressing genes in the initial phase of LR [0.5-4 h after partial hepatectomy (PH)], transition from $G_0$-$G_1$ (4-6 h after PH), cell proliferation (6-66 h after PH), cell differentiation and structure-function reconstruction (66-168 h after PH) was 54, 11, 34, 3 and 54, 49, 70, 49 respectively, illustrating that the associated genes were mainly triggered at the initiation of LR, and worked at different phases. According to their expression similarity, these genes were classified into 41 up-regulated, 21 predominantly up-regulated, 41 down-regulated, 14 predominantly down-regulated, 10 similarly up-regulated and down-regulated genes, respectively. The total up- and down-regulated expression times were 419 and 274, respectively, demonstrating that the expression of most genes was increased while the expression of a small number of genes was decreased. Their time relevance was classified into 14 groups, showing that the cellular physiological and biochemical activities were staggered during LR. According to the gene expression patterns, they were classified into 21 types, showing the activities were diverse and complicated during LR.

**CONCLUSION:** Antigen processing and presentation are enhanced mainly in the forepart, prophase and anaphase of LR. T-cell activation and antigen elimination are enhanced mainly in the forepart and prophase of LR. A total of 127 genes associated with LR play an important role in cellular immunity.



**Key words:** Partial hepatectomy; Rat genome 230 2.0 array; Cellular immune response; Genes associated with liver regeneration

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## INTRODUCTION

After antigenic stimulus, antigens are processed and presented to lymphocytes by antigen-presenting cells, and lymphocytes are activated[1] to proliferate and differentiate into effector cells that eliminate antigens[2]. This process is called immune response which is the self-protective mechanism of organisms developed during a long evolutionary history, closely associated with high animal survival[3]. Usually, the immunoreactions are classified into cellular and humoral immune responses according to different effectors[4]. The cellular immune response performs in brief as following: firstly the antigens were specifically recognized and presented by T-cells[5], then the effector cells, including T-cells, macrophages *etc.*, work by clearing the antigens. Injured cells and cell remnants caused by partial hepatectomy (PH)[6] are harmful to the organism[7], and wound areas are also susceptible to infection with antigen and xenobiotics[8]. Whether the cellular immune system plays a part in this process is worthy of an in-depth study.

In addition, PH[9] also activates the remnant hepatocytes to enter into the cell cycle to compensate for the lost liver mass, which is called liver regeneration (LR)[10]. Usually, based on the cellular physiological activities, the process

is classified into 4 phases: initiation (0.5-4 h after PH), transition from $G_0$ to $G_1$ (4-6 h after PH), cell proliferation (6-66 h after PH), cell differentiation and structure-function reorganization (66-168 h after PH)[11]. According to a time course, it is divided into 4 phases: forepart (0.5-4 h after PH), prophase (6-12 h after PH), metaphase (16-66 h after PH), and anaphase (72-168 h after PH)[12], in which many physiological and biochemical events, such as cell activation, de-differentiation, proliferation and its regulation, re-differentiation, reorganization of structure-function[13], are involved and regulated by many factors such as cellular immune response[14]. The relevance between cellular immune and liver regeneration has been studied at the transcriptional level[12,15,16]. The expression changes of genes in regenerating liver after PH can be detected by rat genome 230 2.0 array[17,18] containing 213 genes participating in the cellular immune response. A total of 127 genes have been identified to be associated with LR[19]. Their expression changes, patterns and action were primarily analyzed in the present study.

## MATERIALS AND METHODS

### Regenerating liver preparation

Healthy SD rats weighing 200-250 g were obtained from the Animal Center of Henan Normal University. The rats were randomly divided into groups, 6 rats in each group (male: female = 1:1). PH was performed as previously described[9], the left and middle lobes of liver were removed. The rats were killed by cervical vertebra dislocation at 0.5, 1, 2, 4, 6, 8, 12, 16, 18, 24, 30, 36, 42, 48, 54, 60, 66, 72, 96, 120, 144 and 168 h after PH and the regenerating livers were observed at corresponding time points. The livers were rinsed 3 times in PBS at 4℃, 100-200 mg was taken from the middle parts of the right lobe. Six samples were collected from each group, mixed into 1-2 g (0.1-0.2 g × 6) liver tissue, and stored at -80℃. The sham-operation (SO) groups underwent the same PH without removal of liver lobes. The animal protection laws of China were strictly followed.

### RNA isolation and purification

Total RNA was isolated from frozen livers according to the manual of the Trizol kit (Invittrogen)[22] and purified the guide for the RNeasy mini kit (Qiagen)[21]. Agarose electrophoresis (180V, 0.5 h) showed that the total RNA samples had a 2:1 ratio of 28S to 18S rRNA intensities. Total RNA concentration and purity were estimated by optical density measurements at 260/280 nm[22].

### cDNA, cRNA synthesis and purification

Total RNA (1-8 μg) was used as a template for cDNA synthesis. cDNA and cRNA synthesis was proceeded as previously described[17]. cRNA labeled with biotin was synthesized using 12 μL synthesized cDNA as a template, and cDNA and cRNA were purified[17]. Measurement of concentration, purity and quality of cDNA and cRNA was conducted as previously reported[22].

### cRNA fragmentation and microarray detection

Fifteen μL (1 μg/μL) cRNA incubated with 5 × fragmentation buffer at 94℃ for 35 min was digested into 35-200 bp fragments. The hybridization buffer was added to the prehybridized rat genome 230 2.0 microarry produced by Affymetrix, and then hybridization was carried out for 16 h at 45℃ on a rotary mixer at 60 rpm. The microarray was washed and stained by GeneChip fluidics station 450 (Affymetrix Inc., USA). The chips were scanned with GeneChip Scan 3000 (Affymetrix Inc., USA), and the signal values of gene expression were observed [18].

### Microarray data analysis

The normalized signal values, signal detections (P, A, M) and experiment/control (Ri) were obtained by quantifying and normalizing the signal values using GCOS1.2[18].

### Normalisation of microarray data

To minimize errors in microarray analysis, each analysis was performed 3 times by rat genome 230 2.0 array. Results with a maximal total ratio (Rm) and an average of three housekeeping genes β-actin, hexokinase and glyceraldehyde-3-phosphate dehydrogenase approaching to 1.0 (Rh) were taken as a reference. Modified data were generated by applying a correction factor (Rm/Rh) multiplying the ratio of each gene in Rh at each time point. To remove spurious gene expression changes resulting from errors in microarray analysis, the gene expression profiles at 0-4 h, 6-12 h and 12-24 h after PH were reorganized by a normalization analysis program (NAP) software according to the cell cycle progression of regenerating hepatocytes. Data statistics and cluster analysis were done using GeneMath, GeneSpring, Microsoft Excel software[18,23,24].

### Identification of genes associated with liver regeneration

First, nomenclature of the cellular immune response was adopted from the GENEONTOLOGY database (www.geneontology.org) and inputted into the cellular immune response at NCBI (www.ncbi.nlm.nih.gov) and RGD (rgd.mcw.edu) to identify the rat, mouse and human genes associated with the activities mentioned above. According to the maps of biological pathways embodied by GENMAPP (www.genmapp.org), KEGG (www.genome.jp/kegg/pathway.html#amino) and BIOCARTA (www.biocarta.com/genes/index.asp), genes associated with blood coagulation were collated. The results of this analysis were codified and compared with those obtained in humans and mice in order to identify human and mouse genes which are different from those of rats. Comparing these genes with the analysis output of the rat genome 230 2.0 array, genes showing more than twofold change in expression level as meaningful expression changes[19], were referred to as rat homologous genes or rat specific genes associated with cellular immune response. Genes displaying reproducible results in three independent analyses with the chip and more than twofold change in expression level at least at one time point during LR with a significant difference ($P \leqslant 0.01 < 0.05$) or an extremely significant difference ($P \leqslant 0.01$) between PH and sham operation (SO), were referred to as genes associated with LR.

**Table 1 Expression of 127 cellular immune response-associated genes during rat liver regeneration**

| Gene abbr. | Associated with others | Fold difference | Gene abbr. | Associated with others | Fold difference | Gene Abbr. | Associated with others | Fold difference | Gene abbr. | Associated with others | Fold difference |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **1 Antigen precessing, presentation** | | | Igf1r | | 0.4 | Tlr4 | 3 | 0.5 | Gadd45g | | 8.0, 0.4 |
| Cd3d | 3 | 4.0, 0.4 | [1]Igh-1a | | 0.3 | Tnf | 3 | 3.2 | Il1rl2 | | 3.2, 0.1 |
| Cd3e | 3 | 2.7 | Il15 | | 0.4 | Tnfsf12 | | 0.3 | Il2 | 2 | 3.5, 0.3 |
| Cd3g | 3 | 0.2 | [1]Il1b | | 0.4 | [1]Tp53 | | 2.9 | Il4 | 2 | 2.6, 0.1 |
| Csf2 | | 0.3 | Il2 | 3 | 3.5, 0.3 | Traf1 | | 0.4 | Inha | | 0.2 |
| Cx3cr1 | 3 | 6.8, 0.4 | Il4 | 3 | 2.6, 0.1 | Usf1 | | 2.0, 0.5 | Itgb1 | 1 | 2.6 |
| Itgb1 | 3 | 2.6 | Il6 | | 6.1, 0.3 | Usf2 | | 3.3 | Klrc2 | 2 | 2.3, 0.4 |
| Lgals3bp | 3 | 10.6 | Irf1 | | 0.3 | Vipr1 | | 2.3 | Klrg1 | | 0.4 |
| Prkra | | 4.2, 0.1 | Itgam | | 0.3 | Xbp1 | | 4.3, 0.3 | Lgals3bp | 1 | 10.6 |
| Tap1 | 3 | 2.2 | Itgb2 | | 0.5 | Zap70 | | 0.4 | Lsp1 | | 0.3 |
| Tlr2 | 3 | 10.6 | Kdr | | 2.4, 0.4 | **3 Antigen elimination** | | | Mif | | 3.2 |
| Tnfrsf4 | 3 | 2.3, 0.3 | Kit | | 0.4 | Adm | | 8.0 | Mnda | | 5.3, 0.5 |
| **2 T-cell activation, proliferation** | | | Klrc2 | 3 | 2.3, 0.4 | Adora2a | | 0.5 | Muc1 | | 6.8, 0.2 |
| Ager | 3 | 0.4 | Map3k7 | | 0.5 | Ager | 2 | 0.4 | Ncf1 | | 3.7, 0.2 |
| Akt1 | | 3.9 | Mapk8 | | 19.7, 0.5 | App | | 6.4 | Ocil | | 9.1, 0.1 |
| Apoe | | 0.1 | Mmp9 | | 9.5, 0.5 | Art2b | | 0.3 | Pfn1 | | 4.2 |
| Arhgdib | | 13.0 | Myd88 | | 2.1 | [1]Bcl10 | | 2.3 | Pla2g4a | | 2.0 |
| B7h3 | | 3.5 | Nfatc1 | | 0.3 | Bdnf | | 2.6, 0.4 | Plaur | | 13.9 |
| [1]Bcl2 | | 0.3 | Nfkb1 | | 2.3, 0.4 | [1]C3ar1 | | 2.3, 0.3 | Pnliprp2 | | 36.8, 0.5 |
| Bcl2l1 | | 2.1, 0.4 | P2rx7 | | 2.5, 0.4 | [1]C5r1 | | 2.6, 0.4 | Prf1 | | 0.2 |
| [1]Bmi1 | | 2.0 | Pawr | | 0.3 | Ccl17 | | 0.1 | Pten | | 0.5 |
| Btk | | 2.0 | Plau | | 3.0, 0.4 | Ccr1 | | 27.9, 0.4 | Ptprc | 2 | 3.0, 0.1 |
| Card11 | | 2.3, 0.2 | Ppbp | | 2.1, 0.1 | [1]Ccr6 | | 4.3, 0.3 | Rela | 2 | 0.5 |
| Cd244 | 3 | 0.3 | Prkca | | 4.6 | Cd244 | 2 | 0.3 | Slc16a1 | | 3.2 |
| Cd80 | | 3.0, 0.3 | Prkcq | | 3.0, 0.4 | Cd3d | 1 | 4.0, 0.4 | Sod2 | | 5.6 |
| [1]Cd86 | | 2.6 | Psme2 | | 4.0 | Cd3e | 1 | 2.7 | Spn | 2 | 4.0, 0.2 |
| Cebpg | | 0.4 | Ptgs2 | | 2.1, 0.1 | Cd3g | 1 | 0.2 | Spp1 | | 2.7, 0.5 |
| Chuk | | 0.3 | Ptk2b | | 3.6 | Cfh | | 2.5 | Tap1 | 1 | 2.2 |
| Cxcl12 | | 0.2 | Ptprc | 3 | 3.0, 0.1 | Crhr1 | | 3.8, 0.5 | Tcrb | 2 | 0.2 |
| Ddx58 | | 11.8 | Rela | 3 | 0.5 | Csf2 | 1 | 0.3 | Tcrg | 2 | 0.3 |
| Erbb2 | | 0.1 | Rhog | | 0.5 | Ctgf | | 13.9 | Tff1 | | 0.1 |
| [1]F3 | | 2.0, 0.2 | Socs1 | | 2.4, 0.5 | Ctse | | 0.4 | Tgfb2 | | 2.9, 0.5 |
| Fcgr1 | | 2.6 | Socs3 | | 2.5, 0.1 | [1]Ctsk | | 10.3 | Tlr4 | 2 | 0.5 |
| Fyn | | 0.4 | Spn | 3 | 4.0, 0.2 | Cx3cr1 | 1 | 6.8, 0.4 | Tnf | 2 | 3.2 |
| Glmn | | 6.0, 0.4 | Tcrb | 3 | 0.2 | E2f1 | | 21.2 | Tnfrsf4 | 1 | 2.3, 0.3 |
| Gzmb | | 7.5 | Tcrg | 3 | 0.3 | [1]Ebi3 | | 0.2 | Umod | | 3.0, 0.4 |
| Icam1 | | 3.0 | Tert | | 5.3, 0.3 | [1]F2 | | 0.3 | Zfp148 | | 2.5 |
| Ifng | | 6.5 | Tgfb1 | | 4.0 | Fosl1 | | 2.3 | | | |

[1]Reported genes associated with liver regeneration; Associated with others: involved in other process of cellular immune response.

## RESULTS

### *Expression changes of genes associated with cellular immune response during liver regeneration*

According to the data from databases at NCBI, GENMAPP, KEGG, BIOCARTA and RGD, 468 genes were involved in the cellular immune response, of which 213 were contained in the rat genome 230 2.0 array. The expression of 127 genes displayed meaningful changes at least at one time point after PH, showing significant or extremely significant differences in expression compared with those after PH and SO, and reproducible results detected by three analyses of rat genome 230 2.0 array, suggesting that the genes were associated with LR. Ranges of the expression of up-regulated and down-regulated genes were respectively 2-37 fold and 2-10 fold of the control (Table 1). Up-regulated, down-regulated and up-/down-regulated expressions were observed in 41, 41 and 45 genes, respectively during LR. The up- and down-regulated expression times were 419 and 274, respectively (Figure 1A). At the initial phase (0.5-4 h after PH), 32 genes displayed up-regulated expression, 19 down-regulated expression, and 2 up-/down-regulated expression. At the transition phase from $G_0$ to $G_1$ (4-6 h after PH), 29 genes showed up-regulated expression, and 11 down-regulated expression. At the cell proliferation phase (6-66 h after PH), 43 genes exhibited up-regulated expression, 44 down-regulated expression, and 29 up-/down-regulated expression. At the cell differentiation and structure-functional reorganization phase (66-168 h after PH), expression was up-regulated in 49 genes, down-regulated in 46 genes, and up-/down-regulated in 15 (Figure 1B).

### *Initial expression time of genes associated with cellular immune response during liver regeneration*

At each time point of LR, the number of initially up-regulated and down-regulated as well as the total number of up-regulated and down-regulated genes were 15 and 6 at 0.5 h; 7, 10 and 17, 13 at 1 h; 7, 1 and 21, 3 at 2 h; 5, 3 and 27, 6 at 4 h; 1, 3 and 19, 10 at 6 h; 0, 0 and 17, 6 at

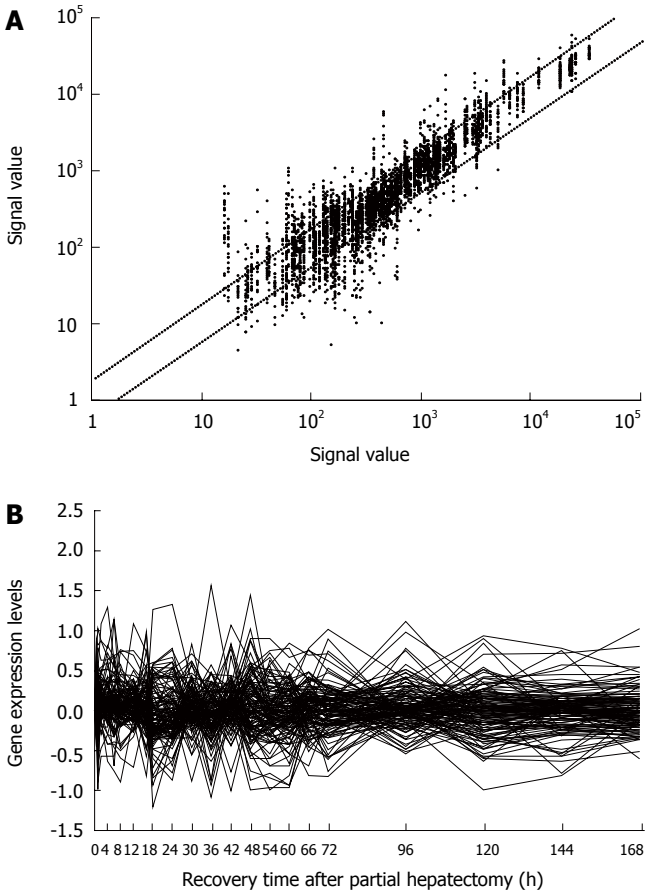


**Figure 1** Expression frequency (**A**) and changes (**B**) of 127 cellular immune response-associated genes during rat liver regeneration. Detection data of rat genome 230 2.0 array were analyzed and graphed by Microsoft Excel. The dots above bias represent the expression of genes up-regulated more than two fold, those under bias represent down-regulated more than two fold; the total times of up-regulated, down-regulated expression under bias are 419, 274, respectively. The ones between biases have meaningless alteration.The expression of 88 genes is up-regulated 2-37 fold and that of 86 genes is down-regulated 2-10 fold.

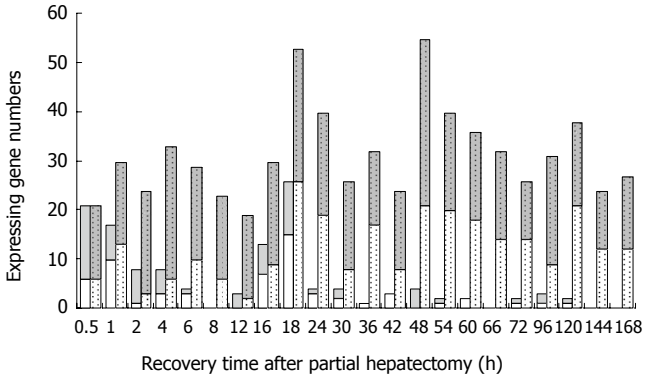


**Figure 2** Initial and total expression profiles of 127 cellular immune response-associated genes at each time point of liver regeneration. Grey bars: up-regulated genes; white bars: down-regulated genes; blank bars: initial expressing genes in which up-regulated genes are predominant in the forepart and prophase and down-regulated genes in the metaphase, whereas very few genes in the anaphase; dotted bars: total expressing genes in which some genes are up-regulated and others are down-regulated during the whole LR.

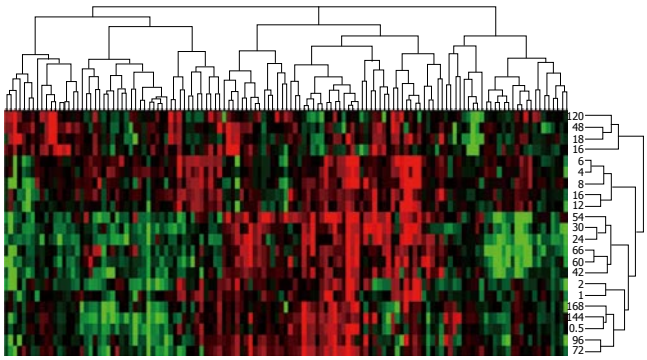

**Figure 3** Expression similarity and time relevance clusters of 127 cellular immune response-associated genes during liver regeneration. Detection data of rat genome 230 2.0 array were analyzed by H-clustering. Red represents up-regulated genes chiefly associated with antigen processing and presentation; green represents down-regulated genes mainly associated with antigen elimination; black represents the genes whose expressions are meaningless. The upper and right trees showing the expression similarity cluster and time series cluster respectively, by which these genes can be classified into 5 and 14 groups, respectively.

8 h; 3, 0 and 17, 2 at 12 h; 6, 7 and 21, 9 at 16 h; 11, 15 and 27, 26 at 18 h; 1, 3 and 21, 19 at 24 h; 2, 2 and 18, 8 at 30 h; 0, 1 and 15, 17 at 36 h; 0, 3 and 16, 8 at 42 h; 4, 0 and 34, 20 at 48 h; 1, 1 and 20, 20 at 54 h; 0, 2 and 18, 18 at 60 h; 0, 0 and 18, 14 at 66 h; 1, 1 and 12, 14 at 72 h; 2, 1 and 22, 9 at 96 h; 1, 1 and 17, 21 at 120 h; 0, 0 and 12, 12 at 144 h; 0, 0 and 15, 12 at 168 h (Figure 2). Generally, gene expression changes occurred during the whole LR. The up- and down-regulated expression times were 419 and 274, respectively. The initially up-regulated genes were predominant in the forepart and prophase and the down-regulated genes were predominant in the metaphase, whereas only a few of down-regulated genes were found in the anaphase.

### *Expression similarity and time relevance of genes associated with cellular immune response during liver regeneration*

A total of 127 genes could be characterized based on their similarity in expression: 41 up-regulated, 21 predominantly up- regulated, 41 down-regulated, 14 predominantly down-regulated, 10 up-/down-regulated genes, respectively (Figure 3). According to the time relevance, they were classified into 14 groups ( 0.5 and 144 h, 1 and 2 h, 4 and 6 h, 8 h, 12 and 16 h, 18 and 48 h, 24 and 30 h, 36 h, 42 h, 54 h, 60and 66 h, 72 and 96 h, 120 h, 168 h). The up- and down-regulated expression times were 21 and 7, 64 and 22, 42 and 8, 72 and 16, 32 and 9, 37 and 34, 62 and 49, 69 and 55, 54 and 28, 57 and 32, 41 and 35, 27 and 25, 20 and 16, 16 and 20, respectively (Figure 3). The up-regulated expression genes were chiefly associated with antigen processing and presentation, and the down-regulated genes were significantly associated with antigen elimination.

### *Expression patterns of genes associated with cellular immune response during liver regeneration*

A total of 127 genes were categorized into 21 patterns according to the changes in their expression: 11 up-regulated genes at one time point (i.e. 4, 18, 30, 48, 54, 96, 120 h) after PH (Figure 4A); 5 up-regulated genes at two

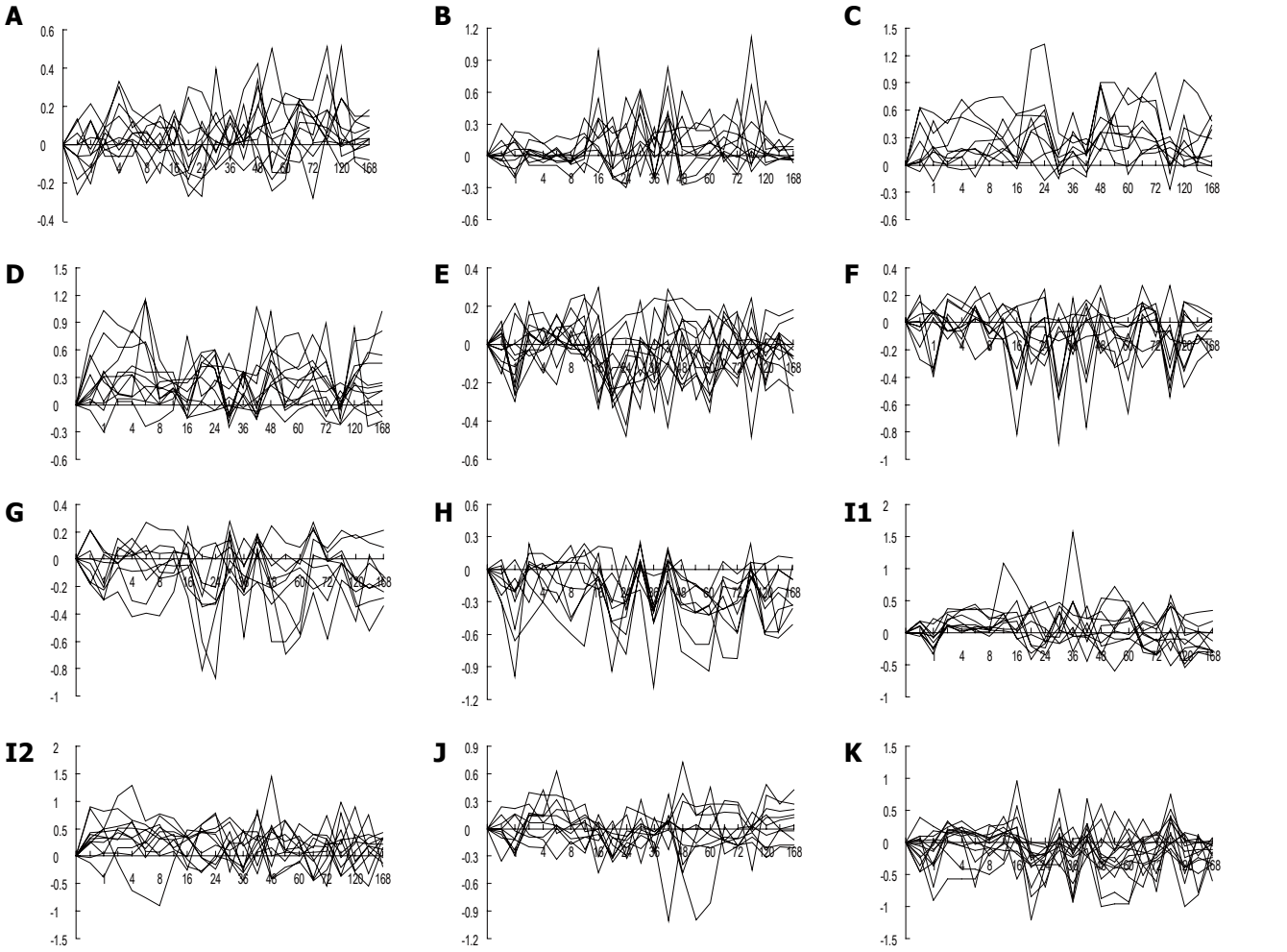


**Figure 4** Twenty-one gene expression patterns of 127 cellular immune response-associated genes during liver regeneration. Expression patterns were obtained by analysis of detection data with rat genome 230 2.0 array using Microsoft Excel. **A-D**: 41 up-regulated genes; **E-H**: 41 down-regulated genes; **I-K**: 45 up/down-regulated genes. X-axis represents recovery time after PH (h); Y-axis shows logarithm ratio of the signal values of genes at each time point to control.

time points (i.e. 12 and 60 h, 18 and 54 h, 24 and 48 h, 30 and 42 h, 72 and 120 h) (Figure 4B); 2 up-regulated genes at three time points (Figure 4B); 3 up-regulated genes at four time points (Figure 4B); 2 up-regulated genes at one time point/two phases (Figure 4C); 4 up-regulated genes at two time points/one phase (Figure 4C); 3 up-regulated genes at two time points/phases (Figure 4C); 4 up-regulated genes at multiple time points/phases (Figure 4D); 13 down-regulated genes at one time point (i.e. 16, 18, 24, 30, 36, 42, 60, 96, 168 h) (Figure 4E); 4 down-regulated genes at two time points (i.e. 16 and 30 h, 18 and 54 h, 30 and 96 h, 42 and 60 h) (Figure 4F); 3 down-regulated genes at three time points (Figure 4F); 3 down-regulated genes at four time points (Figure 4F); 4 down-regulated genes at one phase (i.e. 4-6 h, 4-8 h, 54-60 h and 120-168 h) (Figure 4G); 3 down-regulated genes at one time point/phase (i.e. 48 and 18-24 h, 72 and 120-144 h, 120 and 18-24 h) (Figure 4G); 2 down-regulated genes at one time point/two phases (Figure 4G); 2 down-regulated genes at two time points/one phase (Figure 4H); 2 down-regulated genes at two time points/phases (Figure 4H); 5 down-regulated genes at multiple time points/phases (Figure 4H); 21 predominantly up regulated genes (Figure 4I); 14 predominantly down regulated genes (Figure 4J); 10 similarly up-/down-regulated genes (Figure 4K).

## DISCUSSION

Cellular immune response is a self-protection mechanism formed during the long-term evolutionary processes, closely associated with higher animal. Of the proteins associated with antigen processing and presentation, five proteins including toll-like receptor 2 (TLR2) can activate the immune response by recognizing many kinds of pathogens[25]. Five proteins including lectin galactoside-binding soluble 3 binding protein (LGALS3BP) activate antigen presenting cells[26]. Transporter 1 ATP-binding cassette subfamily B (TAP1) speeds up antigen translocation[27]. The meaningful expression profiles of the genes encoding the above proteins are identical or similar at some time points while different at other time points, indicating that they may co-regulate antigen processing and presentation. In the present study, *tlr2* and *lgals3bp* were all up-regulated at multiple time points, reaching their peaks that were both 10.6 fold of the control respectively at 168 h and 48 h after PH. It is suggested that these genes play a

key role in antigen processing and presentation during LR.

Of the proteins associated with T-cell activation and proliferation, 6 proteins including granzyme B (GZMB) can activate T-cells[28], 4 proteins including transforming growth factor beta 1 (TGFB1) can activate CD4+T cells[29], 10 proteins including Rho GDP dissociation inhibitor beta (ARHGDIB) increase connection between antigen presenting cells and T-cells by promoting T-cell proliferation[30], 16 proteins including protein kinase C alpha (PRKCA) increase cytokine synthesis and expression of IFNγ and IgG[31], apolipoprotein E (APOE) facilitates endocytosis[32], 6 proteins including T-cell receptor beta chain (TCRB) activate the T-cell-dependent signaling pathway[33], 5 proteins including DEAD (Asp-Glu-Ala-Asp) box 58 (DDX58) promote the immunologic response[34]; intercellular adhesion molecule 1 (ICAM1) facilitates leucocyte transport[35]; interferon-gamma (IFN-γ) interacts with IL-12 and TNFα to augment immunological competence [36]; insulin-like growth factor I receptor (IGF1R) conducts signals of autoimmune inflammation[37]; integrin beta 2 (ITGB2) promotes leucocyte adherence and phagocytosis[38]; 4 proteins including Fyn proto-oncogene (FYN) suppress the Th2-mediated immune response[39], 7 proteins including kit oncogene (KIT) inhibit T lymphocyte proliferation[40]; vasoactive intestinal peptide receptor 1 (VIPR1) combines T-cell to enhance HIV infection[41]. In the present study, the meaningful expression profiles of the genes encoding the above proteins were identical or similar at some time points while different at other time points, indicating that they may co-regulate T-cell activation and proliferation. *gzmb* expression was up-regulated at 0.5-8, 36, 48-66 and 168 h and reached 7.5 fold at 48 h after PH. *arhgdib* expression was up-regulated at 16, 42 and 96 h and reached 13 fold at 96 h after PH. *prkca* expression was up-regulated at 16, 30, 42 and 96 h and reached 4.6 fold at 96 h after PH. *ddx58* expression was up-regulated at 1, 16, 30 and 42-48 h, reached 11.8 fold at 42 h after PH. *ifng* expression was up-regulated at 1-6, 18-24, 36, 48-66 and 144-168 h and reached 6.5 fold at 4 h after PH, indicating that they are crucial in T-cell activation and proliferation during LR.

Of the proteins associated with antigen elimination, profilin 1 (PFN1) accelerates antibody processing and modification [42]; 9 proteins including E2F transcription factor 1 (E2F1) and pancreatic lipase-related protein 2 (PNLIPRP2) increase immune response by speeding up the activities of lymphocytes and cytokines[43,44]; zinc finger protein 148 (ZFP148) accelerates the differentiation of monocytes into macrophages by inhibiting the activity of integrin CD11b[45]; complement factor H (CFH) promotes complement activation[46]; cathepsin κ (CTSK) promotes immunologic response *via* bactericidal action[47]; 6 proteins including superoxide dismutase 2 (SOD2) inhibit tumor cell proliferation and migration[48]; lymphocyte specific 1 (LSP1) and uromodulin (UMOD) suppress chemotaxis of macrophages and neutrophils[49,50]; chemokine receptor 1 (CCR1) conducts signals of inflammatory response[51]; 3 proteins including connective tissue growth factor (CTGF) accelerate wound repair by increasing expression of chemotactic factors[52]; transforming growth factor beta 2 (TGFB2) combines IL-10 to suppress immunologic response induced by bacterial infection[53]; myeloid cell nuclear differentiation antigen (MNDA) blocks combination of ligands and receptors[54]; 8 proteins including adrenomedullin (ADM) control the activity of effector lymphocytes and cytokines[55]; plasminogen activator urokinase receptor (PLAUR) promotes cancer cell spread[56]. The meaningful expression profiles of the genes encoding these proteins are identical or similar at some time points while different at other time points, indicating that they may co-regulate antigen elimination. In the present study, *ctsk* expression was up-regulated at 1, 18-24, 48 and 66-168 h and reached its peak at 72 h, which was 10.3 times that of the control and is basically in line with the result reported by Dransfeld[15]. *e2f1* expression was up-regulated at 18-30, 54-72 and 120 h, and reached its peak at 24 h, which was 21.2 times that of the control. *pnliprp2* expression was up-regulated at 12-18 and 36 h, and reached its peak at 36 h, which was 36.8 fold that of the control. *sod2* expression was up-regulated at 0.5, 4-24 and 48 h, and reached its peak at 12 h, which was 5.6 times that of the control. *ctgf* expression was up-regulated at 0.5-8, 18-24, 36, 54 and 72 h, and reached its peak at 1 h, which was 10.7 fold that of the control. *adm* expression was up-regulated at 0.5-24, 36, 48-72 and 168 h, and reached its peak at 54 h, which was 8-fold that of the control. *plaur* expression was up-regulated at 1, 6, 18-24, 48, 72 and 120 h, and reached its peak at 6 h, which was 13.9 times that of the control. These findings suggest that the seven genes are of importance in antigen elimination during liver regeneration.

In conclusion, the expression changes of cellular immune response-associated genes after rat partial hepatectomy can be investigated by high-throughput gene expression analysis. Cellular immune response is enhanced during liver regeneration. Rat genome 230 2.0 array is a useful tool for analyzing the response at the transcriptional level. However, DNA→ mRNA→ protein→ function is influenced by many factors including protein interaction. So we will further analyze the results using such techniques as Northern blotting, protein chip, RNA interference, protein-interaction *etc.*

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*RAPID COMMUNICATION*

# Reversibility of central neuronal changes in patients recovering from gallbladder stones or acute cholecystitis

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## Abstract

**AIM:** To investigate the referred pain area in patients 2-7 years after cholecystectomy in order to test the hypothesis that neuroplastic changes could give rise to post cholecystectomy pain.

**METHODS:** Forty patients were tested. Twenty five were cholecystectomized due to uncomplicated gallbladder stones and 15 because of acute cholecystitis. Sensitivity to pinprick, heat, cold, pressure and single and repeated electrical stimulation was studied both in the referred pain area and in the control area on the contra lateral side of the abdomen.

**RESULTS:** Five patients still intermittently suffered from pain. But in the objective test of the 40 patients, no statistical significant difference was found between the referred pain area and the control area.

**CONCLUSION:** This study does not support the hypothesis that *de novo* neuroplastic changes could develop several years after cholecys-tectomy.



**Key words:** Referred pain; Hyperalgesia; Cholecystitis; Post cholecystectomy syndrome

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## INTRODUCTION

Cholecystectomy in patients with gallstone disease usually relieves the symptoms. However, it has repeatedly been demonstrated that 5%-10% of the patients still suffer from severe pain, and even more patients (25%-40%) have milder symptoms[1,2]. The explanations for continuing symptoms are multiple. An underlying organic disorder can be demonstrated in some patients, but no well-defined diagnosis can be identified in a substantial number of patients. Previous studies have shown that some of the patients suffer from sphincter of Oddi dysfunction (SOD). This condition can be relieved by endoscopic sphincterotomy[3-5]. Others have suggested a more generalized intestinal motor disorder in which SOD may be a component[6-8]. In previous studies we have demonstrated that patients with uncomplicated gallstone disease or acute cholecystitis have an increased sensibility in the referred pain area (RPA), probably reflecting neuroplastic changes and hyperexcitability in the central nervous system[9,10]. Animal studies have shown that such changes are frequently persisting even though the initial cause is eliminated[11]. We therefore hypothesized that continuing central neuronal changes could be the cause of continuing pain in some of the patients. However, we were not able to support this hypothesis in subsequent investigations[12]. Since these studies were carried out shortly after cholecystectomy, the possibility still remains that neuroplastic changes could occur at a later point of time.

Accordingly, the aim of the present study was to evaluate the sensibility in the referred pain area in patients who underwent cholecystectomy some years earlier, and to correlate possible abnormalities to the clinical condition of the patients.

## MATERIALS AND METHODS

### Patients

Two groups of Fifty-five patients were included in this study and divided into two groups. These were patients from the previous gallbladder stones and acute cholecystitis studies[9,10]. The first group consisting of 36 patients was tested prior to their cholecystectomy and then approximately 4 wk following the surgery. These patients were contacted and asked to participate in the follow-up study. Twenty-five entered the study, one man and 24 women, with a median age of 48 years (range 25-68 years). The reason for not entering the study was either that they did not want to (six patients) or we were not able to locate

and thereby get in contact with the patients (five patients). The second group consisting of 19 patients was all cholecystectomized after acute cholecystitis. Two of these patients were excluded and two did not want to participate in the study. The remaining 15 patients who entered the study were seven men and eight women with a median age of 60 years (range 37-84 years). Thus, follow-up testing was done in 40 patients (25 following uncomplicated gallbladder stones and 15 following acute cholecystitis).

### Protocol

The patients were interviewed focusing on any relevant pain or medication. Each patient was asked to express the degree of pain during any attacks similar to what they knew as gallstone-attacks from their experience prior to the cholecystectomy. The pain was described in terms of intensity, localization, number of attacks, duration, physical activity, accompanying symptoms, and use of the visual analogue scale (VAS) anchored at 0 (no pain) to 10 (worst imaginable). After the interview the patients were tested with the same equipment to evoke pain as in the previous studies[9,10]. For testing of somatosensory sensitivity the patients were asked to assign the area under the right curvature/epigastrium where the pain was referred to during the previous disease. This area was assumed to be the area of initial referred pain (RPA). Then a symmetrical area was marked under the left costal margin to represent the control area (CA). The subsequent sensory assessment was performed at the centre of these areas. The experimental sensory testing with different modalities (see below) was used for measuring superficial/deep and sensory/pain thresholds in the RPA and CA. The sensory testing was first applied in the CA and then in the RPA.

### Pinprick

The sensitivity to touch was determined using a Von Frey hair with a bending force of 6.6 g (SenseLab, Somedic AB, Stockholm, Sweden), which was pressed against the skin until bending of the hair occurred. The two skin areas were stimulated consecutively, and the patients were asked if it was painful or if they felt any difference between the two areas. The perception of the pinprick was rated as 'similar', 'greater', or 'painful'.

### Pinching

The sensitivity of pain to the pinching stimulation was determined bilaterally using an electronic pressure algometer (Somedic AB, Stockholm, Sweden) mounted with a 1 $cm^2$ probe, connected to a plastic clamp. A skin fold was placed and pinched between the clamp. The pinching was gradually increased at 10 kPa/s, and the patients pushed a stop button when the pain detection threshold was reached. The mean of three trials, with an interval of a minimum of 20 s between each trial was used in the subsequent calculations.

### Thermal stimuli

The sensitivity to thermal stimuli was tested in the two areas using two 4 $cm^2$ metal rollers, which prior to testing were placed in water baths with temperatures of 0℃ and 40℃, respectively. The cold and warm rollers were held in contact with the skin for 3 s in the two areas, and immediately after the stimulus the patients were asked to rate possible differences in sensation (lesser, similar, greater, or painful) between the two zones. The cold stimulus was applied first.

### Electrical stimuli

A constant current electrical stimulator controlled by a computer (Aalborg University, Denmark) was attached to two electrodes (Neuroline, Disposable Neurology Electrodes, type: 720-01-K; Medicotest A/S, Ølstykke, Denmark). The electrodes were applied to the RPA and CA. Five constant currents, 1ms rectangular pulses applied at 200 Hz, were defined as a 'single burst stimulus'. When this single stimulus was repeated five times at 2 Hz, it was defined as a 'repeated burst stimulus'. This repeated paradigm was used to measure temporal summation [13-15]. The patients were instructed to indicate when the single and repeated stimuli could be felt (sensation threshold), and when pain was perceived (pain detection threshold). The stimulation was started at 0.2 mA, and the current was gradually increased in steps of 0.1-1.0 mA until the actual threshold was reached. The patients could interrupt the stimulation at any time during the experiment.

### Pressure stimuli

The sensitivity of pain to the pressure stimulation was determined bilaterally using an electronic pressure algometer (Somedic AB, Stockholm, Sweden) mounted with a 1 $cm^2$ probe. The pressure was gradually increased at 50 kPa/s, and the patients pushed a stop button when the pain detection threshold was reached. The mean of three trials, with an interval of a minimum of 20 s between each trial, was used in the subsequent calculations.

### General hypersensitivity

Hypersensitivity in general was defined as three or more pain-modalities showing hypersensitivity in the RPA compared to the CA and no modalities demonstrating hypersensitivity in the CA compared to the RPA. All patients were tested in the pain free period.

### Statistical analysis

Data were described as median (range). For comparison of the sensation to pinprick, heat and cold in the RPA and CA, the McNemar test for dichotomous paired data was used. The sensation and pain thresholds for pinching, electrical and pressure stimuli in the two areas were compared using the Mann-Whitney test. Correlation analysis was performed with Pearson's test. $P < 0.05$ was considered statistically significant.

### Ethics

The study was conducted in accordance with the Helsinki Declaration. All patients gave their verbal and written consent following verbal and written information.

## RESULTS

The median time interval between cholecystectomy and the investigation was 35.6 (27.7-58.1) mo for the first group

**Table 1 Characteristics of the 5 patients still intermittently suffering from pain**

| Patient (*n*) | Pain localization | Attack frequency | Pain duration | Physical activity | Accompanying symptoms | VAS | Pain-killers | Anti-depressive |
|---|---|---|---|---|---|---|---|---|
| C (4) | Epigastriel | Daily | h | Depending on activity | Feeling ill and nausea | 9 | Paracetamol | No |
| C (5) | Under the right costal margin | Every 3rd wk | 20-30 s | Sitting down | No | 7 | | SSRI |
| C (14) | Under the right costal margin | Approximately 3 every week | min | Sitting down | No | 4 | No | No |
| C (17) | Epigastriel and under the right costal margin | 2-3/mo | h | Depending on activity | Nausea and reflux for 1-2 d | 7 | NSAID and paracetamol | TCA |
| AC (15) | Under the right costal margin | 2-3/yr | min | Standing | No | 6 | No | |

C: Cholecystholithiasis study[10]; AC: Acute cholecystitis study[9]; VAS: Visual analogue scale; NSAID: Non steroid anti-inflammatory drug; SSRI: Selective serotonin reuptake inhibitor; TCA: Tricyclic anti-depressants.

of patients and 74.9 (62.2-87.8) mo for the second group. The majority of patients no longer had pain in the relevant region. Five patients (12.5%) still intermittently suffered from pain similar to the pain they remembered during the precholecystectomy period localized to the original region (Table 1). The general picture showed some diversity in terms of pain description. One patient had daily attacks of pain and another only two to three attacks every year. One patient had attacks lasting seconds and in another case the duration of pain was days. The mean VAS-score under attacks was 6.6.

Only three patients had general hypersensitivity in the RPA compared to the CA (Table 2). There were no overlaps between the five patients informing of actual intermittent pain and the three patients who had generalised hypersensitivity in the RPA.

### Sensory test

Using Von Frey hair the presence of increased sensitivity in the RPA was detected in three patients (7.5%) but three other patients (7.5%) experienced increased sensitivity in the CA ($P > 0.05$). No patient had allodynia (painful response to a stimulus that does not normally provoke pain).

When cold stimulation was applied increased sensitivity was found in the RPA in 12.5% of the cases and again 12.5% experienced increased sensitivity in the CA ($P > 0.05$). No patient had allodynia. Using heat stimulation, increased sensitivity was found in the RPA in 10% of the cases and 12.5% experienced increased sensitivity in the CA ($P > 0.05$). None of these patients reported that pain was evoked.

### Quantitative assessment

The pinching pain threshold was 176.5 kPa (4-1057 kPa) in the RPA and 213.5 kPa (7-660 kPa) in the CA ($P > 0.05$).

**Sensation to electrical stimuli:** The sensation detection threshold for single burst electrical stimuli was 0.6 (0.3-2.5) mA in the RPA and 0.6 (0.2-1.4) mA in the CA ($P > 0.05$). The pain threshold for single burst electrical stimuli was 13 (0.9-56) mA in the RPA and13.5 (0.7-46) mA in the CA ($P > 0.05$). There was no statistical difference in the pain threshold for single burst electrical stimuli between the RPA and CA ($P > 0.05$). The sensation detection threshold for repeated burst electrical stimuli (temporal summation) was 0.6 (0.2-2.2) mA in the RPA and 0.6 (0.3-1.3) mA in the CA ($P > 0.05$). The pain threshold for repeated burst electrical stimuli was 9 (1.0-30.5) mA in the RPA and 9.3 (0.9-35.4) mA in the CA ($P > 0.05$). Table 3 shows the comparison of results from the present study with result from the initial study.

**Table 2 Sensory findings in the three patients showing generalized hypersensitivity**

| Patient (*n*) | Pinprick | Cold | Heat | Pinching | SES | RES | Pressure |
|---|---|---|---|---|---|---|---|
| AC (2) | - | - | - | ↑ | ↑ | - | ↑ |
| AC (24) | - | - | - | ↑ | ↑ | ↑ | ↑ |
| AC (30) | - | - | - | ↑ | ↑ | ↑ | ↑ |

AC: Acute cholecystitis study[9]; SES: Single electrical stimulus; RES: Repeated electrical stimulus; "-": No difference between referred pain area (RPA) and control area (CA); "↑": Hypersensitivity in the RPA.

**Sensation to pressure:** Pressure gave no indication that the patient still had significant hyperalgesia in the RPA. The pressure pain threshold was 267 (60-890) kPa in the RPA and 270.5 (87-856) kPa in the CA ($P > 0.05$).

## DISCUSSION

In the present study, no evidence for long lasting or *de novo* central neuronal changes were found 2-7 years after uncomplicated gallbladder stones or acute cholecystitis. Somatosensory hyperalgesia in the referred pain area (RPA) has previously been reported in different groups of patients[9,16,10,13]. The phenomenon is the result of direct or indirect convergence of nerve fibres from visceral and somatic tissue at the spinal and/or even the supraspinal levels[17], and the RPA therefore, most likely, reflects central changes in neurons receiving convergent afferent information from both the visceral and somatic systems[18]. Hyperexcitability of neurons in the central nervous system (CNS) may theoretically explain why some patients suffer from pain although the original disease has resolved. In other words, continuous visceral pain stimuli can lead to a hypersensitive cutaneous area, where a stimulus that does not usually cause pain is perceived as painful (allodynia). This central sensitization is characterized by neuroplastic findings such as increased spontaneous activity, decreased firing threshold, and expansion of the receptive fields of

**Table 3 Comparison of results regarding electrical stimuli from the present study with result from the initial study, mean (range)**

| | Present study in mA | | Initial study in mA | |
|---|---|---|---|---|
| | RPA | CA | RPA | CA |
| The sensation detection threshold for single burst electrical stimuli | 0.6 (0.3-2.5) | 0.6 (0.2-1.4) | 0.7 (0.3-1.2) | 0.7 (0.4-1.5) |
| The pain threshold for single burst electrical stimuli | 13 (0.9-56) | 13.5 (0.7-46) | 12.4 (2.6-30.6) | 13.9 (3.7-28.6) |
| The sensation detection threshold for repeated burst electrical stimuli | 0.6 (0.2-2.2) | 0.6 (0.3-1.3) | 0.7 (0.25-1.2) | 0.7 (0.4-1.6) |
| The pain threshold for repeated burst electrical stimuli | 9 (1.0-30.5) | 9.3 (0.9-35.4) | 7.6 (1.5-17.2) | 7.7 (2.7-25) |

RPA: Referred pain area; CA: Control area.

spinal neurons[19,20,16].

The primary objective of this study was to investigate a group of patients who tested twice during and after acute cholecystitis or uncomplicated gallbladder stones. We searched for any persisting changes in the RPA reflecting central hyperexcitability in exactly the same way as in the initial studies shortly after surgery. In these studies, hyperalgesia in the RPA was found and the abnormal sensation disappeared after surgery[9,10]. Other studies have shown that muscular hyperalgesia tends to persist for a long time, outlasting the duration of the initial pain and that muscular hyperalgesia in the RPA could be experienced several years after elimination of renal stones[16,11,18]. These findings by Italian colleagues were not confirmed in our work, where the experimental findings in the RPA in patients with uncomplicated gallstone disease in the pain free period were not dependent on intensity or duration. The statistical analysis showed no significant correlations between the pain thresholds to single and repeated electrical or mechanical stimuli in the RPA. This absence of correlation between hyperalgesia in the RPA in the pain free period, can therefore be caused by too short duration and relatively low intensity of the painful episodes. The duration of the pain attacks was much shorter and the intensity was lower than in above-mentioned studies with unilateral renal/ureteral colics (where the duration was around 5-10 d with high intensity). An alternative explanation for the lack of correlation could be that our method was insufficient for measuring chronic pain caused by neuroplastic changes at the supraspinal level. The method was, however, sufficient to demonstrate abnormalities prior to surgery.

All but five patients no longer suffered from pain similar to the pain they remembered during the preoperative interval. These patients were classified as having postcholecystectomy syndrome[21,22]. In these patients no evidence for hyperalgesia in the RPA was found, reflecting that any neuronal changes at the spinal cord level have resolved. Hence the reason for their symptoms could be either peripheral changes[23,24] relating to the surgical incision of the nerves or more complex supraspinal changes as reorganization in the CNS which have been demonstrated in patients suffering from chronic pancreatic pain[25]. The pathogenesis of chronic pain is poorly understood, but there is a growing body of evidence that neuroplastic changes as seen in neuropathic pain and other chronic pain disorders may be of importance in understanding chronic pain disorders, including post cholecystectomy syndrome.

In conclusion, initial studies in this patient group showed that hypersensitivity is present in the RPA after acute cholecystitis and gallbladder stones[9,10]. After the immediate recovery from the cholecystectomy the hypersensitivity returns to normal[9,10]. The current study has confirmed that the hypersensitivity does not reappear, which does not support the hypothesis that the occurrence of neuroplastic changes could explain pain some years after cholecystectomy. Since five patients in the present study suffered from pain, central changes in a small subset of patients with post cholecystectomy syndrome cannot be ruled out. Therefore, in the future, it would be interesting to study a large group of patients with post cholecystectomy syndrome. Further studies with more advanced equipment for measuring peripheral changes and reorganization in the CNS, along with alternative ways of testing, should be carried out to further explain post cholecystectomy syndrome.

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## COMMENTS

### Background

Cholecystectomy in patients with gallstone disease usually relieves the symptoms. However, it has repeatedly been demonstrated that 5%-10% of the patients still suffer from severe pain, and even more patients (25%-40%) have milder symptoms. Accordingly, the aim of the present study was to evaluate the sensitivity in the referred pain area in patients who underwent cholecystectomy some years earlier, and to correlate possible abnormalities to the clinical condition of the patients.

### Research frontiers

In previous studies we have demonstrated that patients with uncomplicated gallstone disease or acute cholecystitis have an increased sensitivity in the referred pain area (RPA), probably reflecting neuroplastic changes and hyperexcitability in the central nervous system. Animal studies have shown that such changes are frequently persisting even though the initial cause is eliminated. Could this increased sensitivity indicate a later development into post cholecystectomy syndrome?

### Innovations and breakthroughs

There is no indication that changes in the central nervous system persist in patients treated with cholecystectomy after several years of follow-up.

### Applications

This study strips away the hypothesis that central neuronal changes may be responsible for the post cholecystectomy syndrome. Although it does not give sufficient information on the post cholecystectomy syndrome, it is definitely a future puzzle.

### Terminology

Referred pain: Physical pain in a location other than the site of origin. Somatosensory sensitivity: The somatosensory system includes multiple types of sensation from the body-light touch, pain, pressure, temperature, and joint and muscle position sense (also called proprioception). However, these modalities are lumped into three different pathways in the spinal cord and have different targets in the brain.

### Peer review

In order to test the hypothesis that neuroplastic changes give rise to post cholecystectomy pain, the referred pain area after cholecystectomy was studied in 40 patients. However, no statistical significant differences were found between the referred pain area and the control area. Thus, this study does not support the hypothesis that de nova neuroplastic changes cause post cholecystectomy syndrome. The description of materials and methods is sound. However, a major drawback of this study is the small number of patients.

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*RAPID COMMUNICATION*

# Protective effect of melatonin against multistress condition induced lipid peroxidation *via* measurement of gastric mucosal lesion and plasma malondialdehyde levels in rats

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## Abstract

**AIM:** To study the protective effect of a natural antioxidant, melatonin, against multistress condition induced lipid peroxidation *via* determination of gastric damage and plasma malondialdehyde (MDA) level by high performance liquid chromatography in rats.

**METHODS:** We compared indomethacin-induced gastric damage and MDA plasma level in three groups of rats: unoperated, bile duct ligated and sham-operated and evaluated the role of the melatonin on gastric damage and plasma MDA level. Indomethacin and melatonin were injected intraperitoneally in doses of 50 mg/kg and 20 mg/kg, respectively. Animals were killed 4 h after indomethacin injection.

**RESULTS:** Indomethacin induced more severe gastric damage and plasma MDA level in bile duct ligated animals was significantly higher (3.1 ± 0.04 μmol/L) than sham (2.8 ± 0.04 μmol/L) and unoperated animals (1.4 ± 0.08 μmol/L). Pretreatment with melatonin reduced indomethacin-induced gastric damage and plasma MDA level.

**CONCLUSION:** Considering the results of this study, we suggest that in multistress conditions the intensity of gastric damage and the plasma MDA level are great and melatonin reduces the negative effect of lipid peroxidation and cell damage by oxidative stress in multistress conditions due to its antioxidizing activity.



**Key words:** Melatonin; Oxidative stress; Gastric ulcer; Cholestasis; Malondialdehyde; High performance liquid chromatography; Rat

## INTRODUCTION

It has been accepted that the pathogenesis of ulcer is complex and related to different pathogenic factors. The most common side-effect of indomethacin (NSAID) is gastric mucosal damage. The inhibition of the biosynthesis of gastric prostaglandins is accepted as a main mechanism implicated in NSAID-induced gastropathy[1,2]. Oxidative stress is involved in this pathology and it has been suggested that active oxygen metabolites play an important role in gastric damage induced by aspirin[3]. The focal ischemic areas produced as a result of inhibition of prostaglandin synthesis, may eventually produce tissue-damaging mediators such as oxygen-derived free radicals[1]. The break down of lipid peroxide in the biological system produces a number of aldehydes such as malondialdehyde (MDA), which are cytotoxic by reacting with lipids, protein and nucleic acids[4]. It has been shown that gastric mucosa of cholestatic rats is more vulnerable than that of normal animals to NSAIDs and this has been referred to different factors such as increased gastric acid secretion, decreased prostaglandins and increased free radical formation and accumulation of endogenous opioids[5].

Melatonin, a pineal secretory product, was recently found to be a potent free radical scavenger and antioxidant, especially hydroxyl radical (•OH) and peroxyl radical. In *in vitro* and *in vivo* experiments melatonin in different models of oxidative stress has been found to protect tissues against oxidant damage induced by various free radical generating agents[6-9]. It has been shown that melatonin exerts protective and therapeutic effects against cholestatic liver injury and its associated oxidative stress in rats subjected to BDL through its direct and indirect antioxidant actions as well as its anti-inflammatory effects[10-14].

The aim of this study was to investigate the antioxidant effect of melatonin on multistress conditions *via* assessment of gastric lesion and determination of MDA concentration in plasma as a lipid peroxidation biomarker.

We compared gastric damage and plasma MDA level in 3 groups of rats: unoperated (UNOP), bile duct ligated (BDL) and sham-operated (SHAM) and evaluated the antioxidative effect of melatonin in these groups.

## MATERIALS AND METHODS

### Chemicals

The following chemicals were used: 1,1,3,3- tetramethoxypropane (Sigma), butylated hydroxytoluene (Sigma), 2-thiobarbituric acid (Serva), high performance liquid chromatography (HPLC) grade methanol and ethanol (Merck), melatonin (Nature's Bounity), ketamin HCl (Rotex Medica) and Promethazine HCl (Sigma).

### Reagent preparation

Chemical solutions were prepared using distilled deionized water. Butylated hydroxytoluene (BHT) solution was prepared in ethanol to a final concentration of 0.05% BHT. An 0.44 mol/L phosphoric acid solution was obtained by diluting 1 mL concentrated phosphoric acid to 100 mL final volume. 2-thiobarbituric acid (TBA) was dissolved in water on a stirring hot-plate at 50-55℃ to a concentration of 42 mmol/L.

### Experimental design

Male Sprague-Dawley rats weighing 200-250 mg were used in this experiment. All animals were given free access to food and water. The animals were handled in accordance with the criteria and recommendations of the ethics committee on animal experiments of the Medical School Tehran University of Medical Sciences. Animals were divided into three groups.

First group (UNOP, $n$ = 5), second group (BDL, $n$ = 10), third group (SHAM, $n$ = 10). Animals in BDL and SHAM groups were divided randomly into sham plus saline plus indomethacin, bile duct ligation plus saline plus indomethacin, sham plus melatonin plus indomethacin and bile duct ligation plus indomethacin plus indomethacin subgroups, respectively; each subgroup consisted of 5 rats.

### Surgery

Laparotomy was performed under general anesthesia induced by intraperitoneal (IP) injection of ketamin (60 mg/kg) and chlorpromazine (20 mg/kg) in BDL rats, the common bile duct was isolated and doubly ligated. In SHAM rats the bile duct was identified, manipulated and left *in situ*. Then the abdominal wall was closed in two layers[15]. Five days after surgery animals were fasted but with access to water and during this period were housed in individual cages with a wire-mesh floor to prevent coprophagy. Experiments were performed 7 d after surgery, when the BDL group had shown overt jaundice. Rats were divided into 5 groups.

**UNOP group**: which were injected with saline at 0 and 30 min and were killed under ether anesthesia 4 h after last injection and 2 mL blood were drawn from the heart ventricle into a syringe containing EDTA (ethylenediaminetetraacetic acid) with a final EDTA concentration 1 mg/mL and transferred to plastic tubes and centrifuged for 10 min at 4000 rpm at room temperature. The aliquots were kept frozen at -80℃ until analysis. Plasma samples in this temperature are stable for at least one year[16]. Then the stomachs were immediately removed and cut along the greater curvature and the stomach contents were washed out with saline and gastric lesions were fixed with 20% formaldehyde for 5 min.

**BDL group:** which were injected with saline (1 mL/kg, ip) and 30 min later treated with indomethacin (50 mg/kg, ip) and were killed under ether anesthesia 4 h after last injection. Plasma samples and stomachs were prepared like the UNOP group.

**BDL group:** which were pretreated with melatonin (20 mg/kg, ip)[17] and 30 min later treated with indomethacin (50 mg/kg, ip) and were killed under ether anesthesia 4 h after last injection. Plasma samples and stomachs were prepared like the UNOP group.

**SHAM group:** which were injected with saline (1 mL/kg, ip) and 30 min later treated indomethacin (50 mg/kg, ip) and were killed under ether anesthesia 4 h after last injection. Plasma samples and stomachs were prepared like the UNOP group.

**SHAM group:** which were pretreated with melatonin (20 mg/kg, ip) and 30 min later treated with indomethacin (50 mg/kg, ip) and were killed under ether anesthesia 4 h after last injection. Plasma samples and stomachs were prepared like the UNOP group.

### Measurement of gastric lesions and malondialdehyde levels

Gastric lesions were assessed macroscopically and evaluated according to J-score[18]. The J-score was calculated by classifying the lesions by diameter; 0-1 mm = 1; 1-2 mm = 2; greater than 2 mm = 3. The score was defined as the sum of these points in each rat[19]. Plasma analysis was performed immediately after thawing the plasma samples and lipid peroxidation was monitored by MDA measurement by HPLC-based assay of MDA-TBA adduct.

### Standards

Standards and control samples were prepared using TMP with the stock standard solution containing 100 μmol/L TMP in a 40% ethanol solution. Standards were prepared through serial dilution of stock standards with ethanol solution to obtain concentrations of 4, 3, 2, 1, 0.5 and 0 (blank) μmol/L TMP. The concentration of TMP solution did not change after three months at 4℃ (refrigerator)[7]. The standard curve was prepared freshly for analysis each day as well as control samples of 4, 3, 2, 1, 0.5 and 0 (blank) μmol/L TP.

### Sample preparation

Sample derivatization was carried out in 2 mL capacity plastic centrifuge tubes fitted with screw-on caps. To a 150 μL aliquot of sample or TMP standard, 50 μL BHT, 400 μL $H_3PO_4$ and 100 μL TBA solution were added. Sample tubes were capped tightly, vortexed, and then heated for 1 h in a 100℃ water bath. Following heat derivatization, samples were placed on an ice-water (0℃) water bath for 5 min to cool, with 250 μL n-butanol subsequently added to each tube for extraction of the MDA-TBA complex. Tubes were vortexed 5 min and then centrifuged 3 min at

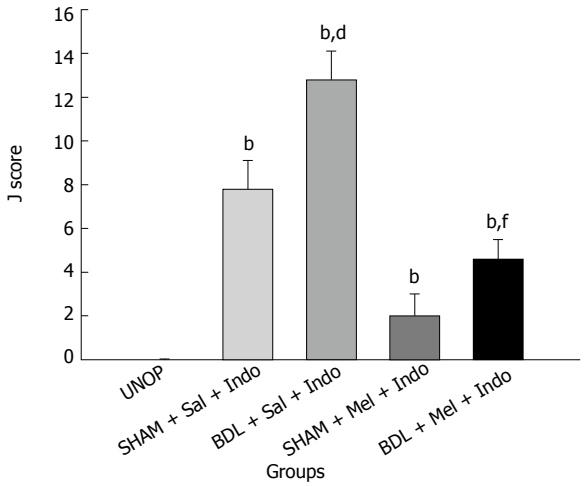


**Figure 1** Effect of melatonin (20 mg/kg) on indomethacin (50 mg/kg) induced gastric damage in BDL and SHAM animals. Five rats were used in each group. Data values are expressed as mean ± SD. $^{b}P < 0.001$ *vs* UNOP group; $^{d}P < 0.001$ *vs* Sham + Sal + Indo group; $^{f}P < 0.001$ *vs* Sham + Mel + Indo group.

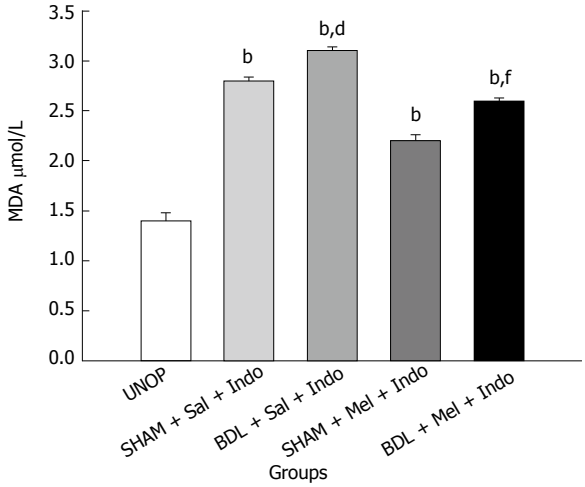


**Figure 2** Effect of melatonin (20 mg/kg) on indomethacin (50 mg/kg) induced plasma malondialdehyde (MDA) levels in BDL and SHAM animals. Five rats were used in each group. Data values are expressed as mean ± SD. $^{b}P < 0.001$ *vs* UNOP group; $^{d}P < 0.001$ *vs* Sham + Sal + Indo group; $^{f}P < 0.001$ *vs* Sham + Mel + Indo group.

14 000 rpm to separate the two phases[20]. Aliquots of 20 μL removed from n-butanol layers of each sample and injected in HPLC for analysis without evaporation[20]. Since MDA degrade approximately 10% per hour, the best performance of assay requires analyzing samples within an hour of derivatization[20].

### HPLC procedure

Chromatographic determination was performed on a Cecil 1100 series high performance- liquid chromatograph equipped with an 1100 series pump and a fluorescence detector which was set at an excitation wavelength of 515 nm and emission wavelength of 553 nm. A HP 3395 integrator was employed to record retention times, chromatograms and evaluate peak areas. The column was a Pefectsil Target (ODS, 150 mm × 4.6 mm, 3-5 μ particles). Elution was performed isocraticaly with a mixture of methanol-buffer (40:60 v/v) at a flow rate of 1.0 mL/min at room temperature. The buffer was 50 mmol/L potassium monobasic phosphate (anhydrous) with an adjusted pH of 6.8 using a 5 mmol/L potassium hydroxide solution. In this study, we used the Agarwal method for determination of MDA[20].

### Linearity

Calibration curves were created for 6 different concentrations (0-4 μmol/L) by plotting the peak areas versus the nominal MDA concentrations. There was a significant linear relationship between MDA concentration in water and plasma and peak area obtained by fluorescence (water: Y = 1 642 598X + 484 484; $r^2 = 0.999$; plasma: Y = 1 888 481X + 2 706 994; $r^2 = 0.999$). Furthermore the slopes of peak areas to MDA levels were parallel in plasma and water, and that no matrix effect existed when n-butanol was added as an extracting solvent, suggesting that n-butanol is superior as an extracting solvent.

### Accuracy and limit of detection

The accuracy was determined by evaluation of analytical recovery after addition of known amounts of standard solution to plasma. After homogenizing the samples, the MDA-TBA adduct was measured as described in the sample preparation section. The limit of detection was computed by standard method[21]. The average of recovery of MDA-TBA adducts was 99%.

### Statistical analysis

Data were analyzed statistically by one-way analysis of variance (ANOVA) and expressed as mean ± SD. The *P* values less than 0.05 were considered statistically significant. If a significant *P* value was obtained, the POST HOC analysis (Tukey-HSD multiple comparison tests) was performed to determine the effect of various treatments on gastric damage and MDA levels. Calculations were performed using SPSS software version 13.

## RESULTS

### J-scores in BDL and SHAM groups

Two days after laparotomy, BDL rats showed signs of cholestasis (jaundice, dark urine and steatorrhea) confirming rise in the level of plasma bilirubin. As shown in Figure 1, 50 mg/kg of indomethacin in BDL and SHAM rats produce gastric lesions with a J-score of 12.8 ± 1.3 and 7.8 ± 1.3, respectively. These results show that gastric mucosal damage is significantly more severe in BDL compared with SHAM and UNOP ($P < 0.001$) animals. This means that cholestasis increased the ulcerogenic effect of indomethacin and in multistress conditions; gastric mucosal damage is significantly more severe. The effect of melatonin on indomethacin-induced gastric damage in BDL and SHAM groups has been shown in Figure 1. As shown in Figure 1, 20 mg/kg of melatonin 30 min before indomethacin reduces the ulcerogenicity of indomethacin both in BDL and SHAM rats. The J-scores in this group were 4.6 ± 0.89 and 2 ± 1, respectively. These results show that the antioxidant effect of melatonin reduces cell damage mediated by oxidative stress.

### MDA levels in BDL and SHAM groups

Plasma MDA levels are shown in Figure 2. MDA level is

significantly higher in BDL compared with SHAM and UNOP rats (BDL = 3.1 ± 0.04; SHAM = 2.8 ± 0.04; UNOP = 1.4 ± 0.08; $P < 0.001$). These results show that the level of MDA is dependent on the number of stressful conditions. The effect of melatonin on MDA level in BDL and SHAM groups has been shown in Figure 2. As shown in Figure 2, 20 mg/kg of melatonin 30 min before indomethacin reduced the lipid peroxidation and MDA levels both in BDL and SHAM groups (BDL = 2.6 ± 0.03; SHAM = 2.2 ± 0.06; $P < 0.001$).

## DISCUSSION

Oxidative stress caused by reactive oxygen species (ROS) damaged cellular lipids, proteins and DNA and is widely recognized as one of the causes of the development of chronic disease. Under normal circumstances, the levels of ROS are low enough to be effectively removed by the natural defense mechanisms of the cell. There are, however, many conditions that enhance the production of ROS to such an extent that cellular defenses are overwhelmed and the cell injured[19]. The evaluation of biomarkers of cellular stress in conditions mediated by oxidative compounds could help to prevent appearance and development of oxidative stress-related diseases. Lipid peroxidation is considered to be important in the development of atherosclerosis, to be involved in aging and other clinical disorders, such as cancer, cardiovascular and liver diseases. An important step in the degradation of cell membranes is the reaction of ROS with double bound polyunsaturated fatty acids to yield lipid hydroperoxide (as primary products). On breakdown of such hydroperoxides a great variety of aldehydes can be formed (as secondary products). MDA, a three carbon compound formed by scission of peroxidized polyunsaturated fatty acids, is one of the main secondary products of lipid peroxidation[16,22]. MDA is reactive toward amino groups of protein and nucleic acid and have cytotoxic and mutagenic effects. Since MDA has been found elevated in various diseases thought to be related to free radical damage, it has been widely used as an index of lipid peroxidation in biological and medical sciences[20,22]. Determination of lipid peroxidation products in the body fluids can be used as a diagnostic tool for detecting increased levels of ROS. The most popular method of MDA detection is derivatization of MDA with different chemicals such as TBA and DNPH (2, 4-dinitrophenylhydrazine) and conversion into derivatives to allow more specific estimation of this compound. We used the HPLC method suggested by Agarwal, a rapid and reproducible method. In this method, protein was not precipitated and samples were derivatized after addition of BHT, and extraction of derivatized sample with n-butanol, found to be a suitable extracting solvent[20]. The plasma MDA levels and J. scores in both BDL animals treated with indomethacin and SHAM animals treated with indomethacin groups were greater than UNOP animals ($P < 0.001$). The plasma MDA levels and J-score in BDL rats treated with indomethacin were greater than SHAM animals treated with indomethacin ($P < 0.001$). The plasma MDA levels and J-score in BDL animals pretreated with melatonin and treated with indomethacin and SHAM animals with melatonin and treated with indomethacin were lower than BDL animals treated with indomethacin and SHAM animals treated with indomethacin. In conclusion, our results suggest that: cholestasis can increase the indomethacin-induced gastric mucosal damage and plasma MDA level. Melatonin is an efficient agent in reducing the negative effect of lipid peroxidation and reduces cell damage by oxidative stress in multistress conditions. According to the calculated detection limit (0.101 μmol/L) and average of recovery (99%) for MDA, sensitive methods for the determination of MDA level in plasma have been used.

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*RAPID COMMUNICATION*

# Listening to Turkish classical music decreases patients' anxiety, pain, dissatisfaction and the dose of sedative and analgesic drugs during colonoscopy: A prospective randomized controlled trial

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## Abstract

**AIM:** To determine whether listening to music decreases the requirement for dosages of sedative drugs, patients' anxiety, pain and dissatisfaction feelings during colonoscopy and makes the procedure more comfortable and acceptable.

**METHODS:** Patients undergoing elective colonoscopy between October 2005 and February 2006 were randomized into either listening to music (Group 1, $n$ = 30) or not listening to music (Group 2, $n$ = 30). Anxiolytic and analgesic drugs (intravenous midazolam and meperidine) were given according to the patients' demand. Administered medications were monitored. We determined their levels of anxiety using the State-Trait Anxiety Inventory Test form. Patients' satisfaction, pain, and willingness to undergo a repeated procedure were self-assessed using a visual analog scale.

**RESULTS:** The mean dose of sedative and analgesic drugs used in group 1 (midazolam: 2.1 ± 1.4, meperidine: 18.1 ± 11.7) was smaller than group 2 (midazolam: 2.4 ± 1.0, meperidine: 20.6 ± 11.5), but without a significant difference ($P$ > 0.05). The mean anxiety level in group 1 was lower than group 2 (36.7 ± 2.2 *vs* 251.0 ± 1.9, $P$ < 0.001). The mean satisfaction score was higher in group 1 compared to group 2 (87.8 ± 3.1 *vs* 58.1 ± 3.4, $P$ < 0.001). The mean pain score in group 1 was lower than group 2 (74.1 ± 4.7 *vs* 39.0 ± 3.9, $P$ < 0.001).

**CONCLUSION:** Listening to music during colonoscopy helps reduce the dose of sedative medications, as well as patients' anxiety, pain, dissatisfaction during the procedure. Therefore, we believe that listening to music can play an adjunctive role to sedation in colonoscopy. It is a simple, inexpensive way to improve patients' comfort during the procedure.



**Key words:** Colonoscopy; Turkish classical music; Sedative medications; Anxiety; Pain; Satisfaction

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## INTRODUCTION

Anxiety and pain are common problems associated with colonoscopy procedure and most endoscopy units prescribe some form of sedation and analgesia for patients undergoing this procedure[1]. Although the use of sedation is both risky and costly[2,3], performing colonoscopy without sedation may adversely affect both the outcome and patients' tolerance and is therefore not universally accepted practice[4-6]. It is imperative that ways be found to reduce the use of sedation without affecting patients' tolerance and satisfaction[1]. To reduce a patient's anxiety during invasive procedures like endoscopy, various approaches have been used to distract the patient's attention, such as therapeutic communication, visualization, aroma therapy, therapeutic touch, and listening to music[7,8].

Use of music to promote relaxation has a long history in medicine[1,9]. Music has been acknowledged as a cheap, safe and effective non-pharmacological anxiolytic agent due to its effect on the perception of pain and anxiety,

reducing the regular pharmacological sedative doses[10]. The role of music as an adjunct to standard treatment has been studied in several disorders[9]. There are a few studies that analyse the effect of music on the anxiety suffered by patients who undergo invasive procedures[10]. Some studies suggest that music alleviates anxiety and improves patients' tolerance during gastrointestinal (GI) endoscopy[9].

In this study, we aimed to assess whether listening to Turkish classical music may decrease the dose of sedative and analgesic medication requried during the colonoscopy procedure and improve anxiety and dissatisfaction of the patients.

## MATERIALS AND METHODS

Between October 2005 and February 2006, 60 patients scheduled for elective colonoscopy consecutively agreed to participate in the study. Patients aged below 18 or above 75 years, those who have hearing problems due to any cause, patients with overt or borderline psychiatric illnesses, presence of senile dementia, treatment with anxiolytic medication in the 72 h prior to the examination and those with considerable cardiopulmonary morbidities were excluded. Patients were randomized into two groups (group 1: listening to music, group 2: not listening to music) using computer-generated random numbers. All examinations were performed by an expert endoscopist who performed at least 200 full-length colonoscopic procedures previously using the Olympus CV-145 video-colonoscope. All patients provided written informed consent for participation in the study. The study protocol was approved by local ethical committee of Gaziantep University.

In this study, in the first part of the questionnaire in order to obtain demographic characteristics of the patients, questions related to age, gender, educational level, marital status, occupation, monthly income, having previous non-formal information about colonoscopy were asked. Doses of sedatives and analgesics were determined by the endoscopist performing the procedure according to patients' demand and anxiety status. Initial intravenous doses of meperidine at 10 mg and initial midazolam at 1 mg were given. Additional doses were titrated based on assessments of the patient by the endoscopist and nurse. Increments of meperidine (10 mg) and midazolam (1 mg) were given if patients showed signs of discomfort, pain, restlessness, or agitation that were not related to hypoxemia. After the procedure, the patients were asked several questions using a visual analog scale ranging from 0 (very much) to 100 (none) for pain (0 = Very much 100 = None), satisfaction (0 = none, 100 = very much), procedure evaluation (0 = unpleasant, 100 = pleasant) and willingness to do the procedure again (0 = never, 100 = happly)[1,11].

The State Trait Anxiety Inventory (STAI) was used to determine the anxiety level of the patients. STAI was developed by Spielberger *et al* in 1970 and adapted to Turkish population by LeCompte *et al* in 1976 and confirmed for reliability and validity by Oner in 1977[12]. This instrument has been used extensively in clinical settings to measure feelings of apprehension, tension, and nervousness. STAI is a two-part 40-item self-report. The Trait Portion (20 items) measures a person's general disposition and the State Portion (20 items) measures how a person feels at the time of the operation. The instrument is rated on a four-point scale. Scores are added to obtain an overall score (higher scores indicate higher levels of anxiety). The internal consistency alpha coefficients of the state portion range from 0.86 to 0.92. It is simple to use, generally taking < 5 min to complete, and easy to score[13,14].

Patients in the study group before and during the procedure were exposed to approximately 30 min of music therapy broadcasting from the central system of the endoscopy unit. The decision about the type of music was given by consultations with the specialist working in the Turkish Music Department of State Conservatory of Gaziantep University. During the research, music of a *ney* (reed flute) that is played using a traditional Turkish classical music instrument was found. It has been theorized that classical Turkish music, a slow and relaxing type of instrumental music, has the most recreative effect on people's psychological state[15] and ney is considered the most suitable instrument for this purpose. It has been known that Uygur Turks use music in the treatment of patients. Especially in the Mevlevi stories, it is told that psychologically ill people become relaxed when they listen to the sound of the ney. This application of music therapy was also seen in the Ottoman Empire period[16,17].

### *Statistical analysis*

Statistical Package for Social Sciences (SPSS 10.0 for Windows) was used for the analysis of the data. We performed Student's *t* test to compare means for two groups of cases. $P < 0.05$ was considered as significant.

## RESULTS

No significant difference was found between the two groups with respect to their age, gender, urban residence or not, educational and marital status, occupation, monthly income and having non-formal information about colonoscopy ($P > 0.05$) (Table 1).

Drug doses in group 1 (midazolam: 2.1 ± 1.4, meperidine: 18.1 ± 11.7) was lower than group 2 (midazolam: 2.4 ± 1.0, meperidine: 20.6 ± 11.5), but the difference was not statistically significant ($P > 0.05$) (Figure 1). Anxiety levels were significantly lower in the study group ($P < 0.05$) (Figure 2)

Patients in group 1 experienced less pain, were more willing to repeat the procedure, perceived the procedure more comfortable and were more satisfied with the procedure. All these parameters in group 1 and group 2 were significantly different ($P < 0.05$) (Figure 3).

## DISCUSSION

Colonoscopy is an uncomfortable and painful endoscopic procedure[4]. The use of various relaxing techniques to reduce pain and anxiety has been reported in medical literature[1]. Music therapy is one of the relaxing techniques and the benefical effects have been recognized for many years[18]. Several studies reported that the use of music

**Table 1 Sociodemographic characteristics of patients**

| | Group | | | | Total | | Significance |
|---|---|---|---|---|---|---|---|
| | Music (+) | | Music (-) | | | | |
| | *n* | % | *n* | % | *n* | % | |
| **Age (yr)** | | | | | | | |
| 20-39 | 10 | 33.3 | 5 | 16.7 | 15 | 25.0 | $\chi^2 = 2.754$ |
| 40-59 | 11 | 36.7 | 11 | 36.7 | 22 | 36.7 | |
| 60 and over | 9 | 30.0 | 14 | 46.6 | 23 | 38.3 | $P = 0.252$ |
| Gender | | | | | | | |
| Female | 16 | 53.3 | 16 | 53.3 | 32 | 53.3 | $\chi^2 = 0.000$ |
| Male | 14 | 46.7 | 14 | 46.7 | 28 | 46.7 | $P = 0.602$ |
| Marital status | | | | | | | |
| Married | 23 | 76.7 | 26 | 86.7 | 49 | 81.7 | $\chi^2 = 1.002$ |
| Single | 7 | 23.3 | 4 | 13.3 | 11 | 18.3 | $P = 0.253$ |
| Educational status | | | | | | | |
| Primary school | 20 | 66.7 | 21 | 70.0 | 41 | 68.3 | $\chi^2 = 1.714$ |
| High school | 8 | 26.7 | 6 | 20.0 | 14 | 23.3 | |
| University | 2 | 6.7 | 3 | 10.0 | 5 | 8.4 | $P = 0.634$ |
| Monthly income | | | | | | | |
| < minimum wage (MW) | 12 | 40.0 | 6 | 20.0 | 18 | 30.0 | $\chi^2 = 4.118$ |
| MW up to 2 times MW | 16 | 53.3 | 18 | 60.0 | 34 | 56.7 | |
| Between 2-3 times MW | 2 | 6.7 | 6 | 20.0 | 8 | 13.3 | $P = 0.128$ |
| Having non-formal information about colonoscopy | | | | | | | |
| Yes | 6 | 20.0 | 10 | 33.3 | 16 | 26.7 | $\chi^2 = 1.364$ |
| No | 24 | 80.0 | 20 | 66.7 | 44 | 73.3 | $P = 0.191$ |
| Total | 30 | 100.0 | 30 | 100.0 | 60 | 100.0 | |

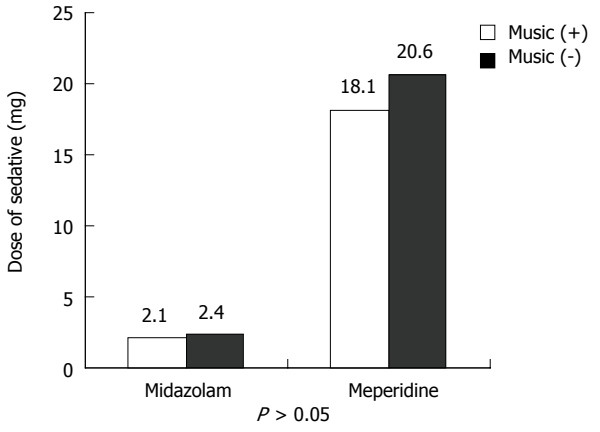


**Figure 1** Patients' mean dose of sedative drugs.

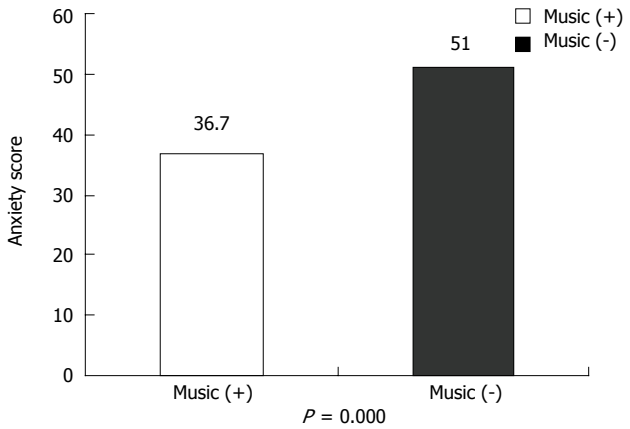


**Figure 2** Patients' mean anxiety scores.

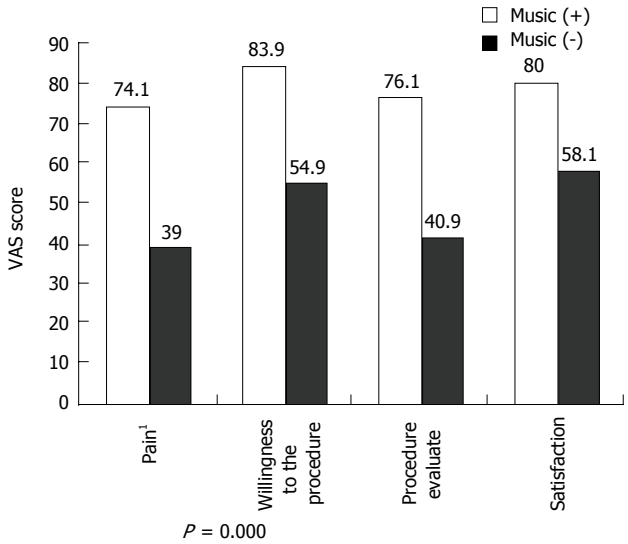


**Figure 3** Patients' mean VAS scores of pain, willingness to do the procedure, procedure evaluation and satisfaction. [1](0 = Very much, 100 = None)

during endoscopy could alleviate anxiety and improve tolerance and compliance[7,18,19]. The results of clinical studies suggest that listening to music has a positive effect on psychological and physiologic status, and thus music has been used as an anxiolytic for relieving discomfort in stress-related interventions[20].

Lee *et al*[20] found that listening to music decreased the dose of sedative drugs that were used during colonoscopy. Harikumar *et al*[9] revealed that the dose of midazolam was lower in music group. In our study, we also found the dose of sedatives was lower; however, probably due to small

sample size, the difference was not statistically significant. Nevertheless, this prospective randomized controlled trial demonstrated that music can decrease the dose of sedative medication required for colonoscopy. The use of music as an adjuvant to sedation can potentially reduce dose-related complications associated with the use of sedative and analgesic drugs.

Many studies in different fields of medicine revealed beneficial effects of music on anxiety and discomfort[7,21-23]. Palakanis *et al*[18] determined that music decreased anxiety levels in sigmoidoscopy patients. Andrada *et al*[10] also found that anxiety levels were lower in music group in colonoscopy patients. In our study, we also clearly showed that listening to music decreased anxiety significantly. Many of the beneficial effects of music are linked to its anxiolytic capacity and its action on the perception of pain. This painful perception, however, is correlated with the levels of anxiety perceived during the procedure; therefore, an increase of this anxiety results in a more painful perception. Our study allows us to affirm that listening to music during colonoscopic procedures decreases the levels of anxiety linked to this invasive procedure.

Music therapy is widely used in treatment of acute and chronic pain[24]. Some studies showed decrease of pain by music[1,24-26]; however, some showed no effect[9,11,20]. In our study, pain perception was significantly decreased by listening to music.

Most studies revealed that music increased patients' satisfaction[1,19,27-29]. Our results are consistent with the literature. Although the doses of sedative drugs were not different between the two groups in our study, the satisfaction score was higher in group 1. The use of music in addition to sedative drugs had a favorable effect on patients' satisfaction. Moreover, most patients who underwent colonoscopy with a combination of music plus sedative drugs were willing to repeat the procedure. Thus, in the present study, music improved patients' acceptance of and tolerance to colonoscopy.

## CONCLUSION

This randomised trial demonstrates that music decreases the dose of sedative and analgesic medication required by patients undergoing colonoscopy, but not significantly. Listening to music decreases anxiety levels and pain scores significantly and increases satisfaction scores and patients' comfort and tolerance. Since it is a readily available, noninvasive, inexpensive, simple, and nonpharmacological method without any side effects, listening to relaxing music is highly recommended as an adjunct to sedatives and analgesics for patients undergoing elective colonoscopy.

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*RAPID COMMUNICATION*

# Glutamine dipeptide for parenteral nutrition in abdominal surgery: A meta-analysis of randomized controlled trials

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**Key words:** Glutamine dipeptide; Parenteral nutrition; Abdominal surgery; Meta-analysis

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## Abstract

**AIM:** To assess the clinical and economical validity of glutamine dipeptide supplemented to parenteral nutrition (PN) in patients undergoing abdominal surgery.

**METHODS:** A meta-analysis of all the relevant randomized controlled trials (RCTs) was performed. The trials compared the standard PN and PN supplemented with glutamine dipeptide in abdominal surgery. RCTs were identified from the following electronic databases: the Cochrane Library, MEDLINE, EMBASE and ISI web of knowledge (SCI). The search was undertaken in April 2006. Literature references were checked by computer or hand at the same time. Clinical trials were extracted and evaluated by two reviewers independently. Statistical analysis was performed by RevMan4.2 software from Cochrane Collaboration. A $P$ value of < 0.05 was considered statistically significant.

**RESULTS:** Nine RCTs involving 373 patients were included. The combined results showed that glutamine dipeptide has a positive effect in improving postoperative cumulative nitrogen balance (weighted mean difference (WMD = 8.35, 95% CI [2.98, 13.71], $P$ = 0.002), decreasing postoperative infectious morbidity (OR = 0.24, 95% CI [0.06, 0.93], $P$ = 0.04), shortening the length of hospital stay (WMD= -3.55, 95% CI [-5.26, -1.84], $P$ < 0.00001). No serious adverse effects were found.

**CONCLUSION:** Postoperative PN supplemented with glutamine dipeptide is effective and safe to decrease the infectious rate, reduce the length of hospital stay and improve nitrogen balance in patients undergoing abdominal surgery. Further high quality trials in children and severe patients are required, and mortality and hospital cost should be considered in future RCTs with sufficient size and rigorous design.

## INTRODUCTION

Glutamine (Gln) is the most abundant free amino acid in the body and plays a vital role in amino acid transport and nitrogen balance. Gln is also a primary fuel for rapidly dividing cells such as enterocytes and lymphocytes, which protect mucosa barricade and enhance immune functions[1]. Patients undergoing elective abdominal surgery usually have malnutrition and Gln concentration is low in blood due to several factors: mechanical obstruction, limitation of food intake, tumor-induced cachexia, obstruction of pancreaticobiliary, malabsorption and ongoing blood loss. Moreover, intravascular and free muscle glutamine pools become depleted in response to perioperative abrosia and operative stress. Free Gln is lack of stability in solution and intravenous adminitration is limited. Glutamine dipeptide (L-alanyl-L-glutamine) can be taken *via* vein and hydrolyzed into glutamine in circulation as Gln substitution. It was given to patients undergoing abdominal surgery in order to improve their postoperative nitrogen balance and immunonutrition[2]. Therefore, it is worth knowing whether routine supplementation of glutamine dipeptide in parenteral nutrition (PN) can amend clinical outcomes.

Meta-analysis has been applied in medical research to improve statistical efficiency, to evaluate the disadvantage of established studies and to draw reliable conclusions from various potentially relevant studies. It is the most promising approach for future research and a guideline for clinical treatment[3]. This meta-analysis aims to enhance our understanding of the clinical and economical validity of glutamine dipeptide for patients undergoing abdominal surgery.

## MATERIALS AND METHODS

### Identification of trials

The meta-analysis included clinical randomized controlled trials (RCTs) of patients undergoing abdominal surgery. The trials compared standard isonitrogen PN and PN supplemented with glutamine dipeptide.

### Search strategy

A computerized literature search was applied from the following electronic databases: the Cochrane Library (April 2006), MEDLINE (PubMed) (1966-April 2006), EMBASE (1980-April 2006) and ISI web of knowledge (SCI) (April 2006). The search was undertaken in April 2006. Literature reference proceedings were hand retrieved at the same time. The subject words for the search were glutamine dipeptide in addition to L-alanyl-L-glutamine. Other useful words were glutamine, parenteral nutrition, operation, surgery, postoperation or perioperation, RCT or clinical trials. Study literature includes abstracts at least in the English language and followed by full text.

### Data collection

Data were extracted independently by two reviewers and decided by the research team. Methodological quality of study was evaluated using the Jadad scale[4] and the trial with a score over 2 was included as high quality. Published studies were identified by the following selection criteria: study design-RCT, population-hospitalized adult patients undergoing abdominal surgery, intervention-parenteral nutrition with glutamine dipeptide and standard parenteral nutrition with isonitrogen and isocalorics. The following data were extracted: quantity and group dividing of patients, different doses and days of glutamine dipeptide used, and the baseline of trials. Outcome variables included: nitrogen balance, length of hospital stay, postoperative infection, immune effects, adverse events and mortality.

### Data analysis

The statistical analysis was performed by RevMan4.2 software, which was provided by the Cochrane Collaboration. $P$ value < 0.05 was considered statistically significant. Heterogeneity was checked by the Chi-square test Meta-analysis was done with fixed effects model when results of the trials had no heterogeneity. If the results had heterogeneity, random effects model was used and causes were analyzed. The result was expressed with an odds ratio (OR) for the categorical variable and weighted mean difference (WMD) for the continuous variables, and with 95% confidence intervals (CI). The Handbook for Cochrane Reviewer (v 1.8.0) from Cochrane Collaboration was used as a guideline for the meta-analysis.

## RESULTS

There were 454 papers relevant to the searching words. Through the steps of screening the title, reading the abstract and the entire article, only nine RCTs[5-13] involving 373 patients were included. Other two studies were excluded because of repeated reports[14-15]. Characteristics of studies included in meta-analysis are presented in Table 1. There were 7 papers published in English and 2 in Chinese. Five studies were done in Europe and 4 in Asia. In the trials, glutamine dipeptide was administered at 0.18-0.5 g/kg per day and lasted 5-7 d.

### Postoperative cumulative nitrogen balance

Six RCTs (involving 238 patients) reported postoperative cumulative nitrogen balance. The result was heterogeneous ($P$ < 0.00001) and the random effects model was used because of different preoperative general nutrition, different PN design and different doses and days of glutamine dipeptide supplementation. Combined analysis indicated that the use of glutamine dipeptide had a positive effect in improving the postoperative cumulative nitrogen balance (WMD = 8.35, 95% CI [2.98, 13.71], $P$ = 0.002) (Figure 1).

### Postoperative morbidity of infection

Five RCTs (involving 215 patients) reported the postoperative morbidity of infection. There was no heterogeneity ($P$ = 0.94). Combined analysis indicated that the use of glutamine dipeptide reduced infective events (OR = 0.24, 95% CI [0.06, 0.93], $P$ = 0.04) (Figure 2).

### Length of hospital stay

Six RCTs (involving 291 patients) reported length of hospital stay. The result was heterogeneous ($P$ < 0.00001) and the random effects model was used because of different severity of primary diseases, different levels of operational injury, different preoperative general nutrition and APACHE Ⅱ scores. Comprehensive analysis indicated that the use of glutamine dipeptide had a positive effect in shortening the length of hospital stay (WMD = -3.55, 95% CI [-5.26, -1.84], $P$ < 0.00001) (Figure 3).

## DISCUSSION

Before operation, abdominal diseases often led to lack of Gln, following malnutrition and immune dysfunctions. After operation, demand for Gln exceeded the supply from diet and from muscle as a response to injury. And it has been shown that intravascular and free muscle glutamine pools become deficient or depleted in response to operative stress[5,16,17]. The deficiency of glutamine is the main cause for protein metabolism disorder, intestinal mucosal injury, enteral wall permeability destruction, bacterial translocation and immunosuppression. All these increase the perioperative infection risk and hinder the postoperative recovery.

Meta-analysis indicates that the use of glutamine dipeptide could improve the postoperative nitrogen balance better than standard PN. Gln, accounting for approximately one third of the translocated nitrogen, plays a major role in supporting vital organ function and repairing wound[18]. Full supplementary glutamine dipeptide not only prevents protein lost, but also makes nutrition metabolism effective.

The study also gives us evidence that use of glutamine dipeptide decreases infective events. Gln is utilized at a high

**Table 1 Characteristics of studies included in meta-analysis on glutamine dipeptide for abdominal surgery**

| Authors | Journals | Year | Study design | Jadad score | Reference | Operation | Patients (Gln/Con) | Gln dipeptide (g/kg) | Days of Gln dipeptide (d) admininistration |
|---|---|---|---|---|---|---|---|---|---|
| Stehle P | Lancet | 1989 | RCT | 3 | 5 | Elective resection of carcinoma of colon or rectum | 12 (6/6) | 0.28 | 5 (postoperative 1-5) |
| O'Riordain | Annals of Surgery | 1994 | RCT | 3 | 6 | Colorectal Resection | 22 (11/11) | 0.18 | 5 (postoperative 1-5) |
| Morlion BJ | Ann Surg | 1998 | Double-blind RCT | 4 | 7 | Major abdominal surgery | 28 (13/15) | 0.3 | 5 (postoperative 1-5) |
| Metes | Clinical Nutrition | 2000 | RCT | 4 | 8 | Major abdominal surgery | 30 (15/15) | 0.5 | 5 (postoperative 1-5) |
| Jiang Z | Zhong guo Yi Xue Ke Xue Yuan Xue Bao | 2000 | Prospective double-blind RCT | 5 | 9 | Gastrointestinal operations | 120 (60/60) | 0.36 | 6 (postoperative 1-6) |
| Neri | Nutrition | 2001 | Multiple centers prospective double-blind RCT | 3 | 10 | Major abdominal surgery | 33 (16/17) | 0.18 | 5 (postoperative 1-5) |
| Fan YP | Zhonghua Wai Ke Za Zhi | 2005 | RCT | 3 | 11 | Abdominal surgery | 40 (20/20) | 0.16 | 7 (postoperative 1-7) |
| Lin MT | World J Gastroenterol | 2005 | RCT | 4 | 12 | Abdominal surgery | 48 (25/23) | 0.417 | 6 (postoperative 1-6) |
| Yao GX | Clin Nutr | 2005 | RCT | 3 | 13 | Gastrointestinal operations | 40 (20/20) | 0.5 | 5 (preoperative 1-postoperative 3) |

RCT: Randomized controlled trial; Gln/Con: Glutamine dipeptide group/controlled group.

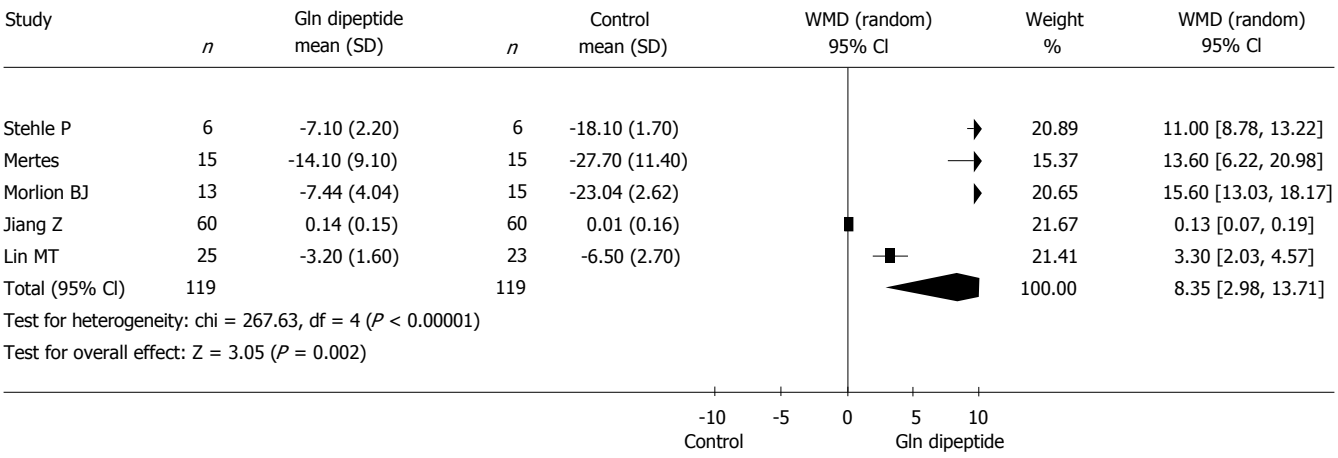


**Figure 1** Effect of glutamine dipeptide on postoperative cumulative nitrogen balance for abdominal surgery. Review: Clinical evidence of glutamine dipeptide for abdominal surgery; Comparision: Gln dipeptide *vs* control; Outcome: Postoperative cumulative nitrogen balance.

rate by cells of the immune system. It supports optimal lymphocyte proliferation, and production of cytokines by lymphocytes and macrophages. Macrophage-mediated phagocytosis is influenced by glutamine availability[1,19]. Hydrolysable glutamine dipeptide can substitute glutamine to support lymphocyte and macrophage functions *in vitro*. Animal studies have shown that intake of glutamine increases survival to bacterial challenge[19]. After operation, immunosuppression resulted in lowered plasma glutamine concentrations. If Gln or its precursors are provided to patients at risk of immunosuppression following surgery, sufficient maintenance of plasma glutamine concentration is of benefit to maintaining immune function. In clinical trials, glutamine dipeptide ameliorates restoration of plasma CD14 levels, improves lymphocyte recovery[6,9] and attenuates plasma IL-6 to relieve immunodepression[12,15]. Moreover, glutamine in circulation can also improve barricade function of gastroenteral mucosa to prevent bacterial translocation[9].

In those studies, mortality and hospital cost were seldomly reported. Length of hospital stay becomes a clinical end point as a surrogate for both clinical and economic efficiency. Meta-analysis shows that the use of glutamine dipeptide has a positive effect on shortening the length of hospital stay. Factors influencing hospital stay by Gln include accelerating wound repair and recovery of intestinal mucosal integrity, preventing potential delay of infection. It implies that such an approach is optimal in clinical practice, especially for severe surgeries[15]. At the same time, no serious adverse effects were found in all the

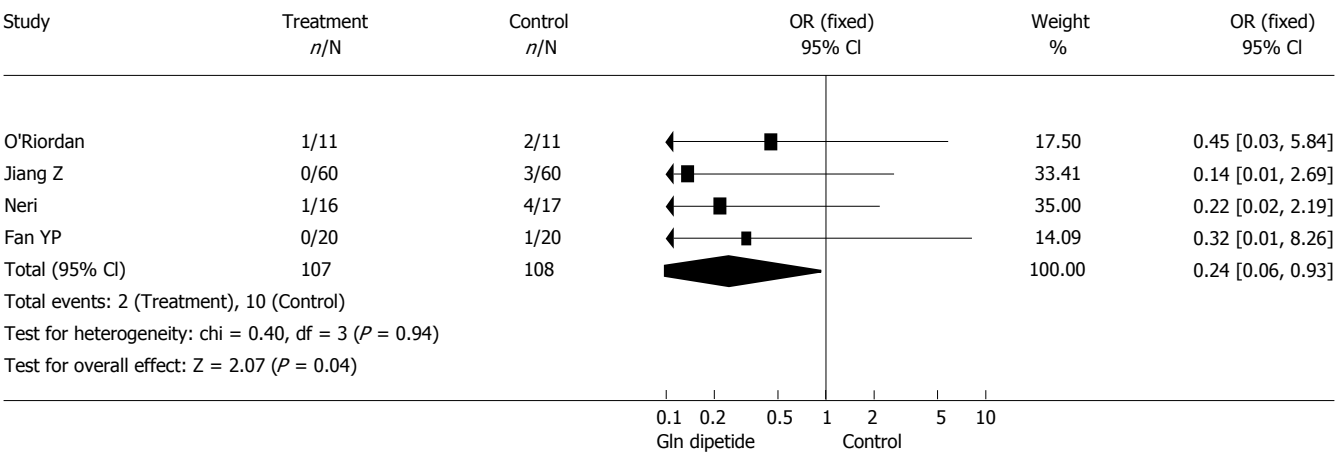


**Figure 2** Effect of glutamine dipeptide on postoperative morbidity of infection for abdominal surgery. Review: Clinical evidence of glutamine dipeptide for abdominal surgery; Comparison: Gln dipetide *vs* control; Outcome: Postoperative infective morbidity.

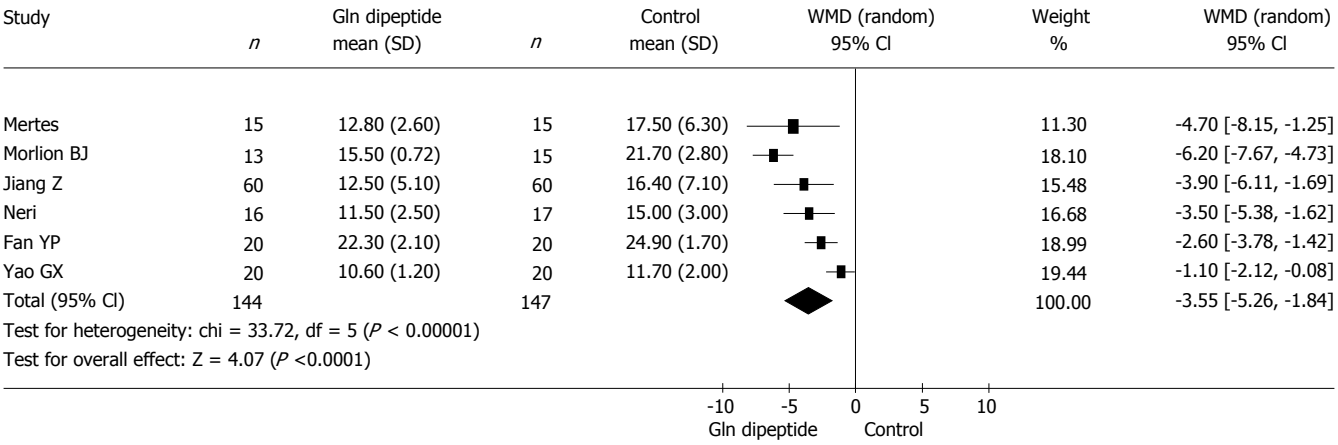


**Figure 3** Effect of glutamine dipeptide on length of hospital stay for abdominal surgery. Review: Clinical evidence of glutamine dipeptide for abdominal surgery; Comparison: Gln dipeptide *vs* control; Outcome: Length of hospital stay.

included studies.

In conclusion, postoperative PN supplemented glutamine dipeptide is effective and safe to decrease the infectious rate, reduce length of hospital stay and improve nitrogen balance in patients undergoing abdominal surgery. Further high quality trials in children and severe patients are required; Mortality and hospital cost should be considered in future RCTs with sufficient size and rigorous design.

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*RAPID COMMUNICATION*

# Endoscopic findings and pathologic characteristics of gastric eosinophilic granuloma: A report of 18 patients

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## Abstract

**AIM:** To investigate the endoscopic findings and pathologic characteristics of gastric eosinophilic granuloma (GEG).

**METHODS:** A retrospective study of 18 cases of gastric eosinophilic granulomas was conducted. Gastroscopy was performed and all specimens of biopsies were stained by H&E and observed under light microscopy.

**RESULTS:** Ulcer was the most frequent endoscopic appearance. The others included deformed pylorus and/or duodenal bulb, esophagitis, mucous hyperemia and/or mucosal erosion. Eosinophilic cell infiltration and generous hyperplasia of arterioles, venules and lymph vessels were found in the lesions of the patients. Interstitium had massive eosinophilic infiltrates and was made up of collagen fibers and fibroblasts. Lymphoid follicles were revealed in some sections of biopsies.

**CONCLUSION:** GEG is lack of specific symptoms and physical signs. It can be misdiagnosed as gastric ulcer in most cases before biopsies. Endoscopy and endoscopic multiple deep biopsies in suspected areas are indispensable for correct diagnosis of GEG.



**Key words**: Gastric eosinophilic granuloma; Endoscopy; Pathology

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## INTRODUCTION

Gastric eosinophilic granuloma (GEG) is characterized by pathological changes including eosinophil cells infiltrated into the submucous layer and muscular layer of the stomach. Its etiology is still unknown. The patients are prone to be misdiagnosed as having gastric carcinoma and gastric ulcer[1]. In order to investigate the endoscopic findings and pathologic characteristics so as to improve the diagnostic accuracy and therapeutic effect for GEG, 18 patients finally diagnosed by endoscopy and pathology are analyzed.

## MATERIALS AND METHODS

### Patients

From 2002 to 2006, a retrospective review of endoscopic mucosal biopsies and pathologic examinations was performed at the Digestive Endoscopic Center of Renmin Hospital of Wuhan University (Wuhan, China). In the routine gastroscopies performed during this period, 18 GEG patients (14 males, 4 females) were found among the 14 396 cases. Their mean age was 44.85 ± 11.70 years (range 22-65 years).

Endoscopic diagnoses were GEG (7 cases; including 5 definite cases and 2 suspected cases), gastric ulcer (5 cases), gastric ulcer accompanying esophagitis (1 case), gastric ulcer accompanying superficial gastritis (2 cases), superficial gastritis (1 case), and superficial gastritis accompanying duodenal ulcer (2 cases).

### Design

Gastroscopy was performed in the 18 cases. All the patients underwent endoscopic mucosal biopsies and pathologic examinations. All specimens of biopsies were stained by H&E and observed under light microscopy. Diagnostic criteria were used in accordance with the literature[2].

### Ethics

This is a retrospective study and data were collected by the common methods used in clinical practice.

## RESULTS

### Endoscopy

Ulcer is the most frequent endoscopic appearance. The endoscopic appearances include ulceration in 15 cases,

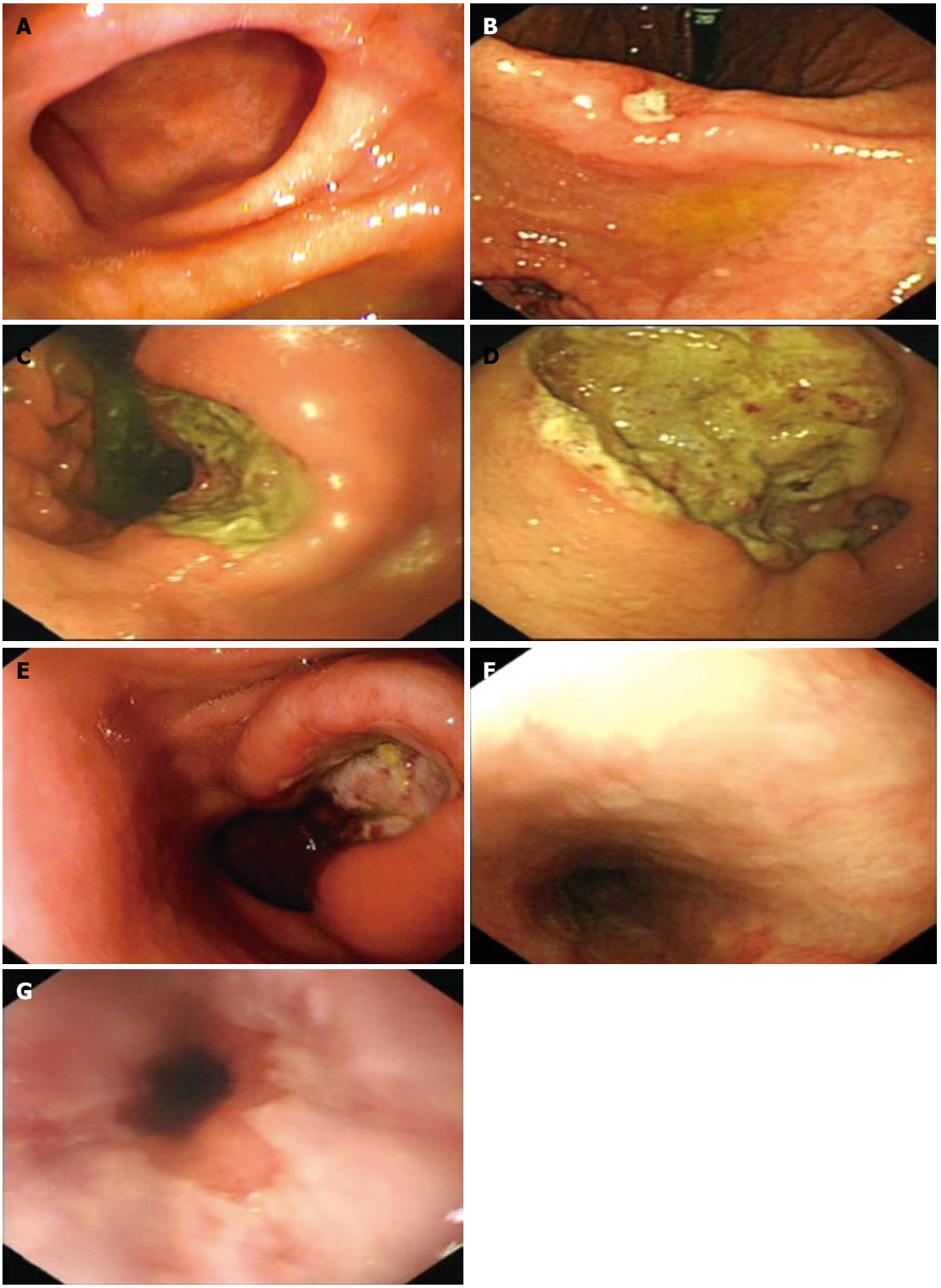


**Figure 1** Endoscopic photographs of GEG. **A**: Retracted ulcer scar on greater curvature and incomplete closure of pylorus; **B**: A 0.3 cm × 0.4 cm ulcer on gastric notch had whitish base; **C**: A 3 cm × 4 cm ulcer on inferior wall of gastric body and lesser curvature side. Yellowish green base and tumid ulcer margin; **D**: A 4 cm × 5 cm giant ulcer on lesser curvature side of gastric antrum and gastric notch had thickened and irregular base. Anterior wall of duodenal bulb was involved; **E**: The deformed gastric antrum and a 2 cm × 3 cm giant ulcer on gastric antrum and posterior wall of pylorus. The ulcer had mignonette base and black spots. The ulcer margin presented indentation-shaped appearance. Ulcer tissue was rigid and prone to bleed; **F**: Rough esophageal mucous membrane, blurred vascular net, clear-cut esophageal Z line; **G**: Higher esophageal Z line.

retracted scar tissue in 1 case (Figure 1A), and erosive gastritis in 2 cases.

**Sites of ulcers:** Gastric notch (3 cases), gastric body (3 cases), gastric antrum (5 cases), complex ulcer (1 case of gastric notch $H_1$+duodenal bulb $A_2$, 2 cases of gastric antrum $H_1$+duodenal bulb $A_1$), multiple ulcers on cardia and gastric antrum (1 case) and duodenal ulcer ( 2 cases).

**Morphologic characteristics of ulcers:** Besides 3 cases of giant peptic ulcers, the diameter of ulcers in most cases was less than 1 cm. Most ulcers were in active stage (7 cas-

es of $A_1$ stage and 3 cases of $A_2$ stage) and had whitish or greyish bases (Figure 1B). Merely 2 ulcers had mignonette bases (Figure 1C). Black spots were seen in the ulcer base in 1 case (Figure 1D). In addition, minute protuberances were frequently seen and caused by hyperplasia of granulation tissues. An irregular base was only found at a giant ulcer on the gastric antrum (Figure 1D). But malignant gastric ulcers, regardless of hemorrhage or not, showed dun or mignonette base, and no minute protuberances occurred. The margin of most ulcers was smooth and regular, and congestion and swelling of the surrounding mucosa were frequently seen, and symmetrically thickened (inflammatory) folds typically radiated to the ulcer base (Figures 1C and D). The surrounding mucosa of one giant ulcer presented with an indentation-shaped appearance (Figure 1E), one (posterior wall ulcer of central gastric body) presented with a crater-like appearance, and one (irregular ulcer of the gastric body) had an obscure boundary and nodular base. These cases should be differentiated from malignant ulcer. In contrast, malignant ulcers characteristically have irregular edges, and the surrounding, asymmetrical folds do not radiate to the ulcer base. Such folds may appear nodular or clubbed. An obvious mass often surrounds the malignant ulcer.

**Texture of ulcers:** Biopsied tissue was relatively rigid but had certain tenacity, ulcer tissues were not fragile, and the sites of biopsies bled less. Nevertheless, ulcer tissues of a giant ulcer on the gastric antrum was rigid and the sites of biopsies were prone to bleed during biopsies (Figure 1E). This case should be differentiated from malignant ulcer. Malignant ulcer is stiff and ulcer tissues are fragile. The sites of biopsies are prone to bleed. Multiple biopsies from the ulcer margin are apt to find malignant cells.

All patients had mucous hyperemia in the gastric fundus and gastric body, and mucosa-mottled congestion in the gastric antrum. Most patients had no gastroduodenal mucosal erosion, except one patient (endoscopic appearance indicative of a complex ulcer) who had mucosal erosion in the gastric notch.

Esophagus was not involved in most patients. Only in 5 cases, esophageal mucosa presented esophagitis-like appearance (Figure 1F). The mucous membrane of esophagus in 4 cases was gray, with blurred vascular net and scattered whitish granulations; 1 case had higher esophageal Z line (Figure 1G), but the mucous membrane of esophagus was normal.

These lesions included 2 cases of deformed pylorus (Figure 1E), 2 cases of incomplete closure of pylorus (Figure 1A) and mucous hyperemia, 1 case of swollen and deformed duodenal bulb, and 1 case of deformed pylorus and duodenal bulb. Anterior wall of duodenal bulb was involved and deformed by a giant ulcer from lesser curvature of gastric antrum to gastric notch in 1 case. Pylorus and duodenal bulb was normal in the rest of the cases.

### *Pathology*

Submucous layer of stomach presented significant inflammatory edema. Massive eosinophil cells and lymphocytes infiltrated into the stomach at full thickness, especially in submucous and muscular layers (Figure 2A-D). In granulated tissues, fibrous tissue proliferation around blood vessels, and fabric scars and hyalinization were found (Figure 2E). Arteriole, veinlets and lymph vessels abundantly proliferated (Figure 2F and G). The muscular layer was crushed and separated by eosinophilic cells, even the serous coat was involved. Lymphoid follicles formed and proliferated in the mucosal base. Intestinal metaplasia emerged in the epithelium of the gastric gland (Figures 2F and G). Chronic inflammation appeared in the peripheral lymph nodes. Ulcers emerged because of gastric mucosa necrosis around the lesions.

Fibroblasts and collagen fibers constituted interstitial substance of the lesions (Figure 2E). Massive eosinophilic cells and lymphocytes infiltrated into the interstitial substance. Occasionally, lymphoid follicles formed in the interstitial substance (Figure 2F and G). Arteriole, veinlets and lymph vessels also existed in the matrix.

## DISCUSSION

Kaijser[3] first described GEG in 1937. In 1950, Polayars formally denominated this syndrome as gastric eosinophilic granuloma. In 1961, Ureles, *et al*[2] systematically synthesized and categorized 47 cases of eosinophil cell infiltration in gastrointestinal tract and described the clinical characteristics of GEG extensively.

The cause of GEG is still unknown, and the pathogenesis is poorly understood. The etiological hypothesis of GEG mainly includes familial inheritance, allergic response, inflammation, foreign body reaction, *H pylori* infection, fungous infection, *etc*.[1,4-6].

There are more male patients than females, and middle-aged patients (11 cases) are frequently seen in this group. Clinical manifestations are lack of specificity and usually include stomachache, belching, abdominal distention, sour regurgitation and pyloric obstruction[7]. Compared with peptic ulcers, epigastric pain usually is irregular and is independent of eating. Abdominal pain is often severe and complicated with bleeding and chronic perforation, and antacids are ineffective. Many patients had anemia of different severities. Eosinophilic cells can increase in the peripheral blood of some patients[1].

Gastric mucosa, thickened mucosal folds, and superficial erosion were seen prominently. Because the lesions were involved in the pylorus in some patients, pyloric deformity and obstruction occurred. A few patients still presented incomplete closure of the pylorus. Most patients have ulcers. The edge of most ulcers is usually smooth and regular, and the ulcers have sharp margins, congestion and hydropsia in the surrounding mucosa, which are frequently seen, and symmetrically thickened (inflammatory) folds typically radiated to the ulcer base. The areas of ulcerations in a few patients are comparatively large; the edges of ulcers are irregular or crater-like in appearance; ulcer tissue is relatively rigid but not fragile, and the sites of biopsies are not prone to bleed[8,9]. But bleeding occurred in the sites of biopsies in 1 patient, which should be differentiated from malignant ulcers. Besides the above-mentioned appearances, GEG still has congestion and swelling of the gastric body and gastric fundus; and a few patients involv-

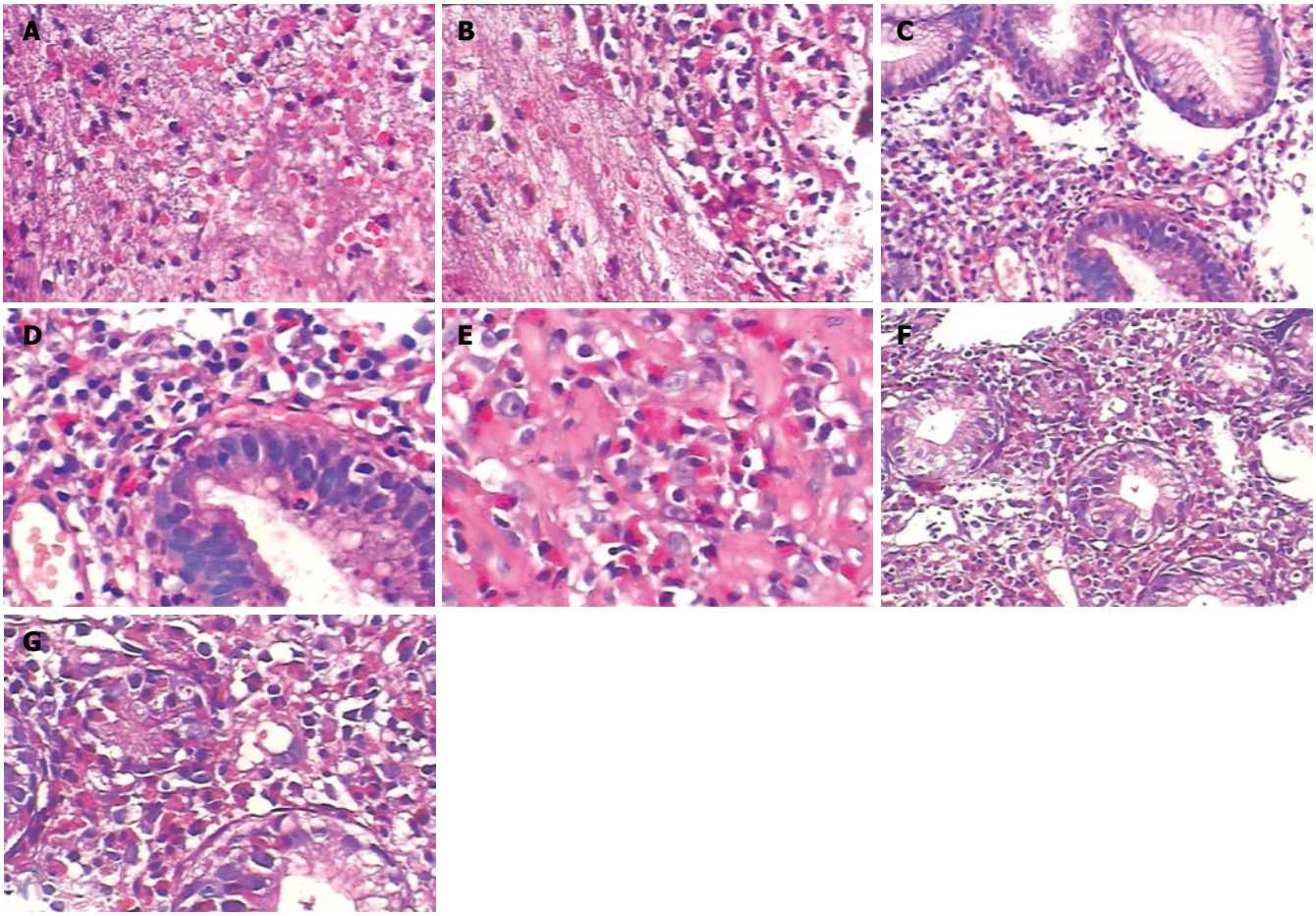


**Figure 2** Histologic photographs of GEG (H&E staining). **A**: Massive eosinophil cells and lymphocytes infiltrated (original magnification x 200); **B**: Same view as A, at different magnification (original magnification x 200); **C**: Massive eosinophil cells and lymphocytes infiltrated, especially surrounding the vessels. Intestinal metaplasia (original magnification x 100); **D**: Same view as C, at different magnification (original magnification x 200); **E**: Massive eosinophil cells and lymphocytes. Fibrous tissue proliferation, fabric scar, intestinal metaplasia and hyalinization (original magnification x 400); **F**: Massive eosinophil cells and lymphocytes. Arteriole, veinlets and lymph vessels abundantly proliferated. Lymphoid follicles (original magnification x 100); **G**: Same view as F, at different magnifications (original magnification x 400).

ing the esophagus present an esophagitis-like appearance.

In this group, all patients had lesions in the gastric antrum, and about 45% ulcers were located in the gastric antrum. The causes of GEG ulceration are suggested in four aspects: (1) Thickened stomach wall or localized masses can induce pylorus dysfunction and ulceration[10]. (2) Food deposition caused by gastric emptying disorder results in persistent food contacting with gastric antrum mucosa, which intensively stimulates gastrin and gastric acid secretion. Gastric mucosa is thus damaged and an ulcer forms. (3) Fibrous tissue proliferation surrounding blood vessels, fabric scars and hyalinization in granulation tissue cause insufficient blood supply to the gastric wall, myxasthenia and H-ion counter-diffusion, thus impairing the gastric mucosa. (4) As mucosa of the gastric antrum and lesser curvature is comparatively friable, mucosal defense to causative agents is weak, which increases the incidence rate of ulceration.

In order to increase the diagnostic accuracy of GEG, endoscopic multiple deep biopsies should be performed in suspected areas because of the eosinophilic cells which mainly infiltrate into the submucosal layer. Accordingly, most patients are firmly diagnosed by this method. With regard to the lesions of widespread infiltrating type, gastric carcinoma must be excluded. Endoscopic biopsies should be performed again when biopsies in suspected areas are negative, but these patients normally have chronic digestive system symptoms and simultaneously combine with increasing eosinophilic cells in peripheral blood, or had histories of hypersensitiveness or anaphylactic disease before. Recently, eosinophilic gastroenteritis is diagnosed by percutaneous puncture biopsies under ultrasound guidance[11].

Vanek[12] suggests the criteria for pathologic diagnosis of GEG as follows: (1) Interstitial substance is composed of fibroblasts and collagen fibers; (2) Eosinophilic cells and lymphocytes infiltrate into interstitium. Lymphoid follicles form occasionally; (3) Arteriole, veinlets and lymph vessels exist in interstitium; (4) Ulcers emerge around the pathologic lesions. While malignant lesions must be carefully precluded, GEG should be differentiated from plasma cell granuloma of the stomach when more plasma cells and Russell bodies are found in pathological lesions of some GEG patients. Exceptional granulomatous lesions such as mycetes, parasite, etc. should also be excluded. The pathological lesions of the 18 cases are consistent with the diagnostic criteria.

Therefore, in order to decrease the misdiagnosis rate, it is extremely important to have an intimate knowledge of endoscopic findings and pathologic characteristics of

GEG. Meanwhile, endoscopic examinations and endoscopic multiple deep biopsies in suspected areas are indispensable for accurate diagnosis. In addition, because endoscopic findings of some GEG patients are similar to that of gastric carcinoma, gastric lymphoma, gastric fibroma, gastric ulcer complicated with fungus infection, *etc*., these diseases must be attentively discriminated.

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*CASE REPORT*

# Acute lower gastrointestinal bleeding from a dieulafoy lesion proximal to the anorectal junction post-orthotopic liver transplant

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## Abstract

A 67-year-old woman underwent an orthotopic liver transplantation for end stage liver disease secondary to chronic autoimmune hepatitis. She developed sudden massive hematochezia on post-operative day 23 with hemodynamic compromise. The source of hemorrhage was found at colonoscopy after careful irrigation and inspection to be a dieulafoy lesion situated just proximal to the anorectal junction. Hemostasis was achieved with epinephrine injection and thermal coagulation.



**Key words:** Bleeding; Anorectal; Dieulafoy; Rectum

Apiratpracha W, Ho JK, Powell JJ, Yoshida EM. Acute lower gastrointestinal bleeding from a dieulafoy lesion proximal to the anorectal junction post-orthotopic liver transplant. *World J Gastroenterol* 2006; 12(46): 7547-7548

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## INTRODUCTION

A dieulafoy lesion is a submucosal ectatic arterial lesion associated with a tiny mucosal defect. It is a rare cause of profuse, but intermittent upper or lower gastrointestinal bleeding. The bleeding is often unresponsive to conservative treatment and is associated with a high mortality rate. The exact process that results in the tortuousity of the superficial artery which erodes through the mucosa and which then may bleed is unknown. Most cases have occurred in the proximal stomach. Less commonly, lesions are encountered in the small intestine, colon and rectum. Cases of rectal dieulafoy-like lesion have been reported in literature. We report a case of massive lower gastrointestinal bleeding from a dieulafoy lesion of the rectum, immediately proximal to the anorectal junction post liver transplantation that was successfully treated with injection of 1:10 000 epinephrine followed by thermal coagulation. Without careful endoscopic examination, this lesion may have been mistaken as bleeding from hemorrhoids.

## CASE REPORT

A 67-year-old woman underwent a cadaveric orthotopic liver transplant for end stage liver disease secondary to chronic autoimmune hepatitis. Immunosuppression consisted of induction with basiliximab (Simulect, Novartis-Canada, Dorval QC) on post operative day 0 and day 4, delayed tacrolimus (Prograf, Astellas-Canada, Markham, ON) started on post operative day 6, mycophenolate mofetil (Cellcept, Hoffman LaRoche, Mississauga ON) and tapering doses of corticosteroids. Her post operative course was complicated and prolonged due to enterococcus faecalis septicemia, requiring intravenous vancomycin for 2 wk and disseminated varicella zoster virus reactivation which was treated with intravenous acyclovir for 10 d followed by oral valacyclovir for 3 mo to prevent recurrence.

On post-operative day 23, she developed sudden massive painless lower gastrointestinal bleeding with hemodynamic compromise. Her hemoglobin level dropped from 97 g/L to 72 g/L within several hours and she required a 2 unit transfusion of packed red blood cells which raised the hemoglobin level to 99 g/L. Her liver allograft was functioning well with a normal coagulogram and her liver blood tests remained stable. She also had normal serum urea and creatinine. There was no history of constipation, abdominal pain or previous gastrointestinal bleeding but occasional diarrhea while she was in the intensive care unit. There was no history of rectal enemas use, rectal digitation, rectal tube or foreign body insertion. On examination, she was not septic, but tachycardic with heart rate of 110/min and blood pressure of 115/70 mmHg and her abdominal examination was unremarkable except for mild tenderness around the incision site. A digital rectal examination revealed the presence of fresh blood with clot and no palpable masses. An urgent colonoscopy to the terminal ileum was performed on the same day and revealed the presence of blood throughout the colon but with abundant fresh clots in the rectum.

No obvious source of hemorrhage could be identified initially. After careful irrigation and inspection, a pulsatile active bleeding site was identified < 0.5 cm proximal to the anorectal junction, just above the dentate line (Figure 1). There was no obvious erosion or ulceration around the vessel and the surrounding mucosa appeared normal. Its appearance was consistent with that of a dieulafoy lesion. Hemostasis was achieved by injection with a total of 4 mL of 1:10 000 epinephrine around the bleeding lesion followed by bipolar electrocoagulation (BICAP). No further episodes of fresh bleeding were noted post-procedure. Passage of a normal brown stool was noted the following day with no further bleeding after a follow-up of 4 mo.

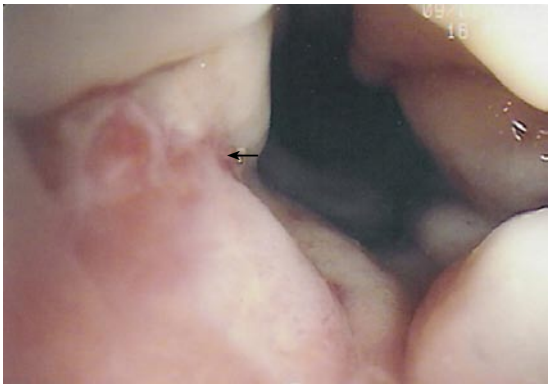

**Figure 1** Rectal dieulafoy (arrow) following injection of 1:10 000 epinephrine solution around the lesion.

## DISCUSSION

Dieulafoy's lesions are uncommon but well recognized life threatening sources of gastrointestinal bleeding. The majority of lesions occur in the stomach, and most lie in the lesser curvature within 6 cm of the gastroesophageal junction[1]. The lesion ruptures spontaneously and bleeds massively for reasons that are unclear. The typical endoscopic appearance of this lesion is a single, round mucosal defect with an artery protruding from its base in the absence of surrounding ulceration. The diagnosis now primarily depends on the endoscopic visual criteria that include active arterial spurting or micropulsatile streaming from minute (< 3 mm) mucosal defects or through normal surrounding mucosa, visualization of a protruding vessel with or without active bleeding within a minute mucosal defect or through normal appearing mucosa, and fresh, densely adherent clot with a narrow point of attachment to a minute mucosal defect or to normal appearing mucosa[2,3]. Microscopic examination of the lesion reveals a small mucosal defect with minimal inflammation and a large, tortuous, thick-walled artery at the base of the defect with rupture of the artery into the lumen with fibrin thrombus in the necrotic artery loop[4,5]. Although the original descriptions and early reports were of lesions in the proximal stomach, similar lesions have subsequently been reported in the esophagus, duodenum, jejunum, colon and rectum[4,6-10]. The unique aspect of this case was the fact that the dieulafoy lesion was immediately adjacent to the anorectal junction and without careful endoscopic lavage, could have been misdiagnosed as a hemorrhoidal bleeding with potentially disastrous sequelae. Since the colonoscopic diagnosis of rectal dieulafoy-like lesion may be difficult in the presence of large amount of luminal blood and clots, we would recommend rapid colonic lavage with a polyethyleneglycol solution either orally or *via* nasogastric tube if oral intake is not tolerated to enable adequate visualization which is crucial in making a diagnosis. A thorough and patient endoscopic examination with lavage is also necessary.

In terms of therapy, historically surgery had been the traditional treatment of dieulafoy lesions. However, the surgical approach has been superceded by endoscopic therapy. Several endoscopic methods have been successfully used including injection of epinephrine in conjunction with coagulation therapy or the application of a Hemoclip.

In summary, rectal dieulafoy lesions are unusual sources of rectal bleeding but must be included in the differential diagnosis of profuse painless rectal bleeding, especially when other definitive lesions are not found. Endoscopic therapy is safe and effective in the treatment of rectal dieulafoy lesions.

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*CASE REPORT*

# Rupture of abdominal aortic aneurysm into sigmoid colon: A case report

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## Abstract

Primary aorto-colic fistula is rarely reported in the literature. Although infrequently encountered, it is an important complication since it is usually fatal unless detected. Primary aorto-colic fistula is a spontaneous rupture of abdominal aortic aneurysm into the lumen of the adjacent colon loop. Here we report a case of primary aorto-colic fistula in a 54-year old male. The fistulated sigmoid colon was repaired by end-to-end anastomosis. Despite inotropic support, the patient died of sepsis and multiorgan failure on the first postoperative day.



**Key words:** Aortocolic fistula; Abdominal aort aneurysm; Rupture; Lower gastrointestinal bleeding; Aneurysm

Aksoy M, Yanar H, Taviloglu K, Ertekin C, Ayalp K, Yanar F, Guloglu R, Kurtoglu M. Rupture of abdominal aortic aneurysm into sigmoid colon: A case report. *World J Gastroenterol* 2006; 12(46): 7549-7550

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## INTRODUCTION

Primary aortocolic fistula is a very rare and catastrophic complication of infrarenal abdominal aortic aneurysms. The incidence of secondary aorto-enteric fistulae has increased to 1.5%-4.0% due to the increase in the frequency of aorto-iliac fistula reconstruction techniques[1]. However, the incidence of primary aorto-colic fistulae is reported to be as low as 0.69%-2.36%[2].

The most common etiology of aorto-colic fistulae is a previous aorto-iliac fistula reconstruction with a synthetic graft for abdominal aortic pathology. An abrasion of the graft or erosion of the wall of the adjacent bowel loop by the suture line may cause a connection between the lumen of both organs[2,3]. Primary aorto-colic fistula is a spontaneous rupture of abdominal aortic aneurysm into the lumen of the adjacent colon loop. Here we report a case of primary aorto-colic fistula.

## CASE REPORT

A 54-year old male was admitted to the Emergency Surgery Department of the Medical Faculty of Istanbul, Istanbul University, Turkey, with a diagnosis of an abdominal pulsatile mass. The patient suffered from hematochezia 4 d prior to admission. His past medical history did not reveal any abnormality besides heavy-smoking. During the physical examination he did not have any abdominal pain or tenderness. However, there was a pulsatile mass in the abdomen. There was blood upon rectal examination. The femoral pulses and lower extremity pulses were bilaterally palpable. The blood pressure was 90/70 mmHg, pulse rate was 112 beats/min, respiratory rate was 24 breaths/min, and the body temperature was 37.3℃. A laboratory investigation indicated hemoglobin of 7.4g/dL, hematocrit of 27%, white blood cells of 14000/mm$^3$, and a normal platelet count. The blood glucose was 160 mg/dL. Computed tomography (CT) scanning showed a 7 cm × 7 cm abdominal aortic aneurysm located infra-renal with gaseous images within the lumen of the aneurysm (Figure 1). Upon CT scanning, a loop of sigmoid colon was in close contact with the aorta at the level of the gaseous images. Emergency laparotomy showed a fistula between the aneurysm and the distal sigmoid colon. The aneurysm was resected and an axillo-bi-femoral bypass with ePTFE graft was carried out. In spite of inotropic support with dopamine and dobutamine, blood pressure was below 90 mmHg and a cardiac arrest (which responded to cardiopulmonary resuscitation) occurred during the operation. The fistulated sigmoid colon was repaired by end-to-end anastomosis. The patient was monitored at the Intensive Care Unit postoperatively. Despite inotropic support, the patient died of sepsis and multiorgan failure on the first postoperative day.

## DISCUSSION

Primary aorto-colic fistula is seldom reported in the literature. Although rarely encountered, it is an important com-

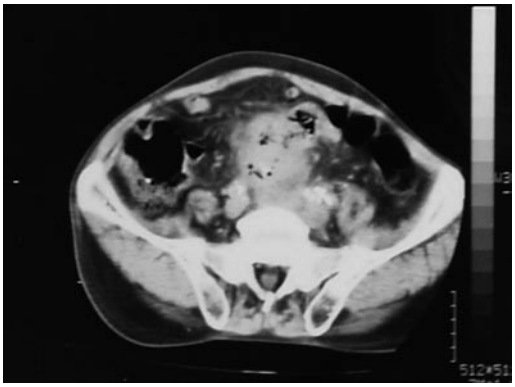

**Figure 1** CT scanning-revealed air bubbles in aortic lumen and fistula tract between the sigmoid colon and the aorta.

plication since it is usually fatal unless detected. Fistulization may occur in the jejunum, ileum, esophagus, stomach as well as colon[4-7]. Although the most frequent site of fistula is the third portion of the duodenum, a classic triad of symptoms including abdominal pain, gastrointestinal bleeding and a pulsatile abdominal mass may present in some cases regardless of the site affected[5,6]. The bleeding may be abundant or intermittent "herald" with hypotension and syncope. Our patient had a history of bleeding four days before he was admitted. However, it did not recur until the day he presented with shock at the Emergency Department. Despite the fact that certain definition of the pathogenesis of primary aorto-enteric fistulae is beyond our knowledge for the time being, mechanical erosion by aneurysms and inflammations such as diverticulitis is generally suggested to be the cause[8,9]. CT scanning and angiographies rarely demonstrate the fistula tract. However, CT scanning is of utmost importance in patients with an initial diagnosis of aorto-colic fistula. CT scanning may reveal an aortic aneurysm, deletion of the plane between the aorta and the adjacent organ, and gas in the wall or even within the lumen of the aorta as it was in this case (Figure 1).

The appropriate treatment of the entity consists of aneurysmectomy, repair of the fistula, and aortic reconstruction with synthetic grafts and omental wrapping or an extra-anatomical bypass. Aggressive antibiotic treatment is required as well to prevent an *in-situ* graft infection, which may be fatal[8]. The mortality rate still remains high and ranges from 30% to 75% depending on the time of surgery. The high mortality is mainly due to the sequelae of hemorrhagic shock and massive blood transfusion and sepsis. It has been stated that early death due to multisystem failure is to be expected and sepsis is an inevitable consequence of aorto-colic fistula[10].

This report emphasizes that rupture of aorto-iliac aneurysm into the sigmoid colon should be included in the differential diagnosis of lower gastrointestinal bleeding, and the high mortality of aorto-colic fistulae may be overcome with prompt diagnosis, immediate surgical intervention, and measures against sepsis. However, it may still cause death of patients in spite of all measures.

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*CASE REPORT*

# Hepatotoxicity induced by cyproterone acetate: A report of three cases

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## Abstract

Cyproterone acetate (CPA) is a steroidal synthetic progestagen and anti-androgenic compound widely administered in prostate cancer which has been evidentially correlated with a severe hepatotoxic potency. Three male patients aged 78-83 years are presented, in whom severe hepatotoxic reactions emerged after CPA administration. Patients were treated with CPA at the doses of 200-300 mg/d for malignant prostate disease for 3-12 mo prior to the acute manifestation of the hepatic disease. Clinical features compatible with mixed hepatocellular and cholestatic liver disease including jaundice, white stools and dark urine, manifested in all three cases whereas encephalopathy and ascites were present in two of the patients. Other primary causes of hepatotoxicity (alcohol consumption and viral hepatitis) were also verified in two cases, and in those patients biopsy findings revealed the presence of cirrhotic lesions in liver parenchyma. Discontinuation of the therapeutic agent led to the amelioration of the clinical profile in all the patients whereas a patient died 40 d after hospital admission due to sepsis, despite acute liver disease improvement. The current article highlights the hepatotoxic potency of a widely administered therapeutic agent and illustrates the importance of clinical surveillance especially in patients with previous hepatic diseases. Three relevant cases are reported and a review of the published literature is made.



**Key words:** Cyproterone acetate; Drug induced hepatotoxicity; Prostate cancer; Idiosyncratic drug reaction; Hepatomitogen action

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## INTRODUCTION

Androgen deprivation therapy in the form of either orchiectomy or treatment with endogenous estrogens or luteinizing hormone releasing hormone (LHRH) analogues alone or coupled with antiandrogens, produces regression of prostate cancer cells through suppression of serum testosterone levels, and is on that ground regarded as an effective adjuvant therapeutic option. Cyproterone acetate (CPA) is a steroidal synthetic progestagen and antiandrogenic compound widely administered not only in prostate cancer but also in breast cancer, severe acne, female hirsutism, precocious puberty, hyper sexuality and sexual deviation in men. CPA inhibits the peripheral actions of testosterone and suppresses gonadotropin secretion by maintaining the negative feedback on the pituitary. Hepatotoxicity is a serious adverse reaction potentially induced by both steroidal and nonsteroidal antiandrogens. The first case of CPA-induced fulminant hepatitis with a fatal outcome was described in 1989[1]. A variety of hepatotoxic reactions have been documented in the literature including immunoallergic cytotoxic reactions[2], cholestasis, autoimmune hepatitis[3], acute hepatitis[4-10] fulminant hepatic failure[1,2,11-17] cirrhosis[18] and finally CPA has been attributed a hepatocellular mutagenic potency leading to hepatocarcinogenesis[8,19-27]. Clinical features include jaundice, fatigue, nausea, elevated serum levels of liver enzymes, different types of necrosis (bridging, confluent, and centrilobular), inflammation and features of hepatic decompensation[19]. This article highlights the hepatotoxic potency of a widely administered therapeutic agent and illustrates the importance of clinical surveillance. Three relevant cases are presented and a review of the published literature is made.

We searched the database PubMed (1995-2005) using the following key words: "cyproterone acetate hepatotoxicity", "drug induced hepatotoxicity". We also included review articles, book chapters, or commonly referenced publications. We reviewed the reference lists of articles identified by the search strategy and selected those we judged relevant. The search was restricted to papers published in English.

**Table 1 Patients' laboratory values**

| Case | Age (yr) | Use of cyproterone acetate (mo) | Daily dose (mg) | Bilirubin (total/direct, g/L)[1] | AST/ALT (IU/L)[2] | Alkaline phospatase (IU/L)[3] | Albumin (g/L)[4] | INR[5] |
|---|---|---|---|---|---|---|---|---|
| 1 | 82 | 12 | 200 | 29.5/18.4 | 935/535 | 300 | 3.9 | 1.4 |
| 2 | 83 | 7 | 300 | 10.1/9.2 | 721/283 | 350 | 4 | 2.24 |
| 3 | 78 | 3 | 300 | 30.0/25.3 | 505/320 | 380 | 4.2 | 1.2 |

[1]Normal values (total) < 1.2 g/L, normal values (direct) < 0.4 g/L; [2]Normal values < 40 IU/L; [3]Normal values < 133 IU/L; [4]Normal values > 3.5 g/L; [5]Normal values < 1.2

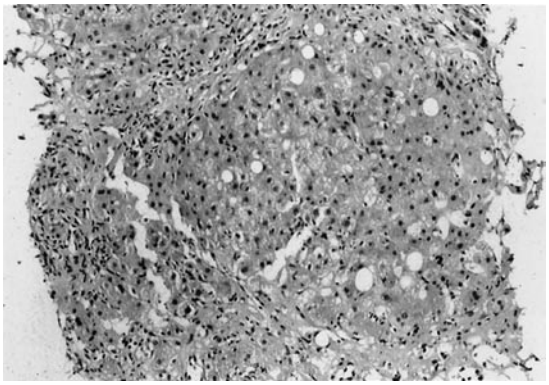

**Figure 1** Hepatocellular degeneration, with ballooning of hepatocytes, portal and lobular inflammation and marked cholestasis with bile plugs were demonstrated in liver biopsy findings (liver biopsy from case 2).

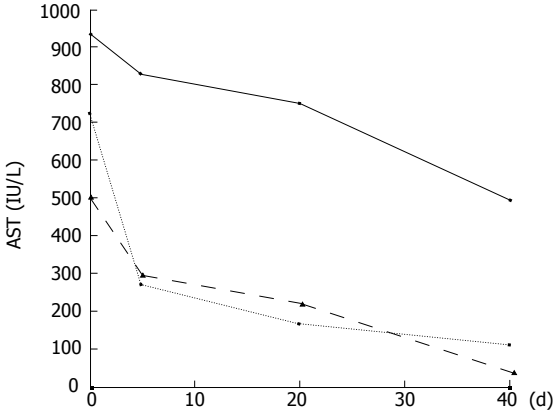


**Figure 2** Serum aspartate aminotransferase levels following withdrawal of cyproterone acetate.

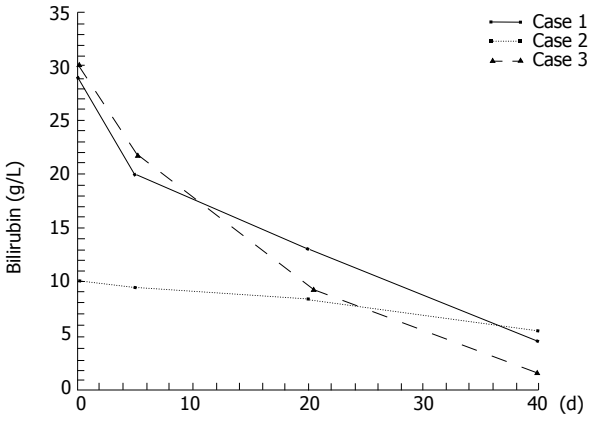


**Figure 3** Serum total bilirubin levels following withdrawal of cyproterone acetate.

## CASE REPORT

Three male patients aged 78-83 years with clinical features compatible with mixed hepatocellular and cholestatic liver diseases, including jaundice, white stools and dark urine were admitted to Hippocration University Hospital of Athens. Clinical manifestations including encephalopathy (Case 2) and ascites were documented in two of the patients (Case 1 and 2) (Table 1). The main characteristics of patients, as well as the values of bilirubin, aspartate (AST) and alanine aminotransferase (ALT), International Normalized Ratio (INR), albumin, and alkaline phosphatase (ALP) at the time of admission are depicted in Table 1. All three patients had been administered cyproterone acetate for 3-12 mo for prostate cancer without metastasis, at a dose ranging from 200-300 mg/d. Extensive clinical evaluation and exclusion of other potential hepatotoxic causes were performed. No serological evidence of recent acute viral hepatic infection was verified whereas in Case 3, pre-existing chronic inactive hepatitis B virus infection was justified based on compatible serological findings (hepatitis B virus surface (HBs) antigen was positive and hepatitis B virus DNA negative by hybridization assay). In addition, a history of excessive alcohol consumption was documented in Case 1. Autoimmunity markers were negative and no evidence of tumor in the liver or pelvis was seen on the computer tomography scan. Yet the presence of cirrhotic lesions was verified in Cases 1 and 3 on tomography. Liver biopsy was performed in all three Cases (Figure 1). In Cases 1 and 3, cirrhosis with fibrosis and loss of the normal lobular architecture were revealed and attributed to chronic hepatitis B and alcohol consumption. In all three cases, hepatocellular degeneration with ballooning of hepatocytes, portal and lobular inflammation and marked cholestasis with bile plugs were also demonstrated. Discontinuation of the therapeutic agent led to the restoration of the pathological clinical profile in Cases 1 and 3 whereas this was not accomplished in the second patient who died 40 d after hospital admission, due to sepsis, despite acute liver disease improvement. (Figures 2 and 3).

## DISCUSSION

The diagnosis of drug-induced hepatotoxicity is usually based on exclusion of other possible causes of hepatic dysfunction and on the temporal association between drug administration and the onset of liver disease while liver biopsy may allow a correct diagnosis[28,29]. Toxic hepatitis in our patients appeared to be causally related to the administration of cyproterone acetate based on a temporal relationship, negative serology for acute viral infection, negative autoantibody markers and exclusion of drugs or other potentially hepatotoxic agents. Proof is not possible in the absence of the rechallenge for ethical reasons. Pathophysiology of CPA-induced hepatotoxicity remains largely hypothetical. The histological features fit with an idiosyncratic reaction to the drug or its metabolites, or possibly an immunologically mediated reaction[2]. In many cases, a direct drug or metabolite effect is implied attributed to the hepatomitogen action of cyproterone, causing an increase of hepatocytes expressing placental glutathione S-transferase, which are considered preneoplastic elements[30-31]. The combination of Transforming Growth Factor-beta 1 (TGF) expression coupled with a strikingly enhanced sensitivity to the induction of apoptosis might be responsible for both the liver damage and the development of liver tumors observed after CPA supplementation since CPA shortens the lag phase of induction of apoptosis by shifting hepatocytes to a point before S phase, where they are highly susceptible to TGF-beta 1-induced apoptosis[19,32].

Hinkel *et al*[33] have conducted a retroprospective analysis of 89 patients who received cyproterone acetate for 4 years (50mg/d po range, 2-152 mo) for prostate cancer. Elevated liver enzymes were documented in 28.2% of the patients, yet CPA-induced liver toxicity and carcinogenesis were implied to be totally irrelevant considering the life expectancy of patients with advanced prostate cancer and the high-dose exposure of CPA required to possibly induce liver malignancies. Since then several large series have assessed the same issue with yet controversial results. In 1996, a retrospective study was conducted in 2506 patients receiving CPA (18-136 mg/d for less than 47.5 mo/patient) and a correlation with hepatocarcinogenesis could not be established. And 9.6% of the patients eventually presented with pathological liver profile, yet discontinuation of the therapeutic agent was not regarded necessary in any case[8]. Lin *et al*[34] assessed 229 patients by retrospective chart review submitted in orchiectomy or LHRH analogues plus antiandrogen for prostate cancer. In 105 patients administered with CPA (150 mg/d), hepatotoxity rates were 9.5%. Serious hepatic injury was documented in 3.8% (4/105) of the patients, mean latency period of hepatotoxity was estimated to be 5.8 ± 1.9 mo and in 9 out of 10 patients CPA-induced hepatotoxity got self resolution after discontinuation of the antiandrogen in an average period of 6.3 ± 4.7 mo[34]. In another study, patients with a preexisting chronic viral hepatitis were recognized as having a higher risk of developing antiandrogen hepatotoxicity[35]. Finally in a series assessing predictive utility of several clinical features, both age and therapeutic indication or the dose prescribed failed to depict with significant accuracy, those patients at risk for presenting hepatic disorders[36].

In the three cases of severe CPA-induced hepatotoxicity presented in this article patients were administered with CPA at the doses of 200-300 mg/d for malignant prostate disease for 3-12 mo prior to the manifestation of hepatic disease. In two of the patients, other primary causes of hepatotoxicity (excessive alcohol consumption and viral hepatitis) were also verified and in those patients, biopsy findings revealed the presence of cirrhotic lesions in liver parenchyma along with acute cholestatic hepatitis. Discontinuation of the therapeutic agent resulted in the amelioration of the hepatic injury in all the patients whereas Case 2 died 40 d after hospital admission from sepsis.

Eleven cases assessing CPA-induced fulminant hepatic failure are presented in the literature[1,2,11-17]. According to Friedman *et al*[13] adverse hepatic reactions occurred more commonly in elderly patients (range, 65-92 years) with malignant diseases who were treated with high doses (range, 100-300 mg) for a prolonged period. Fulminant hepatic failure developed a few weeks to several months after initiation of therapy (range, 2-15 mo). On admission, aminotransferases were 3-27 times that of normal, and a markedly elevated bilirubin of 9-30 times higher than normal were also documented, but the ALP was usually 2 times lower than normal. Clinical features of hepatotoxicity initially included nausea, anorexia and malaise; however, a progressive jaundice eventually ensued. The duration from the onset of jaundice to death was 2-7 wk[13]. Beside acute liver failure, a less severe hepatic reaction can emerge on the ground of CPA-administration. Eight cases of acute hepatitis complicating CPA-treatment have been documented in the literature[4-10]. Doses of 100-150 mg/d are correlated with mild hepatotoxic reactions, mainly elevated transaminase levels, whereas an association between the duration of CPA-treatment and the prevalence of liver enzymes could not be established[8]. Latency before the onset of hepatocellular carcinoma and cirrhosis after CPA therapy is estimated up to several years. Preexisting viral, metabolic, drug-induced or alcoholic liver diseases are found correlated with the development of more severe hepatotoxic reactions. Discontinuation of the antiandrogens results in the resolution of hepatotoxicity. A change to other antiandrogen may be the alternative strategy to CPA-induced hepatic injury although controversy exists upon that issue[9]. After the withdrawal of the causative agent, hepatocellular recovery is achieved within 2-3 mo[19]. Fourteen case reports involving CPA-induced hepatotoxicity are reviewed with regard to the patient's age, dose of CPA, latency before the onset of jaundice and duration between onset of jaundice and death[13,36] (Table 2). Descriptive statistics including means, medians and standard deviations were calculated, and are displayed in Table 2. Age and CPA doses were plotted in correlation with mortality (Figure 4). Relevant statistics were calculated and no statistically significant correlation was demonstrated between age and doses of CPA and the clinical outcome (death or survival) ($P$ = 0.241 and $P$ = 0.789 for CPA doses and age, respectively; not significant).

In conclusion, the aim of this report and review of

**Table 2 CPA-induced hepatotoxicity: review of 14 cases**

| Reference | Age (yr) | Liver injury | Doses (mg) | Latency (mo) | Duration[1] (wk) |
|---|---|---|---|---|---|
| Levesque[1] | 78 | ALF | 200 | 6 | 2 |
| Parys[11] | 65 | ALF | 300 | 12 | 7 |
| Parys[11] | 83 | ALF | 300 | 15 | 2 |
| Bressollette[16] | 79 | ALF | 300 | 10 | 2 |
| Hirsch[14] | 92 | ALF | 100 | 4 | 5 |
| Murphy[2] | 73 | ALF | 100 | 4 | Alive |
| Lombardi[15] | 84 | ALF | | | 1 |
| Friedmann[13] | 81 | ALF | 300 | 6 | 7 |
| Friedmann[13] | 66 | ALF | 300 | 2 | 4 |
| Giordano[10] | 87 | AH | 300 | | Alive |
| Manolakopoulos[9] | 76 | AH | 150 | 7 | Alive |
| Savidou | 82 | AH | 200 | 12 | Alive |
| Savidou | 83 | AH | 300 | 7 | 6 |
| Savidou | 78 | AH | 300 | 3 | Alive |
| Mean | 79.1 | | 242.3 | 7.3 | 4 |
| Median | 80 | | 300 | 6.5 | 4 |
| SD | 7.4 | | 81.2 | 4 | 2.3 |

[1]Duration between onset of jaundice and death; ALF: Acute liver failure; AH: Acute hepatitis.

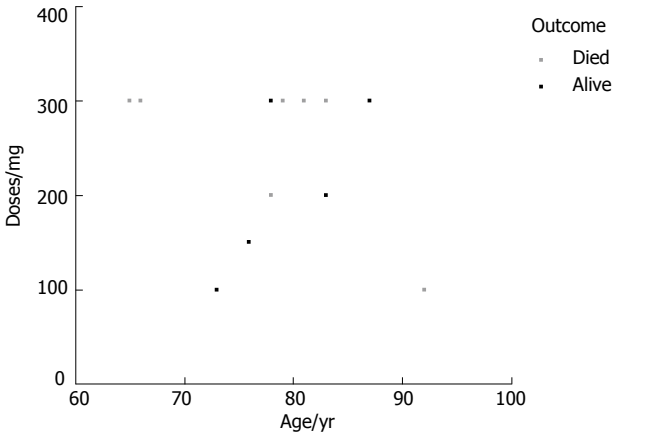


**Figure 4** Scatter plot graphed for patient death events in correlation with patient's age and CPA-doses administered (Table 2).

the literature is to underline the potential hepatotoxicity effect of CPA, which may prove fatal in some cases. Close monitoring of liver function tests is recommended, particularly on the setting of a pre-existing liver disease for which increased risk rate has been implied.

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# Meetings

## MAJOR MEETINGS COMING UP

First Biennial Congress of the Asian-Pacific Hepato-Pancreato-Biliary Association
March 2007
Fukuoka, Japan
<http://www.congre.co.jp/1st-aphpba>/

American College of Gastroenterology Annual Scientific
20-25 October 2006
Las Vegas, NV

14th United European Gastroenterology Week, UEGW
21-25 October 2006
Berlin, Germany

APDW 2006: Asian Pacific Digestive Week 2006
26-29 November 2006
Lahug Cebu City, Philippines

## EVENTS AND MEETINGS IN THE UPCOMING 6 MONTHS

Falk Symposium 151: Emerging Issues in Inflammatory Bowel Diseases
24-25 March 2006
Sydney - NSW
Falk Foundation e.V.
symposia@falkfoundation.de

10th International Congress of Obesity
3-8 September 2006
Sydney
Event Planners Australia
enquiries@ico2006.com
www.ico2006.com

Easl 2006 - the 41st annual
26-30 April 2006
Vienna, Austria
Kenes International

Prague hepatology 2006
14-16 September 2006
Prague
Foundation of the Czech Society of Hepatology
veronika.revicka@congressprague.cz
www.czech-hepatology.cz/phm2006

12th International Symposium on Viral Hepatitis and Liver Disease
1-5 July 2006
Paris
MCI France
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ILTS 12th Annual International Congress
3-6 May 2006
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Internal Medicine: Gastroenterology
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6th Annual Gastroenterology And Hepatology
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World Congress on Gastrointestinal Cancer
28 June 2006-1 July 2006
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icsi2006@stocon.se
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3-7 September 2006
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American Society of Gastrointestinal Endoscopy
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American Society of Colon and Rectal Surgeons
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10th World Congress of the International Society for Diseases of the Esophagus
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VII Brazilian Digestive Disease Week
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Prague Hepatology 2006
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Falk Symposium 152: Intestinal Disease Part I, Endoscopy 2006 - Update and Live Demonstration
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Falk Symposium 153: Intestinal Disease Part II, Immunoregulation in Inflammatory Bowel Disease - Current Understanding and Innovation
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*In press*

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

*Organization as author*

4 **Diabetes Prevention Program Research Group**. Hypertension, insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension* 2002; **40**: 679-686 [ PMID: 12411462 ]

*Both personal authors and an organization as author*

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

*No author given*

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

*Volume with supplement*

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

*Issue with no volume*

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

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

**Books**

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

*Chapter in a book (list all authors)*

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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 Tumour Conference; 2001 Sep 13-15; Leeds, UK. New York: Springer, 2002: 30-56

*Conference paper*

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

**Electronic journal** (list all authors)

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

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

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*EDITORIAL*

# Treatment of hepatocellular carcinoma accompanied by portal vein tumor thrombus

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## Abstract

The prognosis of patients with hepatocellular carcinoma (HCC) accompanied by portal vein tumor thrombus (PVTT) is generally poor if left untreated: a median survival time of 2.7-4.0 mo has been reported. Furthermore, while transcatheter arterial chemoembolization (TACE) has been shown to be safe in selected patients, the median survival time with this treatment is still only 3.8-9.5 mo. Systemic single-agent chemotherapy for HCC with PVTT has failed to improve the prognosis, and the response rates have been less than 20%. While regional chemotherapy with low-dose cisplatin and 5-fluorouracil or interferon and 5-fluorouracil via hepatic arterial infusion has increased the response rate, the median survival time has not exceeded 12 (range 4.5-11.8) mo. Combined treatment consisting of radiation for PVTT and TACE for liver tumor has achieved a high response rate, but the median survival rates have still been only 3.8-10.7 mo. With hepatic resection as monotherapy, the 5-year survival rate and median survival time were reportedly 4%-28.5% and 6-14 mo. The most promising results were reported for combined treatments consisting of hepatectomy and TACE, chemotherapy, or internal radiation. The reported 5-year survival rates and median survival times were 42% and 31 mo for TACE followed by hepatectomy; 36.3% and 22.1 mo for hepatectomy followed by hepatic arterial infusion chemotherapy; and 56% for chemotherapy or internal radiation followed by hepatectomy.



**Key words:** Hepatocellular carcinoma; Portal vein tumor Thrombus; Hepatic resection; Transcatheter arterial chemoembolization; Chemotherapy; Radiation

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## INTRODUCTION

Recent progress in imaging techniques has permitted the diagnosis of hepatocellular carcinoma (HCC) at an early stage. However, portal venous invasion is still found in 12.5%-39.7% of patients with HCC[1-5]. According to the 16th National Survey for Primary Liver Cancer in Japan, 808 of 5130 patients (16%) who received hepatic resection had macroscopic portal venous invasion[6]. Portal venous invasion is a crucial factor that can worsen the prognosis of patients with HCC. It often leads to extensive spreading of the tumor throughout the liver, and can increase portal venous blood pressure, resulting in the fatal rupture of esophageal varices, and can decrease portal flow which causes ascites, jaundice, hepatic encephalopathy, and liver failure. Previous studies have reported that the median survival time of patients with portal venous invasion was 2.7-4 mo if left untreated[7,8]. To improve this short-term prognosis, various treatments have been applied, however, no standard treatment exists. In this paper, we review recent approaches to this disease.

## TRANSCATHETER ARTERIAL CHEMOEMBOLIZATION

Transcatheter arterial chemoembolization (TACE) is a widely used palliative treatment for HCC that is unsuitable for radical treatments. Some investigators have noted that TACE was contraindicated for patients with portal vein tumor thrombus (PVTT), because it carried a potential risk of ischemic liver damage[9]. In contrast, other authors suggested that TACE might be safely performed in patients with PVTT if they have good hepatic reserve and collateral circulation around the portal trunk[10-13]. Yamada *et al*[9] performed TACE in 9 patients with obstruction of the main portal vein. Five of these patients died of hepatic insufficiency within 1 mo after TACE. In autopsy studies of 3 patients who died of hepatic insufficiency, extensive necrosis of tumor tissue and surrounding liver parenchyma was observed[9]. Based on these results, they concluded that TACE was contraindicated in patients with HCC in which the tumor has invaded the major portal vein. This opinion

**Table 1 Transcatheter arterial chemoembolization for HCC with portal vein tumor thrombus**

| Lead author | Yr | *n* | Location of tumor thrombus | Treatment | Mortality within 1 mo (%) | Response rate (%) | Median survival time (mo) |
|---|---|---|---|---|---|---|---|
| Yamada R *et al*[9] | 1983 | 9 | Vp4 | TACE | 55.5 | - | - |
| Okazaki M *et al*[10] | 1991 | 48 | Vp2 | TACE | 6.5 | - | 4.3 |
| | | 56 | Vp3 | TACE | 8 | - | 4 |
| | | 59 | Vp4 | TACE | 5.6 | - | 3.8 |
| Raoul JL *et al*[14] | 1994 | 27 | Vp3, 4 | $^{131}$I-labeled lipiodol | 0 | 40 | 6 |
| Chung JW *et al*[12] | 1995 | 110 | Vp3, 4 | TACE | 2.7 | 28 | 6 |
| Georgiades CS *et al*[13] | 2005 | 32 | Vp3, 4 | TACE | 0 | - | 9.5 |

Vp2: 2nd order branches of the portal vein; Vp3: 1st order branches of the portal vein; Vp4: the main trunk of the portal vein.

is now supported by many clinicians even after some authors have shown that TACE is safe for these patients. One hundred sixty three patients with HCC accompanied by PVTT (48 at the 2nd portal branch, 56 at the 1st portal branch, and 59 at the main portal trunk) received TACE, and the median survival time and mortality rate within 1 mo for these patients were 4.3 mo and 6.5%, 4.0 mo and 8.0%, and 3.8 mo and 5.6% respectively[10]. The authors concluded that there was no difference in risk according to the location of portal invasion and the mortality rate was comparable to that of hepatic resection at the time[10] (Table 1). Based on these results, they suggested that the selection criteria for TACE in patients with HCC that invaded the main portal vein were the presence of cavernous transformation and a serum total bilirubin level of less than 2.0 mg/mL[10,11]. To avoid the interruption of hepatic arterial flow and subsequent hepatic insufficiency by TACE, Raoul *et al*[14] introduced internal radiation therapy using $^{131}$I-labeled Lipiodol in patients with these conditions. Twenty seven patients with HCC, stage Ⅰ or Ⅱ by the classification of Okuda, accompanied by PVTT in the 1st branches of the portal vein or the portal trunk were randomly assigned to an $^{131}$I-labeled Lipiodol group ($n$ = 14) or a Control group ($n$ = 13). The survival rates at 3, 6, and 9 mo were 71%, 48%, and 7% for the $^{131}$I-labeled Lipiodol group and 10%, 0%, and 0% for the Control group ($P < 0.01$): the response rate and median survival time of the treated group were 40% and 6 mo[14]. Chung *et al*[12] performed TACE in 110 patients with HCC that invaded the 1st branch of the portal vein or the main portal vein: the response rate was 28% and the median survival time was 6 mo. In their series, hepatic insufficiency developed in 10 patients and 3 of them died within 1 mo after TACE, and the tumor extended to more than 2 sectors in all 10 of these patients. This tumor extent was a significant factor in predicting the efficacy of therapy, and they concluded that TACE was effective and safe if the extent of the tumor was limited and liver function was preserved[12]. Georgiades *et al*[13] reported that TACE was safe and effective for these patients. Thirty two patients with unresectable HCC that completely occluded the main portal vein or the right, left, or both the right and left portal veins underwent TACE. The mortality rate within 1 mo was zero and there was no evidence of TACE-related hepatic infarction or liver failure. The median survival time was 9.5 mo and the Child-Pugh score was the prognostic factor that was most strongly related to survival[13].

Despite the widespread use of TACE for patients with unresectable HCC and the demonstration that TACE is safe for patients with PVTT, the efficacy of TACE in these patients has long been controversial. Randomized controlled trials performed in the 1990s have found that this approach does not confer any survival benefits compared to conservative management[15-19]. Although tumor growth has been shown to be inhibited, survival has not been shown to be prolonged by this treatment[17]. Llovet *et al*[20] published the results of a randomized controlled trial that was stopped early because TACE provided a statistically significant survival benefit in selected patients; survival rates at 1 and 2 years were 82% and 63% for TACE versus 63% and 27% for supportive care, respectively. Lo *et al*[21] also demonstrated a significant survival benefit in patients with unresectable HCC treated with chemoembolization. The 1-, 2-, and 3-year survival rates in TACE-treated patients were 57%, 31%, and 26%, compared with 32%, 11%, and 3%, respectively, in the controlled group[21]. In a meta-analysis of randomized controlled trials, patients treated with chemoembolization showed a significantly decreased 2-year mortality rate with an odds ratio of 0.53 (95% CI, 0.32-0.89; $P = 0.017$)[22].

## CHEMOTHERAPY

Many chemotherapeutic agents have been studied for their anti-HCC activity. The pyrimidine anti-metabolic agent 5-fluorouracil (5-FU) was the first reported chemotherapeutic agent tested in the treatment of HCC. However, an overall response rate of about 10% and a median survival of 3 to 5 mo have discouraged further use of 5-FU as a single agent[23,24]. Other agents tested include doxorubicin[23,25], which has a reported single-agent activity of 25% and yields a survival advantage when compared with no treatment[25]. There seems to be a general consensus that no single-agent systemic chemotherapy has an objective response rate of more than 25%. Okada *et al* reported the results of systemic chemotherapy in 71 patients with unresectable HCC (Table 2)[26]. The agents were changed from Tegafur to Doxorubicin, Tegafur plus Uracil, Etoposide, Mitoxantrone, Interferon-gamma, Cisplatin, and 5-FU over time and the response rate ranged from 0% to 20%[26]. Among these patients, 22 had tumor thrombus in the main portal vein. The median

**Table 2 Chemotherapy for hepatocellular carcinoma with portal vein tumor thrombus**

| Lead author | Yr | *n* | Location of tumor thrombus | Route | treatment | Response rate (CR+PR) (%) | Median survival time (mo) |
|---|---|---|---|---|---|---|---|
| Systemic chemotherapy | | | | | | | |
| Okada S *et al*[26] | 1992 | 22 | Vp4 | S | Tegaful, doxorubicin, MTX, CDDP, 5-FU, *etc.* | - | 3.9 |
| Low dose CDDP & 5-FU | | | | | | | |
| Ando E *et al*[28] | 1996 | 9 | Vp4 | R | CDDP, 5-FU | 44 | 9.2 |
| Itamoto T *et al*[29] | 2002 | 7 | Vp3, 4 | R | 5-FU + CDDP | 33 | 7.5 |
| Yamasaki T *et al*[30] | 2002 | 6 | Vp3, 4 | R | CDDP, 5-FU/+ leucovorin | 0 | 4.5 |
| Interferon & 5-FU | | | | | | | |
| Patt YZ *et al*[32] | 1993 | 29[1] | - | S | 5-FU + Interferon-α | 22 | - |
| Urabe T *et al*[33] | 1998 | 16 | Vp3, 4 | R | MTX, 5-FU, cisplatin, Interferon-α | 46.7 | 7 |
| Kaneko S *et al*[34] | 2002 | 34 | Vp3, 4 | R | 5-FU, CDDP, MTX, + Interferon-α + Leucovorin | 44 | 11 (CR + PR)<br>3.5 (SD + PD) |
| Sakon M *et al*[35] | 2002 | 8 | Vp3, 4 | R | 5-FU + Interferon-α | 63 | - |
| Ota H *et al*[36] | 2005 | 55 | Vp3, 4 | R | 5-FU + Interferon-α | 43.6 | 11.8 |
| Obi S *et al*[37] | 2005 | 116 | Vp3, 4 | R | 5-FU + Interferon-α | 52 | 6.9 |

[1]Including all patients with HCC. S: Systemic chemotherapy; R: Regional chemotherapy *via* hepatic artery; MTX: methotrexate; CDDP: cisplatin.

survival time of these patients was 3.9 mo, while that of the 49 patients without this condition was 7.3 mo ($P < 0.05$)[26]. Since cisplatin and 5-FU have been reported to exhibit a synergistic effect, this combination has been widely used for various malignancies[27]. The results of the administration of low-dose cisplatin and 5-FU by repeated arterial infusion in nine patients with HCC and PVTT in the main portal trunk was reported by Ando *et al*[28]: the response rate was 44.4% and the median survival time was 9.2 mo. Seven patients with HCC and PVTT in the 1st branches of the portal vein or the main portal trunk received the same regimen: the response rate was 33%, and the median survival time was 7.5 mo[29]. Yamasaki *et al*[30] showed that the addition of leucovorin to this protocol significantly increased the survival time in a randomized study in 19 patients. Among them, 6 had PVTT in the major portal veins: their response rate and median survival time were 0% and 4.5 mo, respectively[30].

Recombinant interferon alpha has been found to be superior to doxorubicin for the treatment of inoperable HCC[31]. Combined treatment with 5-FU and alpha-interferon for HCC patients was first reported by Patt *et al* in 1993[32]. The response rate was reportedly 22%[32]. Urabe *et al*[33] treated 16 patients with HCC and PVTT in the main trunk or the major branches of the portal vein by intrahepatic infusion of methotrexate, 5-FU, and cisplatin, and administered alpha-interferon subcutaneously. The response rate and median survival rate were 46.7% and 7 mo, respectively[33]. The results of combined treatment using alpha-interferon, cisplatin, methotrexate, 5-FU, and leucovorin were reported by the same group: the response rate was 44% and the median survival time was 11 mo for 13 patients who had a complete response or partial response, and 3.5 mo for 16 patients who had stable or progressive disease[34]. Combined intra-arterial 5-FU and subcutaneous alpha-interferon therapy for 8 patients with HCC accompanied by PVTT in the major portal vein was reported by Sakon *et al*[35]: one patient died at 5 mo, the remaining 7 patients were alive after 3-15 mo, and the response rate was 63%. In another study by this group in 2005, 55 patients received this treatment, and 8 (14.5%) showed a complete response, 16 (29.1%) showed a partial response, 4 (7.3%) showed no response, and 27 (49.1%) showed progressive disease[36]. The median survival time and 5-year survival rate were 11.8 mo and 16.4%, respectively[36]. Using this combination protocol, Obi *et al*[37] treated 116 patients with unresectable HCC accompanied by PVTT in the main trunk or the 1st branches of the portal vein: the response rate was 52%, and the median survival time was 6.9 mo.

While the response rate for single-agent systemic treatment for unresectable HCC is less than 25%, combined treatment with cisplatin plus 5-FU or alpha-interferon plus 5-FU using hepatic arterial infusion remarkably increased the response rate from 33% to 63%, and the median survival time was prolonged to 11 mo in patients with an active response, although there appears to be no benefit in patients without an active response.

## RADIATION

Radiotherapy for HCC has been infrequently used in the treatment of HCC because the liver has a low tolerance to whole-organ irradiation[38,39]. However, some authors have reported that the tolerance dose for the liver depends on the volume of liver irradiated, and a small volume of liver tissue can tolerate a higher dose of radiotherapy[40]. Radiotherapy for patients with HCC and PVTT was first reported by Chen *et al* (Table 3)[41]. Ten patients with PVTT in the 1st branches of the portal vein received irradiation for PVTT and TACE for liver tumor, and the response rate was reportedly 100%[41]. The same protocol was used by Tazawa, Yamada, and Ishikura in 24, 8, and 20 patients[42-44]. Objective responses of PVTT ranged from 37.5% to 50%, and the median survival times were 9.7 mo in responders and 3.8 mo in non-responders[42-44]. With advances in three-dimensional planning tools, three-dimensional conformal radiotherapy (3-D CRT) allows clinicians to escalate radiotherapy doses to the tumor and minimize radiotherapy doses to normal tissue, such as normal liver parenchyma, small bowel, and spinal cord. Combined treatment consisting of 3-D CRT for PVTT

**Table 3 Radiation for hepatocellular carcinoma with portal vein tumor thrombus**

| Lead author | Yr | *n* | Location of tumor thrombus | Treatment | Response rate of PVTT (%) | Median survival time (mo) |
|---|---|---|---|---|---|---|
| Chen SC *et al*[41] | 1994 | 10 | Vp2 | TACE for liver tumor + Radiation (30-50 Gy) for PVTT | 100 | - |
| Tazawa J *et al*[42] | 2001 | 24 | Vp3, 4 | TACE for liver tumor + Radiation (50 Gy) for PVTT | 50 | CR PR; 9.7<br>NC PD; 3.8 |
| Yamada K *et al*[43] | 2001 | 8 | Vp3 | Radiation (60 Gy) for PVTT followed by TACE for liver tumor | 37.5 | - |
| Ishikura S *et al*[44] | 2002 | 20 | Vp3 | TACE + Radiation | 50 | 5.3 |
| Yamada K *et al*[45] | 2003 | 19 | Vp3 | 3-D CRT for PVTT (60 Gy) followed by TACE for liver tumor | 57.9 | 7 |
| Hata M *et al*[53] | 2004 | 12 | Vp3, 4 | Proton beam therapy (50-72 Gy) | 100 | 27 (24%: 5-YSR) |
| Nakagawa K *et al*[46] | 2005 | 52 | Vp2, 3, 4 | 3-D CRT for PVTT (60 Gy) | 50 | (15.2%; 3-YSR) |
| Zeng ZC *et al*[47] | 2005 | 44 | | Radiation (50 Gy, 36-60) | 45.5 | 8 |
| Kim DY *et al*[50] | 2005 | 59 | Vp3, 4 | 3-D CRT | 45.8 | CR PR; 10.7<br>NC PD; 5.3 |
| Lin CS *et al*[48] | 2006 | 43 | Vp3, 4 | Conventional: 22<br>3-D CRT: 21 | Con: 75<br>3-D CRT: 83 | Con: 6.0<br>3-D CRT: 6.7 |
| Hsu WC *et al*[49] | 2006 | 53 | Vp3, 4 | 3-D CRT and thalidomide | 50 | - |

3-D CRT: Three-dimensional conformal radiation therapy; YSR: Year survival rate.

**Table 4 Hepatic resection for hepatocellular carcinoma with portal vein tumor thrombus**

| Lead author | Yr | Number of patients | Location of tumor thrombus | Treatment | Operative mortality rate (%) | 5-Year survival rate (%) | Median survival time (mo) |
|---|---|---|---|---|---|---|---|
| Kumada K *et al*[54] | 1990 | 13 | Vp4 | Hx. | - | - | - |
| Yamaoka Y *et al*[55] | 1992 | 29 | Vp3, 4 | Hx. | 11 | (11.6:3YSR) | - |
| Ikai I *et al*[56] | 1998 | 29 | Vp4 | Hx. | - | 4 | - |
| | | 29 | Vp3 | Hx. | - | 11 | - |
| Ohkubo T *et al*[57] | 2000 | 47 | Vp2, 3, 4 | Hx. | 0 | 23.9 | 14 |
| Wu CC *et al*[58] | 2000 | 15 | Vp4 | Hx. | 0 | 26.4 | - |
| | | 97 | Vp1, 2, 3 | Hx. | 3.1 | 28.5 | - |
| Minagawa M *et al*[59] | 2001 | 18 | Vp2, 3, 4 | TACE → Hx. | 0 | 42 | 31 |
| Fukuda S *et al*[60] | 2002 | 19 | Vp3, 4, Vv2, 3, B3, 4 | Hx. → HAI *etc.* | 0 | 36.3 | 22.1 |
| Poon RT *et al*[61] | 2003 | 20 | Vp3, 4, Vv2 | Hx. | 5.7 | 13.3 | 6 |
| Lau WY *et al*[62] | 2004 | 7 | Vp4 | PIAF→Hx. or Yttrium 90 ia + doxorubicin iv → Hx. | 4.1 | 56 | [a] |
| Pawlik TM *et al*[63] | 2005 | 102 | Vp3, Vv2, 3 | Hx. | 5.9 | 10 | 11 |
| Ikai I *et al*[64] | 2006 | 78 | Vp3, Vp4 | Hx. | 3.8 | 10.9 | 8.9 |
| Le Treut YP *et al*[65] | 2006 | 26 | Vp3, 4, Vv2, 3 | Hx. | 11.5 | 13 | 9 |
| Zhou J *et al*[66] | 2006 | 381 | Vp2, 3, 4 | Hx. | - | 12 | - |

[a]Survival curves remain above a survival rate of 50%. PIAF: Doxorubicin, CDDP, 5-FU iv + Interferon-α sc; Hx.: Hepatic resection; HAI: Hepatic arterial infusion chemotherapy; Vv2: The main trunk of hepatic vein; Vv3: The inferior vena cava; B3: 1st order branches of bile duct; B4: The common hepatic duct; YSR: Year survival rate.

and TACE for liver tumor was reported by Yamada *et al*[45]: the response rate and median survival time were 57.9% and 7 mo. Nakagawa *et al*[46] treated 52 patients with HCC and PVTT at the 2nd and 1st branches of the portal vein or the main portal trunk followed by percutaneous ablation therapy, TACE, or both for liver tumor: the response rate was 50%, and the 3- and 5-year survival rates were 15.2% and 5.1%. Five or fewer liver tumors and TACE after radiation independently predicted a favorable prognosis[46]. The response rates of 3-D CRT for PVTT have reportedly ranged from 45.8% to 83%, and the median survival times ranged from 5.3 mo to 7 mo[47-49]. Kim *et al*[50] reported that the median survival time was 10.7 mo in responders and 5.3 mo in non-responders.

Since proton beam irradiation enables excellent dose localization to the target compared to conventional photon irradiation, Matsuzaki *et al* applied it to the treatment of HCC[51,52]. Twelve patients with HCC and PVTT at the 1st branches or the trunk of the portal vein received proton beam irradiation for PVTT and liver tumor: the response rate and median survival time were 100% and 27 mo[53].

## HEPATIC RESECTION

In the early days of a surgical approach for patients with HCC accompanied by PVTT, the goal was to prevent the rapid aggravation of portal hypertension and esophagogastric varices, and to improve the short-term prognosis. Reports of surgical therapy for these patients are shown in Table 4. Kumada *et al*[54] first reported surgical techniques in 13 patients who had tumor thrombus in the portal trunk. They described the resection

techniques; balloon catheter methods, an open method, and resection of occluded portal segment followed by portal reconstruction[54]. Yamaoka *et al*[55] performed tumor thrombectomy combined with hepatic resection in 29 patients with HCC and PVTT in the 1st branch or the portal trunk. While their primary purpose was to avoid impeding the rupture of esophageal varices, they reported an unexpectedly high 3-year survival rate of 11.6%[55]. Ikai *et al*[56] reported the outcome of 150 patients with stage Ⅳ-A HCC who underwent hepatic resection. The 5-year survival rate of 29 patients with tumor thrombi in the 1st branch of the portal vein was 11%, which was not significantly different from that of 29 patients with tumor thrombi in the portal trunk (4%)[56]. Ohkubo *et al*[57] reported the results of 47 patients with portal tumor thrombus in the 1st-2nd branches of the portal vein or the portal trunk who underwent hepatic resection. The 3- and 5-year survival rates were 33.2% and 23.9%, respectively, and the indicators of a favorable prognosis were curative hepatic resection, tumor size less than 10 cm, and absence of intrahepatic liver metastases[57]. Wu *et al*[58] reported that among 368 patients with HCC who underwent curative liver resection, 15 received concomitant liver resection and partial resection of the main portal vein because of apparent tumor thrombi extension to the portal bifurcation, and 97 had HCC which invaded intrahepatic portal branches, as confirmed on pathological examination, but did not involve the portal bifurcation. The 5-year survival rates of the former and latter groups were 26.4% and 28.5% ($P = 0.33$)[58]. They pointed out that intramural invasion was found at the site of thrombi adhesion to the portal vein cuff in 11 of 15 patients in the former group[58]. Minagawa *et al*[59] reported a high survival rate in these patients with the combination of TACE followed by hepatic resection. Eighteen patients who had HCC and gross portal tumor thrombus in the 1st-2nd branch or trunk of the portal vein showed a 5-year survival rate of 42% by preoperative TACE and hepatic resection, while none of the patients who received regional chemotherapy, TACE as monotherapy, or ligation of the portal vein survived more than 1.5 years[59]. The sole independent predictor of a favorable prognosis was hepatic resection[59]. Fukuda *et al*[60] reported that 19 patients with HCC and tumor thrombi in the 1st branch or trunk of the portal vein, inferior vena cava, or extrahepatic bile duct underwent hepatic resection with thrombectomy, and received hepatic arterial infusion chemotherapy after resection as adjuvant therapy. The 5-year survival rate of these patients was 36.3%[60]. Poon *et al*[61] reported that the prognosis of patients with HCC classified as stage Ⅳ in the tumor-node-metastasis classification of the International Union Against Cancer was not homogenous according to the 4 categories. In their paper, the 5-year survival rate of patients with HCC involving the 1st branches or trunk of the portal vein or major branches of the hepatic vein was 13.3%[61]. Lau *et al*[62] reported that 49 patients with initially unresectable HCC received nonsurgical treatment, such as systemic chemotherapy or intra-arterial yttrium-90 microspheres followed by salvage surgery. The 5-year survival rate of these 49 patients was 57%[62]. Their series included 7 patients with HCC involving the main portal vein, and the 5-year survival rate of these 7 patients was 56%[62]. Pawlik *et al*[63] analyzed the prognostic factors in 102 patients with HCC involving major portal or hepatic venous branches who were treated by hepatic resection in 5 hepatobiliary centers, and the significant predictors of a poor prognosis were moderate to severe fibrosis (Ishak grade 3 to 6) and high-grade neoplasm (Edmondson-Steiner grade Ⅲ and Ⅳ), and the operative mortality and 5-year survival rate of these 102 patients were 5.9% and 10%. Ikai *et al*[64] also analyzed the prognostic factors in 78 patients with HCC and tumor thrombus in the 1st branch or trunk of the portal vein, and the independent predictors of a favorable prognosis were absence of ascites, prothrombin activity >/= 75%, and maximal tumor diameter < 5 cm. In their series, the mortality rate and 5-year survival rate were 3.8% and 10.9%[64]. In 108 patients who underwent major hepatic resection for HCC, Le Treut *et al*[65] compared 26 who had HCC with PVTT in the portal or hepatic vein to 82 without PVTT: operative mortality and median survival time were 11.5% and 9 mo in the former group and 8.5% and 41 mo in the latter group. Zhou *et al*[66] reported a large series of hepatic resections: 381 patients with HCC and PVTT at the 1st-2nd branches or the trunk of the portal vein were treated by hepatic resection, and the 5-year survival rate of these patients was 12%.

## CONCLUSION

In these reports, the median survival times were less than 12 mo without hepatic resection. While we can not exclude the possibility that this is the result of a selection bias, the most promising results were obtained with combined treatments that included hepatic resection. This analysis suggests that this disease does not have a homogenous prognosis, and therefore it is important to select patients who have a good prognosis and to treat these patients with combined treatments.

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*EDITORIAL*

# Positions of selective leukocytapheresis in the medical therapy of ulcerative colitis

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## Abstract

Ulcerative colitis (UC) and Crohn's disease (CD) are the major forms of idiopathic inflammatory bowel disease (IBD). Both UC and CD are debilitating chronic disorders that afflict millions of individuals throughout the world with symptoms which impair function and quality of life. The etiology of IBD is inadequately understood and therefore, drug therapy has been empirical instead of being based on sound understanding of IBD pathogenesis. This is a major factor for poor drug efficacy and drug related side effects that often add to the disease complexity. The development of biologicals notably infliximab to intercept tumor necrosis factor (TNF)-α reflects some progress, albeit major concern about their side effects and lack of long-term safety and efficacy profiles. However, IBD seems to be perpetuated by inflammatory cytokines like TNF-α, interleukin (IL)-1β, IL-6 and IL-8 for which activated peripheral granulocytes and monocytes/macrophages (GM) are major sources. Further, in IBD, peripheral GMs are elevated with activation behavior, increased survival time and are found in vast numbers within the inflamed intestinal mucosa; they are suspected to be major factors in the immunopathogenesis of IBD. Hence, peripheral blood GMs should be appropriate targets of therapy. The Adacolumn is a medical device developed for selective depletion of GM by receptor-mediated adsorption (GMA). Clinical data show GMA, in patients with steroid dependent or steroid refractory UC, is associated with up to 85% efficacy and tapering or discontinuation of steroids, while in steroid naïve patients (the best responders), GMA spares patients from exposure to steroids. Likewise, GMA at appropriate intervals in patients at a high risk of clinical relapse suppresses relapse thus sparing the patients from the morbidity associated with IBD relapse. Further, GMA appears to reduce the number of patients being submitted to colectomy or exposure to unsafe immunosupressants. First UC episode, steroid naivety and short disease duration appear good predictors of response to GMA and based on the available data, GMA seems to have an excellent safety profile.



**Key words:** Inflammatory bowel disease; Ulcerative colitis; Crohn's disease; Selective leukocytapheresis; Adacolumn; Interleukin 10; Interleukin-1 receptor antagonist

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## INTRODUCTION

Ulcerative colitis (UC) and Crohn's disease (CD) are the major forms of idiopathic inflammatory bowel diseases (IBD) of the intestine. UC and CD are both debilitating chronic disorders that afflict millions of individuals throughout the world with symptoms which impair function and quality of life. Whereas UC is confined to the colon and the rectum, CD may affect any part of the gut from the mouth to the perianal[1-4]. A multitude of clinical manifestations represent the expressions of IBD. These include diarrhea, rectal bleeding, abdominal discomfort, fever, anemia, and weight loss[1-3]. Both UC and CD tend to run a remitting-relapsing course affected by diverse environmental factors[1,3-5].

Despite the recognition of a genetic background together with environmental factors, which at present are thought to translate into an inappropriate inflammatory response in patients with IBD[3,4,6], currently our understanding on the immunopathogenesis of IBD is inadequate. Hence, up to now drug therapy of IBD has been empirical rather than based on sound understanding of disease etiology. Accordingly, while drug therapy initially appears successful in the majority of patients, it comes at the cost of significant side effects[7,8]. Further, up to now, first line medications for exacerbation of IBD include 5-aminosalicylic acid (5-ASA) or sulphasalazine (SZ) in combination with a corticosteroid with consideration of azathioprine (or 6-mercaptopurine) and nutritional support for some patients[1,9-14]. Treatment failure in patients with severe disease has often been an indication for colectomy in up to 40% of steroid refractory patients[10,15] although in

recent years, cyclosporin A (CysA) has been introduced for corticosteroid refractory UC[15,16]. Despite being moderately effective in this clinical setting in reducing colectomy rates, there remain serious concerns over long-term efficacy and toxicity of CysA[17].

Currently, the view is that IBD is perpetuated by inflammatory cytokines like tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6, IL-8 and others[18,19]. Based on this perception, in recent years, anti-cytokine antibodies, notably the anti-TNF antibody, infliximab have been developed for the treatment of IBD[13], and the seemingly success of infliximab in CD[20,21] is hoped to be realized in patients with UC as well[22,23]. However, the enthusiasm towards biologicals is currently dampened by concerns about their long-term efficacy and safety profiles[24-29]. Taking infliximab as one example (that has been through extensive clinical evaluations), following the initial and subsequent administrations, antibodies to this agent emerge which potentially can reduce its efficacy[29]. Regarding their side effects, the literature on biologic therapy carries headlines like "Tumor necrosis factor antagonist therapy and lymphoma development;"[28] "Serious bacterial infections in patients with rheumatoid arthritis under anti-TNF-α therapy;"[27] "Treatment of rheumatoid arthritis with tumor necrosis factor inhibitors may predispose to significant increase in tuberculosis risk;"[26] "Adverse skin reactions to anti-TNF-α;"[24] "Anti-TNF antibody therapy in rheumatoid arthritis and the risk of serious infections and malignancies;"[30] "Anorectal carcinoma after infliximab therapy in Crohn's disease"[31]. There is no shortage of many more warnings.

In the face of the overwhelming evidence for the involvement of various cytokines in the immunopathogenesis of IBD and the fact that peripheral blood granulocytes and monocytes/macrophages (GMs) are major sources of these cytokines[32,33], GMs appear logical targets in the treatment of IBD. Indeed, histological examination of the mucosal tissue in biopsy specimens from patients with active IBD reveals a spectrum of pathologic manifestations among which presence of an abundance of neutrophils relates specifically to clinical disease activity and severity of the disease[1-3,34-36]. The circulating activated GMs are elevated with increased survival time in active IBD[35-44]. Paradoxically, corticosteroids[45] which are given to most patients with active IBD and inflammatory cytokines[46] increase neutrophil survival time. In this article, the author reviews the therapeutic application of selectively depleting peripheral blood GM by adsorption apheresis (GMA) in patients with IBD with a major focus on UC. The underlying rationale is that selective removal of these cells that are otherwise destined for migration to the intestine reduces the inflammatory intensity, which in turn allows healing to take place. The author also presents arguments why GMA should be likened to an effective and safe biologic therapy in IBD.

## THE STRATEGY FOR SELECTIVE LEUKOCYTAPHERESIS

The Adacolumn which is featured in this editorial is an example of a medical device that can selectively deplete activated myeloid leukocytes from peripheral blood in the GMA therapy of patients with IBD[39,40-58]. In essence, the treatment involves an extracorporeal approach in which patients' blood is passed through a column (Adacolumn) that is filled with specially designed cellulose acetate beads of 2 mm in diameter known as leukocytapheresis carriers. Pre and post column blood cell counts have shown that the carriers adsorb from the blood which passes through the column about 65% of granulocytes, 55% of monocytes and a very small fraction of lymphocytes[41]. These are the leukocytes that bear the so-called FcγR and complement receptors. They include GM, small subsets of $CD19^+$B lymphocytes and $CD56^+$NK (natural killer) cells[59-61]. One novel feature of this treatment is that it involves removing from the body the effector cells rather than administering drugs. It is therefore not expected to induce dependency or refractoriness and the treatment has not been associated with serious side effects in a significant number of patients[39,40,47-58].

## TREATMENT OF PATIENTS WITH SEVERE STEROID REFRACTORY UC

In our first major attempts (albeit without control groups)[39,40] we carefully selected patients with severe UC from a total of 285 patients with active UC who were first given salicylates as the first-line medication and those who did not improve (or worsened) were given intensive prednisolone (PSL) therapy and after a course of intensive PSL therapy, those who improved were not selected; 56 patients who could then be classified as steroid refractory were given GMA with the Adacolumn to deplete their peripheral blood GM. The study design is shown in Figure 1. The patients had a clinical activity index (CAI) of $\geqslant$ 12, a disease activity index (DAI) of $\geqslant$10[40,62,63] and were treated twice weekly for 2 to 3 consecutive weeks and then at one session per week for up to 11 GMA sessions. Assessments within one week after the last GMA session showed a response rate of 85% (Figures 1 and 2). No additional drug therapy was initiated while their ongoing PSL was tapered as symptoms improved. Figure 3 shows typical endoscopic improvements in steroid refractory patients. Pretreatment circulating neutrophil counts were very high, $9.3 \times 10^9 \pm 0.5 \times 10^9$/L, about 3 times the level seen in controls[40] and marked reductions were seen at wk 12 of treatment, $4.9 \times 10^9 \pm 0.4 \times 10^9$/L. Haemoglobin (Hb) at wk 12 relative to baseline increased by 25%, which may relate to the cessation of rectal bleeding following remission or improvements of clinical symptoms. Along with a fall in the patients' CAI and DAI and peripheral blood leukocytes counts, there was a comparable fall in C-reactive protein[40]. A total of 11 non-severe side effects in 7 patients were observed during leukocyte reduction therapy[40]. These were 3 incidences of flushing, 6 incidences of dizziness/light headache, nausea in 1 and mild fever in 1. However, no patient discontinued GMA therapy due to these side effects, all of which lasted from a couple of minutes to 3 h. Further, there was no evidence of opportunistic infection in any patient during or after GMA therapy.

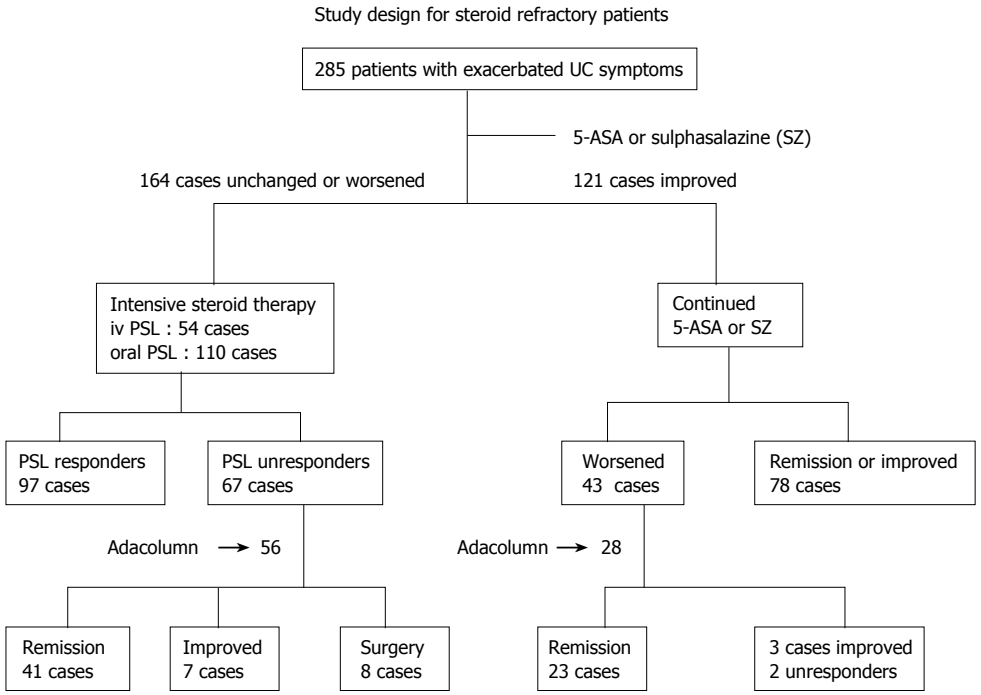



**Figure 1** Study design and patient selection for selective leukocytapheresis with the Adacolumn (GMA) in patients with steroid refractory and steroid naïve ulcerative colitis. iv prednisolone (PSL) indicates intravenous PSL (60 mg/d); oral PSL (40-60 mg/d). The dose of 5-ASA was 1.5-2.25 g/d while the dose of SZ was 2-3 g/d. During GMA course, PSL was tapered or discontinued in patients who improved. As shown, 56 steroid refractory and 28 steroid naïve patients were randomly selected for GMA, and the rest were treated according to Figure 5.

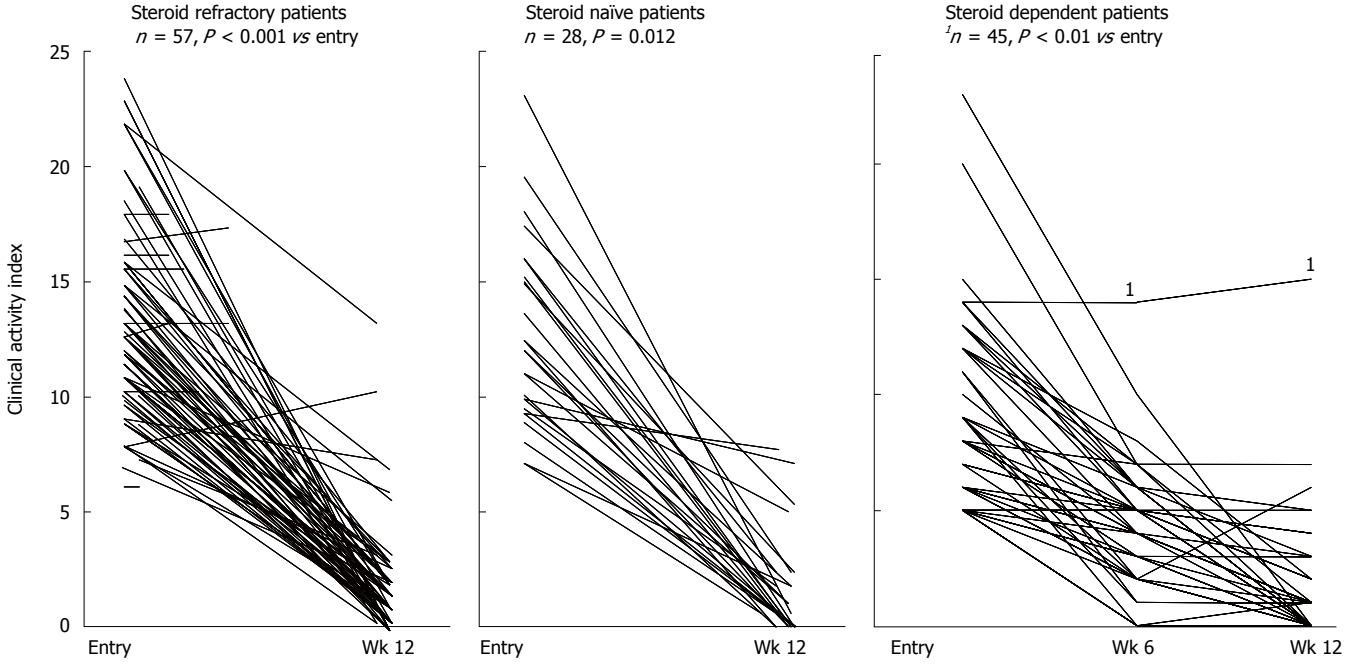
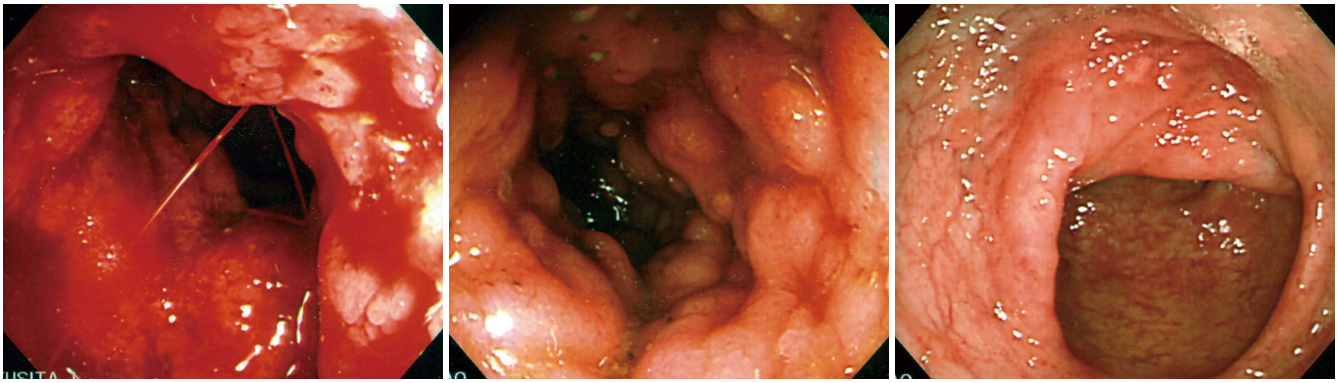



**Figure 2** Fall of clinical activity index (CAI) during the course of Adacolumn GMA in patients with steroid refractory, steroid naïve and steroid dependent ulcerative colitis.

The aforementioned refractory cases represent a subgroup of patients with severe UC who are at a significant risk of serious complications. Indeed treatment failure after 5-10 d of intensive corticosteroid therapy is often considered to be an indication for colectomy, CysA or TNF-α antibodies. Only 8 (14%) patients underwent colectomy. At 12 mo, 79% of patients had maintained their remission. In contrast, the relapse rate in patients who initially respond to CysA has been 60% to 80%[64], and unlike CysA, GMA with the Adacolumn has been without major side effects[39,40-58]. These initial response rates have subsequently been reproduced both in Japan and in Europe[47-58].

## GMA AS A FIRST-LINE MEDICATION FOR STEROID NAÏVE PATIENTS

Together with our steroid refractory patients described above we had a subgroup of steroid naïve patients, all of whom achieved remission by GMA[40]. This has now increased to 28 patients (Figure 1). Most of these steroid naïve patients went into clinical remission by GMA and remained steroid naïve during the study and the 12-mo follow up period. Subsequently, Suzuki *et al* reported treating 20 steroid naïve patients with active UC by GMA[47,48]. The patients treated by Suzuki *et al*[47,48] had moderate to severe UC; mean CAI was 8.8. At entry, all



At entry
(Readily bleeding ulcers)

After 5 adacolumn sessions
(Inflammation exists)

After 10 adacolumn sessions
(Vascular patterns visible)

**Figure 3** Typical endoscopic response in steroid refractory patients. Modified from Hanai *et al*, *Dig Dis Sci* 2002; 47: 2349-2353 with kind permission of Springer Science and Business Media.

patients were on 5-ASA (1.5 to 2.25 g/d). Each patient was to receive up to a maximum of 10 GMA sessions, at a frequency of 2 sessions/wk. Efficacy was assessed 1 wk after the last session. CAI fell to clinical remission levels (CAI $\leqslant$ 4) in the majority of patients after 6 sessions, and only 2 of the 20 patients required all 10 sessions. At post treatment, the mean CAI was 3, with a range from 0 to 12 and 17 of 20 patients (85%) were in clinical remission. The 3 non-responders had deep colonic ulcers at entry. There were significant changes in total peripheral white blood cell counts ($\times 10^9$/L), 9.8 $\pm$ 1.0 *vs* 7.0 $\pm$ 0.6 at post treatment. In contrast, lymphocytes increased dramatically from a pretreatment level of 19% to nearly 30%, attributable to the increase in absolute lymphocyte count[47]. During GMA therapy, 2 incidences of transient mild headache were reported. In both cases, the headache receded within 3 h without medication.

## GMA IN THE TREATMENT OF PATIENTS WITH STEROID DEPENDENT UC

Similarly, we used GMA to treat patients with corticosteroid dependent UC where GMA was used vs PSL[56]. A total of 261 consecutive patients who were initially evaluated were treated with a 5-ASA (1.5-2.25 g/d) or SZ (2-3 g/d). Patients who failed to respond were then treated with steroids and those patients who obtained remission, but relapsed during PSL tapering were given GMA or their steroid dose was increased. Both treatments were added to their ongoing conventional therapy. However, in both groups, PSL was to be tapered or discontinued in line with improvements of CAI. At wk 12, 83% in the GMA group and 65% in the PSL group were in remission (CAI $\leqslant$ 4)[56]. Further, in the GMA group, flushing was seen in 6 cases, nausea in 2 and mild fever in 2. This is in sharp contrast to 40 steroid side effects reported by Shimoyama *et al*[65] in a cohort of 52 patients who were given PSL. Typical remission rate in terms of CAI for GMA in steroid dependent patients is presented in Figure 2.

It is hard to overemphasize the clinical value of GMA in steroid dependent patients because patients in this group are exposed to steroids for most of their active disease lives and therefore, steroid side effects are often additional complications. Thus, one wary physician writes: How to do without steroids in inflammatory bowel disease[8]? Quotations from this article include the followings: "I believe physicians have fallen into an incorrect pattern of using steroids without considering other therapeutic options;" "The toxicity associated with oral steroids occurs so frequently and is so severe that physicians should take another look at administering these agents;" "At a meeting of Crohn's Disease and Ulcerative Colitis Foundation of America, a patient asked 'Why physicians use steroids when they are so destructive to the individual? '" Therefore, Adacolumn GMA might be a safe and perhaps most effective alternative therapy for these patients.

## GMA IN THE TREATMENT OF CD

The vast majority of clinical reports on GMA with the Adacolumn are in patients with UC. However, there is evidence to assume that GMA is effective in patients with CD as well. The first study in CD was reported by Matsui *et al*[54]. In that study, 7 patients with CD refractory to conventional medication including nutritional therapy, each received 5 GMA sessions. Five of seven patients achieved remission. In the study by Fukuda *et al*[55], 21 patients with refractory CD received 5 GMA sessions each. The efficacy rate was 52%. It is imperative to state that the patients Fukuda *et al*[55] included had received conventional medications including 2 wk of optimum nutritional therapy and only patients who remained with a high Crohn's disease activity index (CDAI) were given GMA. Therefore, 52% remission rate in these refractory patients was very encouraging. Domenech *et al*[53] reported treating 12 steroid dependent patients with CD. The remission rate in that clinical setting was 70%. Finally, Muratov and colleagues[66] reported treating 7 patients with CD who were refractory or had relapsed despite medication. Six had received infliximab, but without success. Adacolumn GMA was performed at one session per week for 5 wk. Efficacy was assessed at wk 7 and 12 mo. The median

value of CDAI decreased from 290 at wk 1 to 184 at wk 7 ($P = 0.031$). At the 12 mo follow-up, CDAI had decreased further to 128.5 ($P = 0.0156$).

## WHAT COULD BE THE MOST APPROPRIATE DOSAGE OF GMA?

Since the publications of first papers on GMA in the treatment of patients with UC, the following questions have often popped up in the community of IBD physicians: What is the most effective number of GMA sessions for a patient with severe IBD? What is the most appropriate frequency of GMA, one, two, three sessions per week or more? How long should be the duration of one treatment session and treatment course? The reality is that unlike drugs for which the dosage regimen has been defined, for a non-drug GMA, up to now treatment has been more or less arbitrary. In our centers, we have adopted a strategy of giving patients 2 treatment sessions per week in the first 2-3 wk and then 1 session per week up to 10 or 11 sessions[39,40,56]. We found that although patients with steroid naïve UC achieved remission or improved after 5 sessions, this was not seen in steroid refractory patients who responded better to 10 sessions[40]. Regarding duration of one GMA session, Kanke and colleagues[49], found that 90 min was significantly better than 60 min per GMA session. No data on the duration of one treatment course are available right now. However, the clinical response to Adacolumn GMA may not be immediately evident. For example, in patients with rheumatoid arthritis, there was a sustained increase in CD4+ T lymphocytes up to 12 wk following the last GMA session[41]. Similarly, there was a striking decrease in the expression of the chemokine receptor CXCR3 on leukocytes several weeks after the last GMA session[59,67]. Clearly it seems that much work has yet to be done before the optimum frequency and duration of treatment can be firmly established.

## PATIENTS WHO ARE MOST LIKELY TO RESPOND TO GMA

Currently available data suggest that steroid-naive patients respond particularly well to this treatment[40,47]. Characteristically they respond faster with fewer GMA sessions and have a higher cumulative rate of remission[40,47]. As reviewed above, most steroid naïve patients in our centers achieved remission[40], while the remission rate in the cohort reported by Suzuki *et al*[47] was 85%. In one of the most thorough and retrospective studies by Suzuki and colleagues[58], the authors attempted to determine the responders to Adacolumn GMA. Their findings are summarized as follows. Seven days after the last GMA session, 20 of 28 patients had achieved clinical remission including all 8 patients who had their first UC episode. The mean duration of UC in the 8 first episode cases was 3.4 mo compared with 40.2 mo for all 28 patients and 65.4 mo for the 8 non-responders. The response to GMA seemed to be independent of basal CAI. The 8 non-responders were given conventional medication (CM) or CysA if the former failed. Two patients responded to CM, 3 to CysA and 3 underwent colectomy. The authors' conclusions are as follows. First UC episode and short disease duration appear good predictors of response to GMA. Further, GMA might be an effective first line medication[57]. It would appear that the clinical response in patients with chronic continuous UC[40] and patients with deep colonic lesions[47] might be somewhat less satisfactory than in those experiencing their first UC.

## GMA IN PATIENTS AT A HIGH RISK OF CLINICAL RELAPSE

Bjarnason and colleagues in London (Guy's King's and St Thomas' Medical School) are currently evaluating the efficacy of GMA with the Adacolumn to suppress IBD relapse in patients at a high risk of experiencing one. The ongoing work was presented at the UEGW 2005 in Copenhagen[68]. The approach represents a fundamental change in the philosophy of treating IBD. Instead of treating active disease, asymptomatic patients are identified solely on the basis of a very high fecal calprotectin concentration, a neutrophil selective protein that provides quantitative measure of intestinal inflammatory activity[34-36]. The high calprotectin levels (over 250 μg/g) place them in a very high-risk group for clinical relapse[32]. This multi-center, prospective, randomized controlled study, assigned patients to Adacolumn, undergoing 5, once weekly, outpatient GMA sessions, or to unchanged treatment. Both patients with UC and CD were included. Follow up was monthly for 6 mo for a clinical relapse. In the Adacolumn group, 62% maintained their remission compared to 24% in the control group ($P < 0.04$, Pearson Chi squared test). Life table analysis demonstrated the mean survival in the Adacolumn group, 181 d *vs* 104 d in the control group ($P = 0.016$, Mantel Chi-squared test). This study represents a new approach to the treatment of IBD, namely targeting the inflammatory component of the disease at an asymptomatic stage. It seems likely that the 5 weekly sessions of GMA in such patients will have a significant effect and potentially avoid the morbidity associated with severe clinical relapses and the subsequent drug therapy in most patients.

## EFFECTS OF GMA ON LEUKOCYTE-DERIVED INFLAMMATORY AND ANTI-INFLAMMATORY FACTORS

Although the primary target of GMA with the Adacolumn is to deplete activated peripheral blood leukocytes principally granulocytes and monocytes/macrophages, it has been difficult to explain why some patients continue to improve long after the treatment has been ceased. Also the low relapse rate during follow-up we have reported[40] can not be fully explained by our current understanding of neutrophil function or the effects of GMA on peripheral blood levels of leukocytes *per se*. Alternative mechanisms of action have therefore been sought. Adacolumn is filled with cellulose acetate beads to which leukocytes that bear

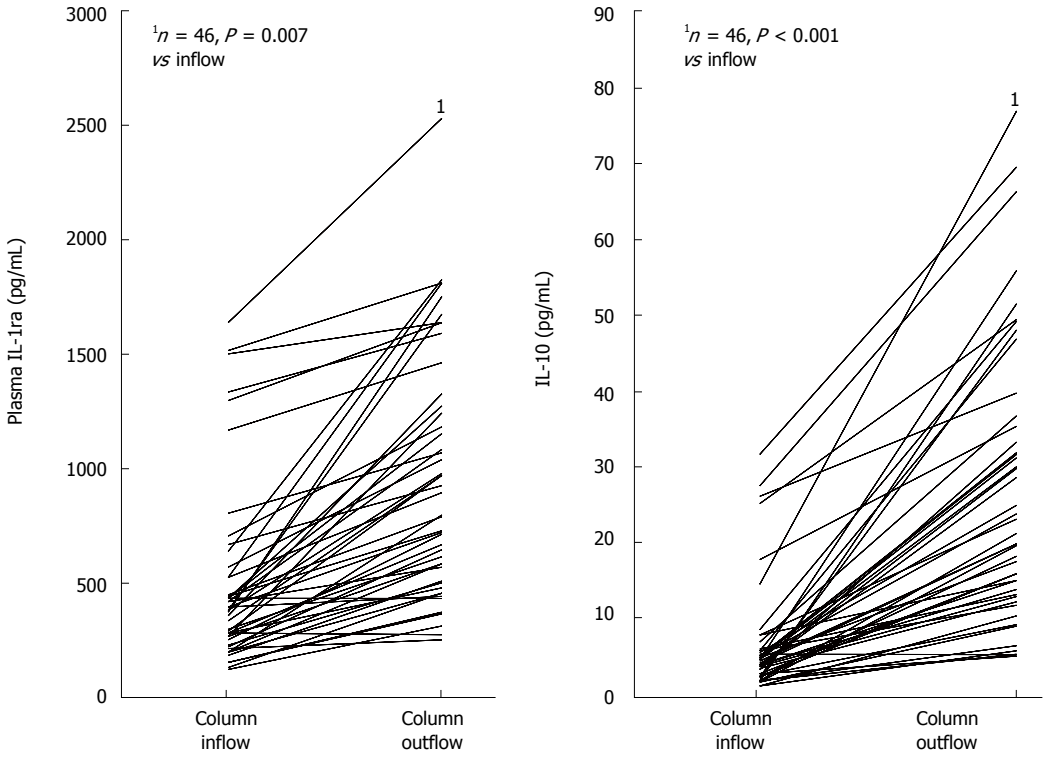



**Figure 4** Release of interleukin-1 receptor antagonist (IL-1ra) and IL-10 in the Adacolumn during Adacolumn GMA in patients with active ulcerative colitis. Column outflow returns to the patients. Elevated IL-1ra and IL-10 (both anti-inflammatory) in the column outflow is potentially very significant (see text for comments on IL-1ra and IL-10).

the FcγR and complement receptors adhere[59-61]. The adsorbed leukocytes release an array of active substances both toxic and non-toxic, but anti-inflammatory as well[47,69-71]. Some of these like cytokines, complement fragment C3a and C5a are of short half-life and may not reach the patients' circulation in fully active form. In view of this background, several investigators have carried out analysis on blood samples taken from the Adacolumn inflow and outflow (blood return line to patients) during GMA procedure. We[69,70] as well as Suzuki *et al*[47] found a significant increase in blood levels of soluble TNF-α receptors I, Ⅱ. Soluble TNF receptors are believed to neutralize TNF without invoking TNF-like actions[72]. Further, several studies report a marked decrease in the capacity of peripheral blood leukocytes to generate inflammatory cytokines (TNF-α, IL-1β, IL-6 and IL-8) following Adacolumn GMA[39,41,59,65,71,73,74]. Also, GMA procedure appears to produce a similar effect on leukocyte trafficking receptors. Thus the expressions of both L selectin[59,74] and the chemokine receptor CXCR3[59,67] were dramatically reduced, while the expression of the leukocyte integrin, Mac-1 (CD11b/CD18) was up-regulated[41,59,73]. These observations indicate that the procedure has a suppressive effect on leukocyte extravasation. Similarly, *in-vitro* studies showed that incubation of human whole blood with the Adacolumn leukocytapheresis carriers for 60 min resulted in the generation of significant amounts of IL-1 receptor antagonist (IL-1ra) and hepatocyte growth factor (HGF)[75]. However, the authors did not detect significant amounts of TNF or IL-1. IL-1ra has an essential role in the control of inflammation in the mucosa[76,77] while HGF is known to promote mucosal epithelial cell regeneration, which is an essential step in ulcer healing[78]. These observations on cytokines and adhesion molecules are perhaps of more academic significance than therapeutic value, but still are related to the effects of the GMA on neutrophils. Figure 4 shows elevated IL-1ra and IL-10, another major anti-inflammatory cytokine[79] in the blood at the Adacolumn outflow.

There is evidence that GMA suppresses cytokine profiles within the mucosal tissue as well. Thus, Muratov *et al*[66] found a very marked decrease in tissue interferon (IFN)-γ positive T-cells in clinical responders ($P = 0.027$) after GMA. In parallel, significantly lower levels of IFN-γ producing lymphocytes were detected in peripheral blood. IFN-γ positive cells in pretreatment biopsies completely disappeared or decreased in post-treatment biopsies sampled 2 wk after the last GMA session in responders ($P = 0.027$) and appeared to predict the maintenance of long-term remission or response after 12 mo. In another study by Yamamoto and colleagues[80] , the authors found that at entry the mucosal concentrations of IL-1β, IL-1, IL-1ra, IL-6, IL-8 and TNF-α were significantly higher compared with healthy persons, while IL-1ra/IL-1β ratio was significantly lower. In patients who achieved clinical remission by GMA, but not in those without remission, the mucosal tissue concentrations of IL-1β, IL-1ra, IL-6, IL-8 and TNF-α significantly decreased, whereas the IL-1ra/IL-1β ratio significantly increased.

## GMA INCREASES PERIPHERAL BLOOD LYMPHOCYTES

As stated above, the Adacolumn leukocytapheresis carriers selectively adsorb FcγR and complement receptors bearing leukocytes[59-61]. Indeed, Adacolumn not only spares the prevailing lymphocytes, it also induces an increase in *de novo* lymphocytes[41,47,81]. This is very intriguing and is in line with the original thinking for the design and development of the

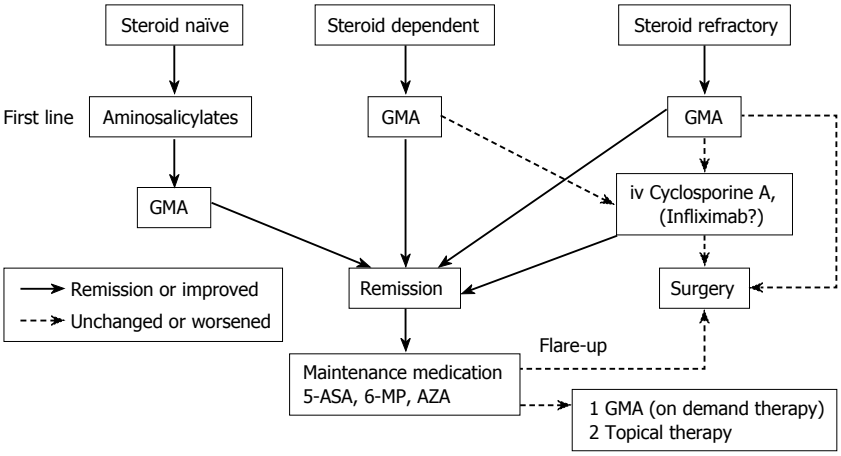



**Figure 5** Treatment algorithm for ulcerative colitis without corticosteroids. Based on this scheme, patients at any stage of their disease might benefit from GMA and avoid corticosteroids or other drug based medications. Intensive leukocytapheresis (2 or more GMA sessions per week in the first 3 wk) is recommended in severe or fulminating cases. GMA: Granulocyte and monocyte adsorption apheresis; 5-ASA: 5-aminosalicylic acid; 6-MP: 6-Mercaptopurine; AZA: Azathioprine.

present Adacolumn, to tame the exuberant immune system in patients in whom an elevated peripheral neutrophils level was thought to promote disease progression[41]. One likely question could be "What is the merit of sparing lymphocytes? " The precise role of lymphocytes in the relapse of IBD is uncertain and evidence presented below indicates that indiscriminately removing peripheral lymphocytes even for a short period might in fact be pro-inflammatory in patients with IBD[71,82]. The majority of patients with active IBD have very low lymphocyte counts[47,81,83] and a low lymphocyte count has been associated with relapse of CD[83]. Hence depleting the already compromised lymphocytes potentially could impair adequate immune function. To our knowledge, there is no published data showing elevated peripheral lymphocytes in patients with active IBD. Further, in one of the best controlled studies on lymphocytapheresis in IBD reported by Lerebours and colleagues[84], the authors selectively depleted peripheral lymphocytes in patients with CD with the aim of suppressing clinical relapse. At the end of an 18-mo follow-up, the rate of relapse was 83% in the lymphocytapheresis group and 62% in the control group; the clinical outcome in the lymphocytapheresis group being 21% inferior to that of control. The increase in lymphocytes associated with Adacolumn GMA indicated above is primarily attributable to an increase in CD4+ T cells. Of these, the CD4+CD25+ T-cell subset suppresses intestinal inflammation through mechanisms that involve interleukin 10 and transforming growth factor beta[85,86].

## CONCLUDING REMARKS

There have been significant recent advances in the medical therapy of IBD represented in part by the availability of biologicals which are developed to intercept the inflammatory cytokines or related inflammatory cells. However, the introduction of biologicals (albeit representing progress), has added new dimensions to the spectrum of treatment related adverse side effects like tuberculosis and lymphoma, to mention just two. Biologicals on which there rest great hope bear long term efficacy and safety concerns. Given that IBD is often associated with elevated and activated myeloid leukocytes which are major sources of inflammatory cytokines (the very agents that biologicals are expected to intercept), selective depletion of these leukocytes with the Adacolumn should represent a natural biologic therapy, and based on the available data, GMA seems to have an excellent safety profile. GMA in patients with steroid refractory UC (albeit mostly uncontrolled studies) has been associated with impressive clinical efficacy together with tapering or discontinuation of steroids, while in patients with steroid dependent or steroid naïve, GMA spares patients from exposure to steroids. Likewise, GMA at appropriate intervals in patients with IBD at a high risk of clinical relapse suppresses relapse thus sparing the patients from the morbidity associated with IBD relapse. Additionally, the procedure appears to reduce the number of patients being submitted to colectomy or exposure to potent immunosupressors like CysA. First UC episode, steroid naivety and short disease duration appear good predictors of response to GMA. This might be in line with the finding that corticosteroids support granulocytes. The author believes that GMA should be given as a first line medication. A treatment algorithm reflecting the author's current opinion for the medical therapy of UC is presented in Figure 5.

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*EDITORIAL*

# Aging and the intestine

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## Abstract

Over the lifetime of the animal, there are many changes in the function of the body's organ systems. In the gastrointestinal tract there is a general modest decline in the function of the esophagus, stomach, colon, pancreas and liver. In the small intestine, there may be subtle alterations in the intestinal morphology, as well as a decline in the uptake of fatty acids and sugars. The malabsorption may be partially reversed by aging glucagon-like peptide 2 (GLP2) or dexamethasone. Modifications in the type of lipids in the diet will influence the intestinal absorption of nutrients: for example, in mature rats a diet enriched with saturated as compared with polysaturated fatty acids will enhance lipid and sugar uptake, whereas in older animals the opposite effect is observed. Thus, the results of studies of the intestinal adaptation performed in mature rats does not necessarily apply in older animals. The age-associated malabsorption of nutrients that occurs with aging may be one of the several factors which contribute to the malnutrition that occurs with aging.



**Key words:** Aging; Gastrointestinal tract; Intestine; Absorption; Malnutrition

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## INTRODUCTION

Although each and every one of us is familiar with inevitable age-related changes, the task of clearly defining the term is challenging. Aging is a multi-factorial process which includes both intrinsic and extrinsic factors. To further complicate matters, in humans the term can be considered from sociological, physiological, psychological and molecular perspectives.

While we tend to use the term "ontogeny" to describe development in early life, and "aging" to describe development or sometimes presumed degeneration in later life, it is likely that the better concept is that of development over the lifetime of the animal. Thus, "young" and "old" become descriptive terms describing a process over time. Aging then may be considered to be a continuum that begins at conception and proceeds until death. The definition of aging can be further refined as "chronological age" according to the passage of time. Although advancing age is associated with increases in morbidity and mortality in general, this approach fails to consider the health of the individual. Determining a specific age at which an individual becomes "old" is arbitrary, and the concept of aging has changed over the centuries with humans now experiencing increased longevity and quality of life.

"Biological age" reflects the presence or absence of disease. Because there is not always a direct relationship between age and disease, this definition is considered to be a better marker of health status. The term "functional aging" has also been used to emphasize the limitations of defining health based on chronological age. This definition characterizes people based on what they can do in relation to others in society, but may also be used to characterize the level of functioning of organs and systems in the elderly. Finally, the concept of "successful aging" takes this idea one step further, and suggests that the aging process is variable, and may be characterized as a balance between gains and losses[1]. The compression of morbidity and an enhanced quality of life are cornerstones of the concept of successful aging.

## THEORIES OF AGING

A number of theories have been proposed to describe the process of aging. Longevity genes have been identified in many species, suggesting that aging may be at least partially under genetic control. In yeast, overproduction of the enzyme Sir2 prolongs the life of yeast grown under normal nutrient conditions[2]. It has been suggested that increases in Sir2 (seen in response to caloric restriction or resveratrol, a polyphenol found in red wine) may increase gene silencing, and thereby result in greater genomic stability[2]. Research undertaken in Drosophila has identified single gene mutations that extend life span. These include the gene Methuselah (*mth*), a secretion-type receptor that provides resistance to stress[3], and *Indy*

(I'm Not Dead Yet), whose gene product is homologous to Kreb's cycle intermediates[4,5]. In humans, a genetic component to aging has also been suggested. Werner's syndrome, a disorder characterized by an apparent accelerated senescence, has been associated with a single gene locus on chromosome 8[6]. On the other hand, a genome wide scan of elderly subjects suggested that there is a locus on chromosome 4 which influences a person's genetic susceptibility to age well and to achieve exceptional longevity[7].

Cellular theories emphasize that the environment as well as intrinsic properties of the cell, often referred to as a "cellular clock", may limit survival. Pivitol research by Hayflick and Moorhead[8] found that normal human fetal cell strains were limited to 40-60 doublings before they entered senescence. From this finding, they developed the concept of the "Hayflick limit" to explain determination of longevity. From this early work, the concept of telomere shortening was then established as another mechanism of longevity determination. These repetitive DNA sequences found at the end of chromosomes are progressively depleted with age, and may represent a method by which cells enter senescence. However, this theory is not universally accepted, largely due to a lack of correlation between telomere length and life span in many animal species[9].

Aging may be the consequence of oxidative damage. Oxidative damage to DNA, protein, carbohydrates and lipids contribute to degenerative diseases in aging, due to a disruption in cellular homeostasis. The activation of specific stress signalling pathways results in alterations in gene expression mediated by a variety of transcription factors including NF-κB, p53, and heat shock transcription factor 1 (HSF1)[10]. While levels of antioxidants correlate with longevity in primates[11,12], there is no evidence that antioxidant supplementation affects life span. It has been suggested, but not proven conclusively, that the success of calorie restricted diets in extending the lifespan of rodents is related to a reduction in free radical formation[13-15].

The role of insulin/IGF-1 signalling in the regulation of lifespan has been studied. The gene *daf*2, an insulin/IGF-1 receptor homolog has been shown to affect the lifespan of *C. elegans*[16]. Similarly, a related tyrosine kinase receptor, InR, regulates lifespan in Drosophila[17]. Holzenberger *et al*[18] demonstrated the importance of this pathway in mammals. In this study, heterozygous knockout mice ($Igf1r^{+/-}$) were used, as null mutants were not viable. These $Igf1r^{+/-}$ mice had IGF-1 receptor levels that were half of those seen in wild-type animals. These mice lived an average of 26% longer than did their wild-type littermates, without developing dwarfism, or showing adverse changes in physical activity, fertility or metabolism. This suggests that the link between insulin signalling and longevity seen in lower order organisms may also exist in mammals. Furthermore, the $Igf1r^{+/-}$ mice showed a greater resistance to oxidative stress, a known determinant of aging[10]. This lends support to the theory that oxidative stress plays an important role in the aging process.

In addition to increasing resistance to oxidative stress, insulin/IGF-1 signalling may affect aging via effects on Forkhead transcription factors of the FOXO class. Overexpression of FOXO extends life span[19]. Insulin/IGF-1 receptor binding, and subsequent activation of the PI3K/Akt pathway, results in the phosphorylation of Akt, which inactivates FOXO by sequestering it in the cytoplasm[20]. This alters the effects of FOXO on resistance to stress, apoptosis and longevity, and provides another potential link between insulin/IGF-1 and aging.

Other theories of aging focus on neuro-endocrine changes, including reductions in the levels of the steroid hormone dehydroepiandrosterone (DHEA). Both animal and human studies have demonstrated that oral replacement of DHEA may prevent or reduce age-associated events such as cancer and cardiovascular disease, and may stimulate immune function[21-25].

## A SOCIETAL PERSPECTIVE

Seniors constitute the fastest growing segment of Canada's population. In fact, the proportion of seniors has risen from one in twenty in 1921, to one in eight in 2001. Within this group, the number of Canadians aged 85 or more is anticipated to increase substantially, up to 4% of the total population by the year 2041 (Health Canada, 2002). Women make up the majority of seniors, with gender differences becoming more pronounced in the oldest age groups.

The aging of the population may be thought of as a modern day success story. For the first time in history human beings have been afforded the opportunity to live an unprecedented number of years, with a reasonable quality of life. This accomplishment is not without challenges, however, as society struggles to adapt to a changing demographic, with a unique set of physiological, psychological, and social needs of the elderly themselves as well as their caregivers.

Several non-genetic factors may influence life expectancy, including improvements in sanitation and nutrition, as well as reductions in maternal mortality and the rates of infectious diseases (An Aging World: 2001, U.S. Census Bureau). These changes, coupled with lower fertility rates, result in a changing demographic that presents society with the challenges of providing quality health care to an aging population, and facilitating the social, economic and community involvement of seniors.

Although most seniors rate their health as "good" or "very good", seniors are more likely to visit health care professionals, to take medication, and to be hospitalized when compared to their younger counterparts. Therefore, increases in this population and the associated increased health care utilization may place a burden on the system. Indeed, health expenditures for seniors in 2000-2001 represented 43% of total health care expenditures (Health Canada, 2002). Of course we recognize that these persons have contributed greatly to our society, and it is our responsibility to provide ready access to quality of healthcare for these special persons, who must be treated with respect and allowed to age with dignity.

## MALNUTRITION IN THE ELDERLY

The elderly are at a high risk for malnutrition, yet

unfortunately it is often underdiagnosed[26]. Poor nutritional status is a key determinant of morbidity and mortality in the elderly[27-30]. Because nutrition is a modifiable risk factor, attempts should be made to design preventative nutritional strategies aimed at improving the quality of life and consequently minimizing the use of health care resources.

Why are the elderly malnourished? There are a number of contributing factors including: (1) inadequate intake, attributed to a lack of appetite, or difficulty in preparing food; (2) psychological factors, including depression; (3) social factors, including isolation and low income; and (4) physiological factors, such as reduced sense of smell and taste, drug-nutrient interactions and reductions in nutrient absorption[31]. Sullivan *et al*[29] demonstrated that hospitalization was a risk factor for inadequate food intake in seniors, possibly due to the unattractive and monotonous food choices, or due to the side effects of drug therapies. Reduced food intake is generally accepted as the main cause of undernutrition in the geriatric population, and as such therapies should be aimed at increasing food intake. Many researchers also feel that malnutrition in the elderly is indicative of prevailing social conditions, and that therapies should be aimed at alleviating poverty, isolation and depression in this age group.

Because one of these several factors which may contribute to malnutrition relates to possible age-associated changes in the physiology of the gastrointestinal tract, this topic will be reviewed.

## THE AGING PROCESSES AND THE GASTROINTESTINAL TRACT

The aging of the population, coupled with the potential impact on the health care system, has focused attention on the physiological processes associated with aging. Only with an increased understanding of the aging process can we work towards improving the quality of life for the elderly, and reducing disease morbidity in this population.

There are age-related alterations in the gastrointestinal tract but the difficulty lies in excluding concomitant pathological factors as the cause of these changes. Certainly with aging, conditions such as diabetes, pancreatic or liver disease, cancer, or drug-induced enteropathy will have potential adverse effects on the form and function of the intestine. It is necessary to exclude these pathological factors, to consider the physiological changes that occur in the healthy elderly, and to understand how these factors influence the nutritional status of this population.

Dysphagia is more common in the elderly than in younger persons[32]. Selective neurodegeneration may occur in the aging enteric nervous system (reviewed in Saffrey)[33], and may contribute to gastrointestinal symptoms such as dysphagia, gastrointestinal reflux and constipation. Interestingly, caloric restriction in rodents can prevent the neuronal losses that occur with aging, suggesting that diet may influence gastrointestinal aging[34]. Alterations in esophageal motility may be due to reductions in the number of neurons in the myenteric plexus of the elderly[35]. While gastric motility may be impaired with aging[36,37], small intestinal motility is unaffected[38-40]. Aging may affect the signal transduction pathways and cellular mechanisms controlling smooth muscle contraction, which may influence colonic motility and thereby contribute to the development of constipation (reviewed in Bitar and Patil)[41].

The data regarding aging and gastric acid secretion is inconclusive, as early studies were likely confounded by the presence of *H pylori* in some persons. Achlorhydria or hypochlorhydria may result from atrophic gastritis, as a result of the use of medications such as proton pump inhibitors, or as a result of *H pylori* infection[42-44]. This reduction in gastric acidity may increase the risk of small bowel bacterial overgrowth, potentially leading to malabsorption[45]. For example, McEvoy *et al*[46] found that 71% of patients in a general geriatric ward had bacterial overgrowth of the small intestine, while 11% were found to be malnourished. Indeed, bacterial overgrowth in older adults is associated with reduced body weight, which is paralleled by reduced intake of several micronutrients[47].

Although structural changes in the pancreas are seen with aging, no functional age-related alterations are seen using the fluorescein dilaurate test[48]. Some studies demonstrate reduced secretagogue-stimulated lipase, chymotrypsin and bicarbonate concentrations in pancreatic juice with aging[49]. Other research suggests that there is little evidence of reduced pancreatic secretions with age, independent of other factors including the presence of disease and the effect of drugs[50].

There are age-related reductions in liver mass and blood flow, yet microscopic changes are subtle[51-53]. While structural and functional changes do not correlate well, there is evidence that liver function declines with age. For example, Cao *et al*[54] used microarrays to show that aging in mice is accompanied by changes in the expression of genes in the liver involved in inflammation, cellular stress and fibrosis, all of which are linked to age-related liver pathologies. Interestingly, caloric restriction in mice starting at weaning reversed the majority of the age-related changes, once again emphasizing the ability of the diet to influence the aging process.

Holt *et al*[55] looked at age-related changes in the intestinal morphology of Fischer 344 rats. Increases in villous width were noted throughout the small intestine, while increases in villous height were limited to the ileum. Other studies in rats have shown age-related losses in villous and enterocyte heights[56]. Age-related declines in mucosal surface area have also been reported in rabbit jejunum[57]. Human studies generally show no changes in intestinal morphology, as determined from measurements of villous height, crypt depth, crypt-to-villous ratios and enterocyte size[58-60]. Warren *et al*[61] showed a decrease in villous height with age. Martin *et al*[62] described histological changes that occur in aging mice: when old mice were compared to young mice, there were larger villi, a reduced number of crypts, and fewer villi and crypts per mm along the small intestine. These changes were most pronounced in the distal, as opposed to the proximal small intestine. However, even if there are minor age-associated alterations in intestinal morphology with aging, there is not a clear association between intestinal morphology and nutrient

uptake with aging. For example, despite reductions in mucosal surface area, aged rats demonstrated increases in the jejunal uptake of saturated fatty acids[57]. So, while it remains controversial as to whether or not aging is associated with morphological changes, even if such changes were to occur, the impact on nutrient uptake may not be clinically relevant.

Ciccocioppo *et al*[63] suggested that intestinal architecture is maintained with aging by increases in proliferation and differentiation rates. This agrees with work done by Corazza *et al*[64] that showed increased expression of proliferating cell nuclear antigen (PCNA) in older subjects when compared to their younger counterparts.

## NUTRIENT DIGESTION AND ABSORPTION

Age-related alterations in the abundance of brush border malease (BBM) enzymes may also impact upon the digestion and subsequent absorption of nutrients. BBM lactase phlorizin hydrolase (LPH) and sucrase-isomaltase (SI) activities fall with age in rats[65]. Bacterial overgrowth, which is common in the elderly, may also negatively impact upon disaccharidase activity, and thereby possibly reduce carbohydrate absorption[66].

Hollander and colleagues demonstrated that intestinal permeability to medium sized probes (mannitol, polyethylene glycol) increased in 28-month old rats when compared to 3-month old rats[67]. However, the lactulose: mannitol (LTM) ratio was not different between young and old subjects, indicating that intestinal permeability to these sugars does not change significantly with age in humans[68]. A study using breath hydrogen analysis following a carbohydrate meal showed evidence of malabsorption with aging. Elderly patients (ranging from 65-89 years, mean age, 79 years) were compared to control subjects (ranging from 20-64, mean age, 35 years). Significantly more subjects in the elderly group (7 out of 21) excreted excess $H_2$ when compared to controls (0 out of 19)[69]. This suggests that there may be malabsorption of carbohydrates in the elderly. *In vitro* transport experiments using BBM vesicles also demonstrated a reduction in $Na^+$-dependent D-glucose uptake in patients over the age of 70[70]. In contrast, Wallis and co-workers[71] did not find changes in $Na^+$-dependent glucose transport in BBM vesicles isolated from duodenal biopsies from patients whose ages ranged from 55 to 91 years.

Experiments using rodent models of aging also demonstrate conflicting results. Several studies show reductions in D-glucose absorption in aged rats[72-74]. Depending upon the intestinal site studied, a normal or increased absorptive capacity was also found in a study using everted intestinal segments from old versus young rats[75]. Results from studies in mice also do not offer conclusive results on the effect of aging on nutrient absorption. Ferraris *et al*[76] showed in aged mice a reduction in uptake and site density of the $Na^+$-dependent glucose transport in the BBM, SGLT1. This is in contrast to the findings of Thompson *et al*[77] who showed an increase in intestinal glucose uptake in aged mice. Our lab has recently investigated the effect of age on intestinal glucose uptake in Fischer 344 rats using the *in vitro* intestinal sheet method[78]. Glucose uptake was reduced in 9 mo old and 24 mo old rats when compared to 1 mo old animals. When changes in mucosal surface area were accounted for, only ileal glucose uptake was reduced in the older animals. These age-associated changes in glucose uptake were not explained by alterations in the abundance of SGLT1, GLUT2 or $Na^+K^+$-ATPase.

The variations in the results from human, rat and mouse studies may be due to the differences in the methodologies that were used. While some investigators studied uptake using BBM vesicles[70-74], others used everted intestinal rings[75,77,79] or intestinal sheets[78]. As well, the method of expressing results may influence qualitative differences between studies. Uptake is often expressed on the basis of intestinal weight, and does not taken into account any potential age-associated changes in mucosal weight or surface area. The strain and ages of the animals, and the site of the intestine used also differ between studies, and may explain the variability in the results.

The uptake of fructose has been studied in aging mice. Ferraris and Vinnekota[79] showed that D-fructose uptake per milligram of tissue was higher in the jejunum of young as compared to old animals. Adaptive increases in uptake, in response to increases in carbohydrate levels, were blunted in these mice, and were restricted to more proximal regions of the small intestine

While it is reasonable to speculate that the complexity of lipid absorption may make it susceptible to the effects of aging, experimental findings do not consistently support this notion. While a number of animal studies demonstrate reduced *in vitro* lipid absorption with aging[80,81], others have shown increases in lipid absorption in aged rats using an *in vivo* perfusion model[82]. Aging is associated with a decrease in the thickness and resistance of the unstirred water layer[80], which could partially explain the finding of increased absorption with aging in the *in vivo* model.

Early work using human subjects demonstrated reductions in lipid absorption with age[83]. There also appears to be reduced intestinal absorption of bile acids with age[84], although it is not clear if this negatively impacts lipid absorption in the elderly. When healthy elderly human subjects were studied, however, no correlation between age and 72 h fecal fat excretion was found[85].

More recently, a study by Woudstra *et al*[86] showed that the ileal uptake of several fatty acids including 16:0, 18:0, 18:1 and 18:2, was reduced in 24 mo old rats, when compared to 1 mo old animals. However, when mucosal surface area was considered these differences disappeared, suggesting that the age-related changes in lipid uptake were largely due to non-specific reductions in intestinal surface area. After considering the results of all of these studies, Holt[87] suggested that no important changes in lipid absorption with aging have been described.

## MODIFICATION OF AGE-ASSOCIATED DECLINES IN INTESTINAL ABSORPTIVE FUNCTION

Holt *et al*[55] have shown that the intestine of elder rats is capable of adopting its function in response to changes

in dietary protein levels. In adult rats, a diet enriched with saturated fatty acids (SFA) results in increased intestinal sugar uptake when compared to an isocaloric diet enriched with polyunsaturated fatty acids (PUFA)[88-91]. Similarly, Vine *et al*[92] studied the effect of various fatty acids on the passive and active transport properties of rat jejunum, and found that an SFA diet increased $Na^+$-dependent glucose uptake when compared to a diet enriched with n6 PUFA. Of importance, Woudstra *et al*[93] showed that the intestinal response to dietary lipids may differ with age. In this study, in contrast to what is seen in younger animals, feeding a PUFA diet increased lipid uptake when compared to feeding a SFA diet. Drozdowski *et al* (unpublished observations) have also shown that PUFA rather than SFA increases intestinal sugar uptake in older rats. The mechanism responsible for the age-related alteration in adaptation to daily lipids is not known. But clearly, the results of adoptive studies in young rats do not necessarily apply to older animals. Other factors which may enhance the reduced uptake of sugars that occurs in older animals indicates glucagon-like peptide 2 (GLP2) at the glucocorticosteroid, dexamethasone (Drozdowski *et al*, unpublished observations, 2006).

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# Persistent CXCR4 expression after preoperative chemoradiotherapy predicts early recurrence and poor prognosis in esophageal cancer

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## Abstract

**AIM:** To study the effect of CXC chemokine receptor-4 (CXCR4) expression on disease progression and prognosis in esophageal cancer.

**METHODS:** CXCR4 expression was evaluated in 37 patients with histologically confirmed esophageal squamous carcinomas (ESCC) undergoing preoperative chemoradiotherapy (CRT) by immunohistochemical staining.

**RESULTS:** Eleven out of 37 ESCC patients showed a pathological complete response (CR) after CRT. CXCR4 protein expression was observed in cell cytoplasms of 13 tumors, and null expression was seen in 13 tumors. Distant recurrence was significantly more common in patients with positive CXCR4 expression ($P$ = 0.0318). After a median follow-up time of 31.6 mo, 19 patients progressed (12 of 19 expressed positive CXCR4) and 11 died (10 of 11 expressed positive CXCR4). Overall survival was significantly correlated with lymph node metastasis (952.1 ± 53.8 d in negative group *vs* 475.1 ± 56.2 d in positive group, $P$ = 0.023), distant metastasis (874.0 ± 60.4 d in negative group *vs* 434.9 ± 75.2 d in positive group, $P$ = 0.014) and CRT (811.5 ± 51.2 d in responder group *vs* 459.6 ± 94.0 d in non-responder group, $P$ = 0.00038) and further with an absence of CXCR4 expression or no residual tumor (959.8 ± 51.0 d in null expression or no tumor group *vs* 412.0 ± 57.1 d in positive expression group, $P$ = 0.0001).

**CONCLUSION:** Persistent positive CXCR4 expression is implicated in tumor aggressiveness and poor prognosis in ESCC after CRT, and preoperative CRT may improve the prognosis of ESCC *via* CXCL12-CXCR4 signaling pathway.



**Key words:** CXC chemokine receptor-4; Metastasis; Chemoradiotherapy; Esophageal cancer

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## INTRODUCTION

The migration of tumor cells to a secondary site from their primary location is a crucial issue in cancer metastasis. Recently, a novel "homing" signaling mechanism has been proposed, in which target organs produce and release specific chemokines that attract cancer cells bearing corresponding receptors[1,2]. This mechanism was originally characterized for organogenesis, hematopoiesis and inflammation, and draws on the principles of the "seed and soil" hypothesis advocated by Paget more than one century ago[3]. Signaling results in directional, site-specific cancer cell migration leading to implantation in the favorable "soil" of organs. A large number of studies support this "homing" mechanism by demonstrating that malignant cells can target specific organs or tissues using select chemokine receptors, mainly through the CXCL12-CXC chemokine receptor-4 (CXCR4) pathway[4-8].

Chemokines are signaling molecules that function in myriad cell trafficking events. They have been classified into four subgroups (C, CC, CXC, and $CX_3C$) based on the positioning of their cysteine residues[9]. CXCL12, also

known as stromal cell-derived factor-1 (SDF-1), belongs to the CXC chemokine family and CXCR4 is the only known physiological receptor for SDF-1[10]. Chemokine receptor activation can lead to growth, adhesion and, most importantly, directional migration[11]. In hematopoiesis and development, stem cells and progenitor immune cells migrate to and from various organs and tissues under the directional guidance of chemokines[12]. Chemokine migratory activation also plays a role in integrating T-cell migration during immune and inflammatory responses[13]. The discovery that chemokine receptors are expressed on nonhemopoietic cell types, such as endothelial and epithelial cells, will inevitably lead to the receptors being implicated in other biological and disease processes such as angiogenesis, organ development, metastasis and tumorigenesis. Indeed, clinical studies have already revealed that CXCR4 expression is associated with increased metastasis and decreased survival of some cancer patients[14-18].

Esophageal cancer is one of the most aggressive forms of cancer with rapid growth. Common to other cancers, the presence of lymph node metastasis and vascular invasion indicate a highly malignant potential[19]. Surgery is the treatment of choice for patients with locoregionally confined esophageal cancer, but the five-year survival rate is only 10%-30%, even after curative surgery[20]. The reason for this is that esophageal cancer shows extensive local invasion or frequent regional lymph node metastasis, often at the time of initial diagnosis. Since 1996, we have introduced preoperative chemoradiotherapy (CRT) combined with radical surgery for the treatment of esophageal cancers, and have reported that adjuvant preoperative CRT increased resectability, reduced the incidence of both local recurrence and distant metastasis, and achieved better prognosis for CRT responders[21]. However, no data are currently available on the role of CXCR4 expression in esophageal cancer progression, and the prognosis of patients undergoing CRT has not as yet been reliably estimated. In this study, therefore, we retrospectively investigated the expression of CXCR4 protein in human esophageal squamous cell carcinoma (ESCC) tissues and evaluated the clinical implications of these patients who underwent preoperative CRT and radical surgery.

## MATERIALS AND METHODS

### Patients and therapy

Thirty-seven patients, seven women and 30 men with a mean age of 60.32 (range 44-78) years and surgically excised ESCC were studied at the Hyogo College of Medicine, Hyogo, Japan, between April 1996 and June 2003. Preoperative CRT was performed as follows (schedule shown in Figure 1): 5-flurouracil (5-FU) (500 mg/m$^2$ per day) was administered as a 120-h continuous intravenous infusion starting on d 1, and cisplatin (CDDP) (15 mg/m$^2$ per day) as a 2-h iv infusion on d 1-5. Radiation therapy was performed on d 1-5, after CDDP infusion, using a linear accelerator (Mevatron KD2, Siemens, Germany). The radiation method has been previously reported[16]. Chemotherapy was combined with radiation therapy during the first week, and then radiation therapy alone was repeated for the next 3 wk (d 8-12, 15-19, and 22-26). A single dose was 2 Gy/d, with a total dose of 40 Gy.

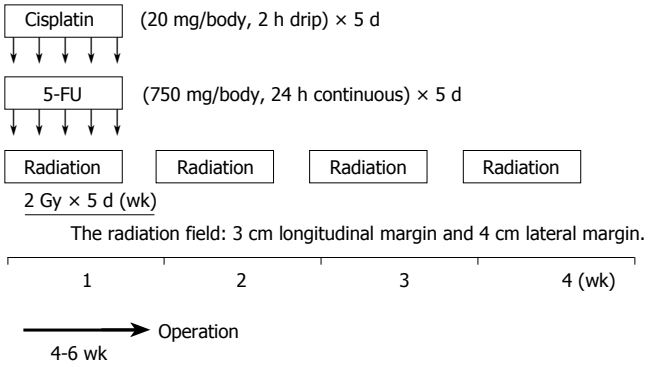


**Figure 1** Schedule of preoperative chemoradiotherapy (CRT).

Surgery was usually performed 4 to 6 wk after the completion of CRT. Resected specimens were cut open longitudinally and fixed with formalin. Follow-up information was obtained from office charts, hospital records, and telephone interviews.

### Immunohistochemistry

ESCC tissue specimens were processed using conventional procedures for paraffin embedding, cut into 4-μm sections, and mounted onto poly-L-lysine-coated slides. Sections were dewaxed in xylene, rehydrated in descending alcohols, and heated twice by microwave oven for 5 min each time in 10 mmol/L sodium citrate (pH 6.0) for antigen retrieval. They were then blocked for endogenous peroxidase with 30 mL/L $H_2O_2$ in methanol, and blocked again for non-specific antibody binding with normal rabbit serum. After then they were incubated for 2 h at room temperature with mouse mAb against human CXCR4 (R&D Systems, Minneapolis, MN, USA), followed by a standard avidin-biotin-peroxidase complex method. The slides were developed with 3, 3'-diaminobenzidine tetrahydrochloride solution containing 1 mL/L $H_2O_2$ and were lightly counterstained with hematoxylin. Normal mouse IgG was substituted for the primary antibody as a negative control. The sections were finally counterstained with Lillie-Mayer's hematoxylin and mounted.

The sections were examined microscopically by three of the authors (R.Y., T.T., and Y.F.) without knowledge of clinicopathologic features. CXCR4 expression was categorized into four grades according to staining intensity in comparison with interstitial infiltrates[18]: score 3 (strong), staining intensity greater than interstitial infiltrates; score 2 (moderate), staining intensity equal to interstitial infiltrates; score 1 (mild), staining intensity less than interstitial infiltrates; and score 0 (negative), no staining. Additional CXCR4 expression scores were also assigned: score 3, CXCR4 high; scores 0-2, CXCR4 null or low.

### Statistical analysis

Overall survival (OS) was defined as the time from the date of initial diagnosis to patient death or the date of the last available information on vital status. In univariate analysis, the difference between the cumulative survival rates of

**Table 1 Patient characteristics**

| Characteristics | *n* |
|---|---|
| Sex (M/F) | 37 (30/7) |
| Mean age (yr) | 60.32 |
| Location of tumor | |
| Cervical | 1 |
| Upper thoracic | 6 |
| Middle thoracic | 19 |
| Lower thoracic | 11 |
| T-classification | |
| $T_3$ | 14 |
| $T_4$ | 15 |
| N-classification | |
| $N_0$ | 24 |
| $N_1$ | 13 |
| M-classification | |
| $M_0$ | 28 |
| $M_1$ | 9 |
| Disease stage (UICC TNM stage) | |
| Ⅱa | 17 |
| Ⅲ | 13 |
| Ⅳa | 2 |
| Ⅳb | 5 |

patient groups was calculated by the log-rank test for comparison using Kaplan-Meier survival curves. Statistical significance was considered at $P < 0.05$. Statistical analyses were carried out using Statistica statistical software, version 06J (Statistica, Tulsa, OK, USA).

## RESULTS

### Patient and tumor characteristics

Patient and tumor characteristics are summarized in Table 1. The patient gender bias was male (M:F, 30:7). Histology of all tumors was shown to be ESCC by histological examination. Thirty-six tumors originated in the thorax. According to the TNM system of the American Joint Committee on Cancer, stage Ⅱ tumors were seen in 17 patients (46%), stage Ⅲ in 13 (35.1%), and stage Ⅳ in seven (18.9%). Thirteen (35.1%) patients had lymph node metastasis ($N_1$ in six, $M_{1a}$ in three, and $M_{1b}$ in four patients) at the time of diagnosis. All lesions before CRT presented with a $T_3$ or $T_4$ extent of invasion. Three-quarters of patients had tumors between 6 and 8 cm in diameter. M+ classification was described in seven tumors. Two patients had distant metastasis of the liver. All patients experienced a disease-free period. During the follow-up period, four (10.8%) patients developed local recurrence or residual tumors, six (16.2%) developed neck or celiac lymph node recurrence, and seven (18.9%) developed distant metastasis. Fifteen (40.5%) patients died during follow-up: 13 (35.1%) died from their tumors and the remaining two (5.4%) were tumor free and died of intercurrent diseases.

### Prognostic value of persistent CXCR4 expression

Eleven tumors were totally eradicated by CRT, resulting in an absence of visible tumor cells: a pathological complete response (CR). CXCR4 expression was absent from 13 tumors (35.1%). Another 13 tumors (35.1%) were positive for CXCR4 expression: 11 scored 1, one scored 2, and the last scored 3. The patient who scored 3 died of multiple lung metastasis two months after surgery. Staining was observed predominantly in the cytoplasm of tumor cells (Figure 2). In the positive CXCR4 expression group ($n$ = 13), recurrences were found in 10 patients: two locally

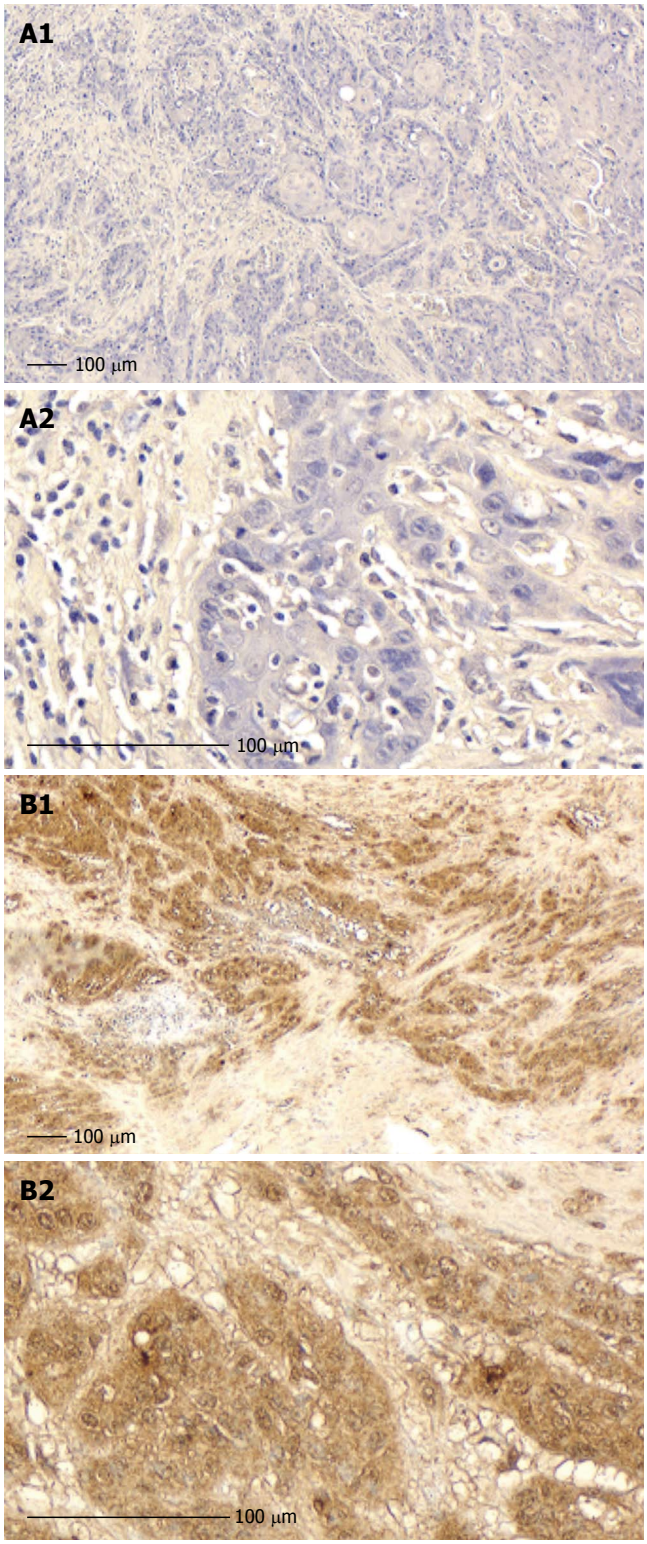


**Figure 2** Immunohistochemical detection of CXCR4 in ESCC specimens. **A**: Null expression (**A1**: × 40; **A2**: × 200); **B**: Strong expression (**B1**: × 40; **B2**: × 200).

**Table 2 Univariate analysis of prognostic factors for overall survival**

| Covariate | | *n* | *P* |
|---|---|---|---|
| Age (yr) | < 70 | 31 | |
| | ⩾ 70 | 6 | NS |
| Gender | Male | 30 | |
| | Female | 7 | NS |
| CRT | Effective | 28 | |
| | Not effective | 9 | 0.00038[b] |
| Lymph node metastasis | Positive | 13 | |
| | Negative | 24 | 0.023[a] |
| Distant metastasis | Positive | 14 | |
| | Negative | 23 | 0.014[a] |
| Depth of tumor invasion | $T_3$ | 23 | |
| | $T_4$ | 14 | NS |
| Tumor location[1] | Upper | 14 | |
| | Lower | 23 | NS |
| CXCR4 expression | Positive | 13 | |
| | Negative | 24 | 0.0001[b] |

[1]Upper or lower: Above or below the tracheal bifurcation. NS: Not significant. [a]$P < 0.05$, [b]$P < 0.01$ comparison between two corresponding groups.

and eight distant in the liver, bone, thyroid gland, lung and neck lymph nodes. By contrast, in the null CXCR4 expression group ($n = 11$), recurrences were found in only two patients, in the liver and bone. Statistical analysis showed that distant recurrence was more common in those patients with positive CXCR4 expression ($P = 0.0318$).

After follow-up, disease progression had occurred in 12 of the 13 patients with positive CXCR4 expression, and 11 of them died. Univariate analysis of prognostic factors for OS is summarized in Table 2. Lymph node metastasis and distant metastasis had a significant prognostic value (952.1 ± 53.8 d in lymph node metastasis negative group *vs* 475.1 ± 56.2 d in positive group, $P = 0.023$, and 874.0 ± 60.4 d in distant metastasis negative group *vs* 434.9 ± 75.2 d in positive group, $P = 0.014$, respectively). Furthermore, there was a very significant effect of CRT on OS prognosis (811.5 ± 51.2 d in responder group *vs* 459.6 ± 94.0 d in non-responder group, $P = 0.00038$). Kaplan-Meier analyses (Figures 3 and 4) suggest that prognosis was particularly unfavorable for patients with persistent positive CXCR4 expression in their primary tumors compared with CXCR4 null expression patients or those with no residual tumor (959.8 ± 51.0 d in CXCR4 null expression or no tumor group *vs* 412.0 ± 57.1 d in positive expression group, $P = 0.0001$). In addition, CXCR4 null expression patients showed an improved OS compared with positive expression patients ($P = 0.0005$).

## DISCUSSION

The understanding of the molecular basis of tumor development has progressed dramatically in the last few decades. It is well known that esophageal cancer shows poor prognosis because of its aggressiveness; therefore, it is important to understand the role of molecular factors in the acquisition of malignant potential. The presence and number of metastases in ESCC is important both

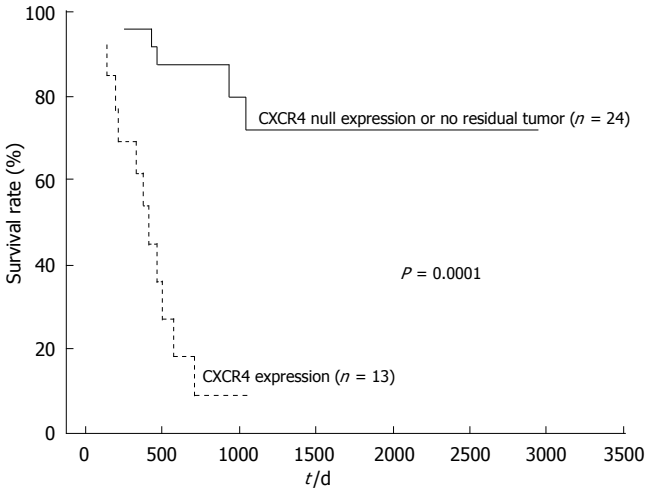


**Figure 3** Overall survival in ESCC patients with no residual tumor or null CXCR4 expression and those with positive CXCR4 expression.

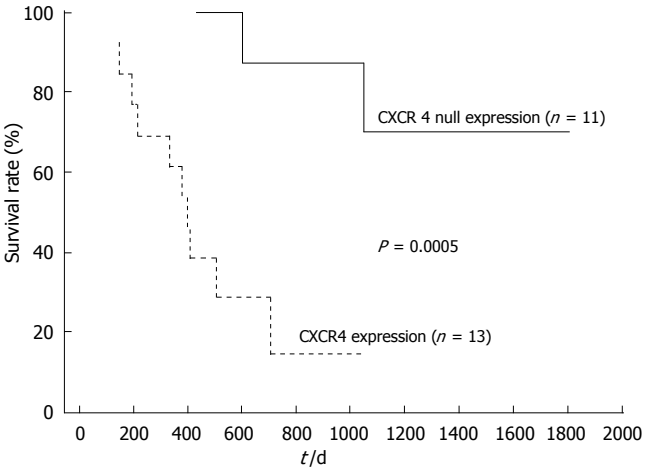


**Figure 4** Overall survival in ESCC patients with null or positive CXCR4 expression.

for staging and prognosis[22]. The metastatic potential of primary ESCC is considerably higher than other primary solid tumors when the size of the primary lesion is compared. Notably, in the present study, univariate analyses demonstrated that persistent CXCR4 expression was the most influential factor for OS in operable ESCC patients who underwent CRT. Patients with advanced ESCC, even in T4 stage, demonstrated improved prognosis when preoperative CRT was successful. Persistent CXCR4 expression after CRT may therefore be a useful biomarker for screening and management of high-risk patients with poor prognosis. To date, this is the first report demonstrating the prognostic role of CXCR4 expression in ESCC patients undergoing CRT.

It is likely that several mechanisms are involved in cancer metastasis including those of the "homing" signaling process described earlier. In ESCC, the signaling mechanism together with a mechanical drainage pattern might facilitate specific metastasis to lymph nodes, liver and lung. After distant metastatic cells have passed through vascular channels and implanted, a favorable "soil" might be responsible for further growth and proliferation.

Frequently, these esophageal metastases occur in multiple foci, but little is known about these patterns. Furthermore, the concept that cancer implantation enacts an immune response, thus accentuating chemoattraction of CXCR4-positive tumor and immune cells, cannot be discounted[23]. It has recently been reported that CXCR4 is highly expressed in malignant but not normal breast tissue, and that CXCL12 is expressed in those organs where breast cancer metastasis is frequently found such as bone marrow, lymph nodes, lung, and liver[1]. Moreover, neutralizing the interactions of CXCL12-CXCR4 by administration of an anti-CXCR4 antibody significantly impairs the metastasis of breast cancer cells to the lung and regional lymph nodes in mice[1]. Taken together with our results, these findings indicate that the CXCL12-CXCR4 interaction is important for the metastasis of solid tumors that fail to respond to CRT. Persistent CXCR4 expression even after CRT appears to have a prognostic value, although the alteration of CXCL12-CXCR4 signaling by CRT was not directly analyzed in our study. CXCR4 antagonists have already been studied in phase I clinical trials in multiple myeloma and non-Hodgkin's lymphoma[24-26]. These preliminary data regarding hematopoiesis suggested that AMD3100, a CXCR4 antagonist, is safe and effective in reversibly inhibiting CXCL12-CXCR4 binding and in mobilizing WBCs, CD34+ cells and hematopoietic progenitor cells (HPCs) into the circulation. Utilization of CXCL12-CXCR4 pathway may be a promising therapeutic approach in the prevention and treatment of metastasis.

In conclusion, we have demonstrated that positive CXCR4 expression after CRT correlates with distant recurrence and provides an independent prognostic factor for ESCC survival. These findings strongly suggest that CXCR4 plays an important role in ESCC progression and could provide a novel molecular target for the treatment of ESCC. Large cohort studies in a multicenter setting will be necessary to validate our findings and explore the potential use of CXCR4 antagonists in the treatment of ESCC patients with a high risk of early relapse following CRT.

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## COMMENTS

### Background

The chemokine CXCL12, also known as stromal cell-derived factor-1 (SDF-1), and its receptor CXCR4 have been implicated in organ-specific metastases of several malignancies. Recently, we have reported that adjuvant preoperative chemoradiotherapy (CRT) reduced the incidence of both local recurrence and distant metastasis. However, the impact of CXCR4 expression on disease progression and prognosis in esophageal cancer patients undergoing preoperative CRT remains unknown.

### Research frontiers

It has recently been reported that CXCR4 is highly expressed in malignant but not normal breast tissue, and that CXCL12 is expressed in those organs where breast cancer metastasis is frequently found such as bone marrow, lymph node, lungs, and the liver. Moreover, neutralizing the interactions of CXCL12-CXCR4 by administration of an anti-CXCR4 antibody significantly impairs the metastasis of breast cancer cells to the lungs and regional lymph nodes in mice.

### Innovations and breakthroughs

We have demonstrated that positive CXCR4 expression after CRT correlates with distant recurrence and provides an independent prognostic factor for ESCC survival. These findings strongly suggest that CXCR4 plays an important role in ESCC progression and could provide a novel molecular target for the treatment of ESCC. To date, this is the first report demonstrating the prognostic role of CXCR4 expression in ESCC patients undergoing CRT.

### Applications

Persistent CXCR4 expression after CRT may be a useful biomarker for the screening and management of high risk patients with poor prognosis. CXCR4 antagonists have already been studied in phase I clinical trials in multiple myeloma and non-Hodgkin's lymphoma. Potential use of CXCR4 antagonists should be explored in the treatment of ESCC patients with a high risk of early relapse following CRT.

### Terminology

Chemokines are signaling molecules that function in myriad cell trafficking events. They have been classified into four subgroups (C, CC, CXC, and $CX_3C$) based on the positioning of their cysteine residues. CXCL12, also known as stromal cell-derived factor-1 (SDF-1), belongs to the CXC chemokine family and CXC chemokine receptor-4 (CXCR4) is the only known physiological receptor for SDF-1. Chemokine receptor activation can lead to growth, adhesion and, most importantly, directional migration.

### Peer review

The paper is scientific and innovative contents as well as readability can reflect the advanced levels of the clinical research in gastroenterology both at home and abroad.

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# Folate levels in mucosal tissue but not methylenetetrahydrofolate reductase polymorphisms are associated with gastric carcinogenesis

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## Abstract

**AIM:** To evaluate whether folate levels in mucosal tissue and some common methylenetetrahydrofolate reductase (MTHFR) variants are associated with the risk of gastric cancer through DNA methylation.

**METHODS:** Real-time PCR was used to study the expression of tumor related genes in 76 mucosal tissue samples from 38 patients with gastric cancer. Samples from the gastroscopic biopsy tissues of 34 patients with chronic superficial gastritis (CSG) were used as controls. Folate concentrations in these tissues were detected by the FOL ACS: 180 automated chemiluminescence system. MTHFR polymorphisms were analyzed by PCR-RFLP, and the promoter methylation of tumor-related genes was determined by methylation-specific PCR (MSP).

**RESULTS:** Folate concentrations were significantly higher in CSG than in cancerous tissues. Decreased expression and methylation of c-myc accompanied higher folate concentrations. Promoter hypermethylation and loss of $p16^{INK4A}$ in samples with MTHFR 677CC were more frequent than in samples with the 677TT or 677CT genotype. And the promoter hypermethylation and loss of $p21^{WAF1}$ in samples with MTHFR 677CT were more frequent than when 677CC or 677TT was present. The 677CT genotype showed a non-significant higher risk for gastric cancer as compared with the 677CC genotype.

**CONCLUSION:** Lower folate levels in gastric mucosal tissue may confer a higher risk of gastric carcinogenesis through hypomethylation and overexpression of c-myc.



**Key words**: Folate; Methylenetetrahydrofolate reductase; Polymorphism; DNA methylation; Gastric cancer

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## INTRODUCTION

Methylation of gene regulatory elements is a well-documented epigenetic change that can lead to gene inactivation. Human gastric carcinogenesis is suggested to be associated with the decrease of total genomic DNA methylation, hypomethylation of certain specific oncogenes such as c-myc, and hypermethylation of promoter of some tumor suppressor genes containing $p16^{INK4A}$ and *hMLH1* gene[1]. Folate (or folic acid) is essential for normal DNA methylation and synthesis. We have performed a series of studies to investigate the interrelationship between DNA methylation and folate status in plasma of patients with gastric cancer[2,3]. The plasma folic acid concentration in patients who showed hypomethylation of c-myc was lower than that in patients showing normal methylation. Low plasma levels of folate have been associated with an increased risk for gastric cancers[4,5].

Methylenetetrahydrofolate reductase (MTHFR) is a key enzyme in folate metabolism that regulates the intracellular folate pool. Two MTHFR polymorphisms, C677T and A1298C, are known to be risk factors for gastric cancer in Chinese[6], but not in Korean[7]. The MTHFR 677T allele was significantly associated with gastric cancer risk with an odds ratio (OR) of 2.49 [95% confidence interval (CI): 1.48-4.20] in heterozygous MTHFR 677CT carriers and an OR of 2.85 (95% CI: 1.52-5.35) in homozygous MTHFR 677TT carriers in a high risk Italian population[8]. These findings suggest that common variants of MTHFR may play a role in the etiology of gastric cancer, particularly

gastric cardia adenocarcinoma. Future studies using large sample sizes and incorporated detailed data on dietary folate intake and related serological measurements are needed to confirm these findings[9].

The extent to which tissue folate levels and MTHFR 677 (C→T) polymorphism interact to affect DNA methylation in gastric carcinogenesis is uncertain. It is even not clear that there is a relationship between folate concentrations and DNA methylation in gastric mucosal tissue. In the current study, we hypothesized that folate levels and some common MTHFR variants are associated with the risk of gastric cancer through DNA methylation. Our data show that decreased folate in tissues is associated with a higher risk of gastric cancer. However, MTHFR gene polymorphisms are not independent risk factors for initiation and progression of gastric cancer, although the 677CT genotype shows a non-significant higher risk for gastric cancer as compared with the 677CC genotype.

## MATERIALS AND METHODS

### Subjects

Thirty-eight consecutive patients with gastric cancer underwent resection at Shanghai Renji Hospital between May and December 2004. Clinicopathological factors, tumor histologies and disease stages were evaluated according to the General Rules for Clinical and Pathological Studies on gastric cancer. Paired samples (76) of histologically verified primary gastric cancer and corresponding non-cancerous gastric mucosa (> 5 cm away from cancerous margin) of 38 patients were obtained immediately after surgical resection. HE-stained sections were examined for pathological diagnoses, and were categorized according to the WHO histological classifications of gastric cancer. The histological characteristics of non-cancerous tissues were chronic atrophic gastritis, intestinal metaplasia, or dysplasia. All of tumors were located in gastric antrum or corpus, and not in fundus or cardia. There were 23 cases of tubular adenocarcinoma, 4 cases of mucinous adenocarcinoma and 11 cases of tubular-papillary adenocarcinoma. The mean age of the patients at resection was 61 (range 31-81) years and it included 25 men and 13 women. A portion of each tissue (approximately 3-5 g) was snap-frozen on dry ice and kept in liquid nitrogen until use for DNA or RNA extraction. Another 34 patients with chronic superficial gastritis (CSG) were studied as sex, age and *H pylori* infection (by histology, urease test or breath test, as well as alcohol and tobacco intake matched controls to the gastric cancer group). Three endoscopic biopsy tissue samples were obtained from each control. All controls were subjected to clinical assessment, upper gastrointestinal endoscopy, histopathology of antral mucosa. No chronic atrophic gastritis, intestinal metaplasia or dysplasia was detected in any of the controls. Complete written consent was obtained from all patients and controls.

### Determination of folate concentrations in tissues

One milliliter of PBS was added to 10 mg of mucosal tissues. Lysates were sonicated, and the debris was removed from samples by centrifugation for 10 min at 15 000 × *g*, 4℃ in a microcentrifuge. Folate levels in gastric mucosa were measured with an ACS: 180 automated chemiluminescence analyzer (Chiron Diagnostics Corporation, East Walpole, MA). The ACS: 180 Folate assay is a direct chemiluminescence competitive immunoassay. Folates in the patient sample competed with acridinium ester-labeled folates in the Lite Reagent for a limited amount of biotin-labeled folate binding proteins. Biotin-labeled folate binding proteins bind to avidin that is covalently coupled to paramagnetic particles in the Solid Phase. The sample was pretreated to release the folates from endogenous binding proteins.

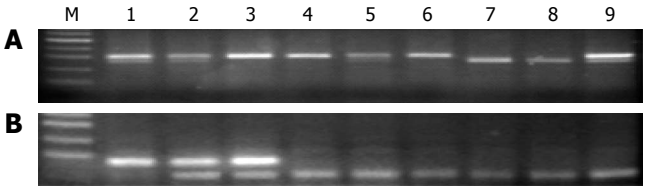


**Figure 1** **A**: *Hinf* I distinguishes MTHFR 677 (C→T) PCR products. M: 50 bp Ladder; L1, 2, 5 and 9: CT genotype; L3, 4 and 6: CC genotype; L7 and 8: TT genotype; **B**: *Mbo* II distinguishes MTHFR (A→C) PCR products. M: 50 bp ladder; L1: CC genotype; L2 and 3: AC genotype; L4-9: AA genotype.

### Analysis of MTHFR polymorphisms using PCR-RFLP

Genomic DNA was isolated from gastric mucosal tissue using QIAamp DNA Mini Kit (Qiagen Inc., Valencia, CA) according to the manufacturer's instructions. MTHFR C677T and A1298C mutations were detected after PCR amplification with the corresponding primers. The restriction enzyme *Hinf* I (New England Biolabs, Beverly, MA) was used to distinguish the 677 (C→T) polymorphism. The variant allele (677TT) gained an *Hinf* I restriction site and resulted in two fragments of 175 bp and 23 bp after digestion. The wild-type allele (677CC) had a single band representing the entire 198 bp fragment (Figure 1A). The restriction enzyme *Mbo* II (New England Biolabs) was used to distinguish the 1298 (A→C) polymorphism. The restriction site was absent in the variant allele (1298CC), and digestion yielded four fragments of 84, 31, 30 and 18 bp, whereas the wild-type allele (1298AA) generated 56, 31, 30, 28 and 18 bp bands (Figure 1B). The restriction products were analyzed by electrophoresis in a 3% agarose gel stained with ethidium bromide. PCR amplifications were run for 35 cycles, with each amplification cycle consisting of 30 s at 94℃, 30 s at 62℃ or 51℃ (for polymorphic sites at positions 677 and 1298, respectively), and 45 s at 72℃. PCR products were visualized on 3% agarose gels[10].

The MTHFR 677 (C→T) allele in gastric cancer and CSG tissues, but not the MTHFR 1298 (A→C) allele in gastric cancer samples, was in Hardy-Weinberg equilibrium ($P > 0.05$).

### Real-time RT-PCR for tumor-related genes

The transcription levels of tumor-suppressor genes, $p16^{INK4A}$ and $p21^{WAF1}$; proto-oncogene, c-myc; and mismatch repair (MMR) genes, *hMLH1* and *hMSH2*; were detected using real-time RT-PCR. Total RNA was isolated using a commercial kit (Trizol) according to the manufacturer's instructions (Invitrogen Gibco BRL, Carlsbad, CA).

**Table 1 Sequences of primers and probes for real-time PCR, and primers for MSP**

| Gene | Primer (forward) (5'-3') | Primer (reverse) (5'-3') | Probe | Genbank number |
|---|---|---|---|---|
| β-*actin* | CTG GCA CCC AGC ACA ATG | GGA CAG CGA GGC CAG GAT | ATC ATT GCT CCT CCT GAG | BC016045 |
| $p16^{INK4A}$ | CAT AGA TGC CGC GGA AGG T | CAG AGC CTC TCT GGT TCT TTC AA | CCT CAG ACA TCC CCG | NM_058197 |
| $p21^{WAF1}$ | CTG GAG ACT CTC AGG GTC GAA | GGA TTA GGG CTT CCT CTT GGA | ACG GCG GCA GAC CAG CAT GA | NM_078467 |
| c-myc | ACA CCG CCC ACC ACC AG | CCA CAG AAA CAA CAT CGA TTT CTT | AGC GAC TCT GAG GAG G | V00568 |
| *hMLH1* | GGC CAG CTA ATG CTA TCA AAG AG | CTT TAA CAA TCA CTT GAA TAC TTG TGG A | ATT GAG AAC TGT TTA GAT GCA | U07418 |
| *hMSH2* | ATC CAA GGA GAA TGA TTG GTA TTT G | CAA AGA GAA TGT CTT CAA ACT GAG AGA | CAT ATA AGG CTT CTC CTG GC | U04045 |
| $p16^{INK4A}$(M) | TTA TTA GAG GGT GGG GCG GAT CGC | GAC CCC GAA CCG CGA CCG TAA | | X94154 |
| $p16^{INK4A}$(U) | GGG GGA GAT TTA ATT TGG | CCC TCC TCT TTC TTC CTC | | X94154 |
| $p21^{WAF1}$(M) | TGT AGT ACG CGA GGT TTC G | TCA ACT AAC GCA ACT CAA CG | | NM_007592 |
| $p21^{WAF1}$(U) | TTT TTG TAG TAT GTG AGG TTT TGG | AAC ACA ACT CAA CAC AAC CCT A | | NM_007592 |
| c-myc (M) | TAG AAT TGG ATC GGG GTA AA | CGA CCG AAA ATC AAC GCG AAT | | AF002859 |
| c-myc (U) | TAG AAT TGG ATT GGG GTA AA | CCA ACC AAA AAT CAA CAT GAA T | | AF002859 |
| *hMLH1* (M) | ACG TAG ACG TTT TAT TAG GGT CGC | CCT CAT CGT AAC TAC CCG CG | | AB017806 |
| *hMLH1* (U) | TTT TGA TGT AGA TGT TTT ATT AGG GTT GT | ACC ACC TCA TCA TAA CTA CCC ACA | | AB017806 |
| *hMSH2* (M) | TCG TGG TCG GAC GTC GTT C | CAA CGT CTC CTT CGA CTA CAC CG | | AB006445 |
| *hMSH2* (U) | GGT TGT TGT GGT TGG ATG TTG TTT | CAA CTA CAA CAT CTC CTT CAA CTA CAC CA | | AB006445 |

M: Methylation PCR primers; U: Unmethylation PCR primers.

Reverse transcription reactions using 5 μg of total RNA in a total reaction volume of 20 μL were performed with Superscript Ⅱ reverse transcriptase (Invitrogen Life Technologies, Inc.). Relative quantitation using the comparative Ct method with data from the ABI PRISM 7700 Sequence Detection System (version 1.6 software) was performed according to the manufacturer's protocol. The primers and fluorogenic probes for these genes were provided by Shenyou Company, Shanghai. The sequences of the probes and forward and reverse primers are shown in Table 1. Real-time PCR was also performed with primers and a probe for *β-actin* to normalize each of the extracts for amplifiable human DNA. The results were expressed as the ratio of copies of target genes to *β-actin*. Ct values were measured, and the average Ct of triplicate samples was calculated. An alteration of mRNA expression was defined as a 3-fold difference in expression level[11].

### Bisulfite modification and methylation-specific PCR of promoters of tumor-related genes

To address whether DNA methylation of tumor-related genes is associated with the folate level in mucosal tissue and MTHFR polymorphisms, methylation-specific PCR (MSP) was performed in CpG-rich regions of $p16^{INK4A}$ and $p21^{WAF1}$, c-myc, and *hMLH1* and *hMSH2*, in order to detect changes in DNA methylation of the genes due to drug treatments. Bisulfite modification protocols were adopted as described by Xiong and Laird[12]. Genomic DNA treated with bisulfite was amplified with promoter specific primers of each gene (Table 1). The primers were designed without CpG dinucleotides to enable the amplification of both methylated and unmethylated alleles.

The 50 μL PCR reactions consisted of 100 ng of bisulfite-treated DNA, 0.1 mmol/L dNTPs, 2.0 mmol/L $MgCl_2$, and 0.5 μmol/L of each primer. PCR products were directly loaded onto 3% agarose gels and electrophoresed. The gel was stained with ethidium bromide and directly visualized under UV illumination. Wild-type $p16^{INK4A}$ and $p21^{WAF1}$ primers were used to verify that complete conversion of the DNA occurred in the bisulfite reaction. A positive control for complete methylation was also amplified.

### Statistical analysis

Data were presented as means ± SD. Comparisons between groups were made using Student's paired *t* test. The differences between cancerous, non-cancerous and CSG tissues, and their relationships were analyzed by Fisher's exact test using SAS v.6.12. All statistical tests were two tailed and considered significant at $P = 0.05$. A case-control study was performed and the allelic frequency of the polymorphism was calculated for both cases and controls. Odds ratios (OR) and 95% CI were calculated to evaluate the association between CSG or cancer and the presence of MTHFR C677T polymorphism. The Mantel Haenszel $\chi^2$ procedure was used to assess the linear trend between lesion severity and magnitude of the association with MTHFR C677T genotype.

**Table 2 Folate level and transcription and methylation of c-myc**

| Methylation status | Transcription | Case number (*n*) | *P*[1] | Average folate concentration (μg/L) | *P*[2] |
|---|---|---|---|---|---|
| Hypomethylated | Up-regulated | 6 | | 2.25 ± 1.23 | |
| | Unchanged | 0 | 0.02 | | 0.06 |
| Methylated | Up-regulated | 13 | | 3.91 ± 2.01 | |
| | Unchanged | 19 | | | |

[1]Transcription and DNA methylation of c-myc; [2]Folate concentration and DNA methylation of c-myc.

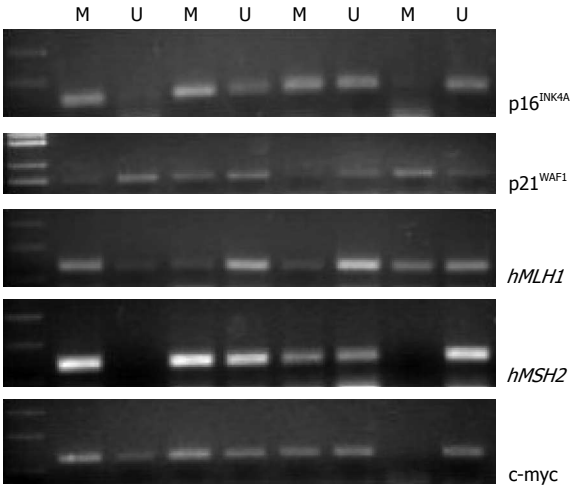


**Figure 2** Methylation-specific PCR (MSP) for tumor-related genes. MSP was performed on DNA from gastric cancerous tissue with primers designed to specifically detect methylated and unmethylated promoter regions. The data shown are representative of three replicate MSP experiments. Marker: 100 bp ladder; U: Unmethylated-MSP; M: Methylated-MSP.

## RESULTS

### *Folate levels were lower in cancerous tissue than in non-cancerous tissue or CSG*

Folate concentrations in CSG tissue (5.48 ± 2.15 ng/mL) were significantly higher ($P < 0.05$) than in the other two groups (gastric cancerous tissue, 3.65 ± 1.97 μg/L and corresponding non-cancerous gastric mucosa, 4.01 ± 2.11 μg/L). There were no significant differences in folate concentrations between different sites of the stomach including antrum and corpus. No correlation was found between folate levels and the degree of infiltration, lymph node metastasis, tumor size, or TNM clinical stage of gastric cancer (data not shown).

### *c-myc expression and methylation were associated with folate concentrations*

As shown in Table 2 and Figure 2, decreased expression of c-myc accompanied higher folate levels. However, there was no definite association between folate levels and different expression levels of tumor suppressor genes including $p16^{INK4A}$, $p21^{WAF1}$, *hMLH1* and *hMSH2* in mucosal tissue (data not shown). Furthermore, hypomethylation of c-myc was found in cancerous tissues, which showed up-regulated expression of c-myc, and folate levels in tissues with a hypomethylated c-myc gene showed a downward trend compared with folates in unmethylated samples in advanced gastric cancer (tubular-, mucinous-, and tubular-papillary adenocarcinoma).

**Table 3 MTHFR gene polymorphisms and the transcription of tumor-related genes**

| Genes | MTHFR | Up-regulation *n* (%) | Normal (*n*) | Down-regulation *n* (%) | mRNA ratio of cancerous to non-cancerous tissue (mean ± SD) | *P* |
|---|---|---|---|---|---|---|
| $p16^{INK4A}$ | CC | 1 | 3 | 10 (71.43) | 0.63 ± 1.05 | 0.84 |
| | CT | 1 | 8 | 10 (52.63) | 0.7 ± 0.88 | 0.69 |
| | TT | 0 | 2 | 3 (60) | 0.85 ± 0.96 | - |
| | AA | 1 | 9 | 16 (61.54) | 0.57 ± 0.96 | 0.51 |
| | AC | 1 | 4 | 7 (58.33) | 0.81 ± 1.15 | - |
| $p21^{WAF1}$ | CC | 1 | 7 | 6 (42.86) | 1.06 ± 1.06 | - |
| | CT | 1 | 5 | 13 (68.42) | 0.47 ± 0.69 | 0.062 |
| | TT | 0 | 2 | 3 (60) | 0.55 ± 0.55 | 0.32 |
| | AA | 2 | 10 | 14 (53.85) | 0.75 ± 0.85 | 0.57 |
| | AC | 0 | 4 | 8 (66.67) | 0.58 ± 0.85 | - |
| c-myc | CC | 6 (42.86) | 7 | 1 | 2.26 ± 2.17 | 0.048 |
| | CT | 11 (57.89) | 6 | 2 | 4.32 ± 3.25 | - |
| | TT | 2 (40) | 2 | 1 | 2.15 ± 1.8 | 0.17 |
| | AA | 15 (57.69) | 8 | 3 | 3.84 ± 3.17 | 0.076 |
| | AC | 4 (33.33) | 7 | 1 | 2.06 ± 1.64 | - |
| *hMLH1* | CC | 0 | 3 | 11 (78.57) | 0.46 ± 0.67 | 0.053 |
| | CT | 1 | 8 | 10 (52.63) | 0.57 ± 0.82 | 0.13 |
| | TT | 0 | 3 | 1 (25) | 1.3 ± 1.28 | - |
| | AA | 2 | 10 | 14 (53.85) | 0.72 ± 0.96 | 0.28 |
| | AC | 0 | 4 | 8 (66.67) | 0.39 ± 0.55 | - |
| *hMSH2* | CC | 0 | 5 | 9 (64.29) | 0.31 ± 0.32 | - |
| | CT | 1 | 10 | 8 (42.11) | 0.88 ± 0.97 | 0.04 |
| | TT | 0 | 3 | 2 (40) | 0.94 ± 1.12 | 0.064 |
| | AA | 1 | 13 | 12 (46.15) | 0.8 ± 0.98 | 0.19 |
| | AC | 0 | 5 | 7 (58.33) | 0.4 ± 0.42 | - |

### *MTHFR polymorphisms might affect the methylation status of tumor-suppressor gene promoters*

As indicated in Table 3, the 677CC, CT, CC, CT and CC genotypes of MTHFR were most frequently detected with aberrant methylation of $p16^{INK4A}$, $p21^{WAF1}$, *hMLH1*, *hMSH2* and c-myc, respectively. The 1298AA genotype was found associated with aberrant methylation of all tumor-related genes studied.

Compared to the 677CC genotype, expression of *hMSH2* was significantly down-regulated in cases with the 677CT genotype, and showed a large but non-significant decrease in the presence of 677TT. Cases with the 677CT genotype showed a lower level of $p21^{WAF1}$ expression than did cases with the wild-type 677CC genotype, while *hMLH1* coupled with the 677CC genotype displayed a trend of decreased expression compared with the 677TT genotype. No differences were observed in $p16^{INK4}$ mRNA levels between 677CC, 677CT or 677TT. c-myc transcription in cases with the 677CT genotype was significantly higher than when the 677CC genotype was present.

Compared with the 1298AC genotype, c-myc

transcription showed an increase in cases with the 1298AA genotype. There was no difference in the expression of $p16^{INK4A}$, $p21^{WAF1}$, *hMLH1* or *hMSH2* between the three MTHFR genotypes.

### *MTHFR polymorphisms might not be an independent factor affecting initiation and progression as well as biological characteristics of gastric cancer*

There were no differences in either the genotype distribution or allele frequency for alleles 677T between gastric cancerous tissue and CSG ($P > 0.05$), although the 677CT genotype showed a non-significant higher risk for advanced gastric cancer as compared with the 677CC genotype (Table 4). In addition, due to the limitation of sample size, we failed to find any significant association between MTHFR polymorphisms, 677 (C→T) and 1298 (A→C), and the degree of infiltration, lymph node metastasis, tumor size, or clinical stage of gastric cancer (Table 5).

### *MTHFR polymorphisms were not associated with folate levels in gastric cancerous tissues*

The genotypes of 677 and 1298 sites were not associated with folate concentrations in gastric cancerous tissue and CSG (data not shown).

**Table 4 MTHFR polymorphisms of gastric cancerous tissue and CSG**

| Polymorphic site | MTHFR genotype | Cancerous tissue *n* (%) | CSG *n* (%) | OR (95% CI) | *P* |
|---|---|---|---|---|---|
| C677T | CC | 14 (36.84) | 15 (44.12) | 1 (reference) | - |
| | CT | 19 (50.00) | 11 (32.35) | 1.85 (0.65-5.24) | 0.24 |
| | TT | 5 (13.16) | 8 (23.53) | 0.67 (0.17-2.57) | 0.55 |
| A1298C | | | | | |
| | AA | 26 (68.42) | 22 (64.71) | 1 (reference) | - |
| | AC | 12 (31.58) | 11 (32.35) | 0.92 (0.34-2.52) | 0.87 |
| | CC | 0 | 1 (2.94) | - | - |

## DISCUSSION

Gastric cancer is a common malignant tumor worldwide, with a much higher incidence in Asian than in Western countries. Multiple genetic and epigenetic alterations are involved in gastric carcinogenesis.

Folate is an important constituent of fruits and vegetables and may confer protection against cancer. An important biological function of folate is to provide methyl groups required for intracellular methylation reactions and *de novo* deoxynucleoside triphosphate synthesis; therefore, folate deficiency is thought to be carcinogenic through disruption of DNA methylation and synthesis and impaired DNA repair[13]. However, folate requires metabolic transformations catalyzed by several enzymes including MTHFR, which irreversibly converts 5, 10-methylenetetrahydrofolate to 5-methyltetrahydrofolate. The MTHFR gene is highly polymorphic, of which the two most common variants are located at nucleotides 677 (C→T) and 1298 (A→T). Low MTHFR activity may prevent the shunting of methyl groups from *de novo* dTMP synthesis, a rate-limiting step for DNA synthesis, to methylation pathways.

It has been suggested that the cancer risk associated with MTHFR polymorphisms may be modulated by folate intake[14]. When folate intake is sufficient, individuals carrying the variant MTHFR genotypes may have a decreased risk because under these conditions, while adequate provision of methyl donors is still ensured, enhanced genomic integrity would be achieved *via* conservation of folate within a cyclic intracellular pathway by shunting methyl groups toward nucleotide synthesis due to diminished MTHFR activity. However, when folate intake is low, both DNA methylation and DNA synthesis and repair might be impaired in carriers of variant MTHFR genotypes, which, in turn, results in increased risk of carcinogenesis. This hypothesis of a gene-nutrient interaction may explain the conflicting reports showing either reduced[10,15,16] or elevated[17,18] risk of cancers.

Many previous studies have investigated the relationship between folate status and MTHFR gene polymorphisms in carcinogenesis. However, because they were mainly focused on blood plasma and blood cells, little is known about folate status in mucosal tissues. Gastric cancer originates from epithelial cells; therefore, a study performed in mucosal tissues is more accurate and effective than those performed using blood. In the present study, we examined folate concentrations and MTHFR gene polymorphisms in gastric mucosal tissues, and found that folate levels were significantly lower in cancer cases (including cancerous and corresponding non-cancerous tissues) than that in the CSG controls (no chronic atrophic gastritis, intestinal metaplasia or dysplasia), suggesting that folate deficiency may increase the risk of cancer[4]. However, we failed to find the association between folate levels and the degree of infiltration, lymph node metastasis, tumor size, or clinical stage of gastric cancer. Possibly folate levels in mucosal tissue influence the initiation but not the progression of gastric cancer.

Transcriptional silencing of tumor suppressor genes by DNA hypermethylation and over-expression of proto-oncogenes by DNA hypomethylation play crucial roles in the progression of gastric cancer. Many genes involved in the regulation of cell cycle, tissue invasion, DNA repair and apoptosis have been shown to be inactivated by this type of epigenetic mechanism. The loss of $p16^{INK4A}$ expression, and hypermethylation in the promoter region is the mechanism of loss of $p21^{WAF1}$, and hypermethylation of the *hMLH1* gene promoter has been associated with a transcriptional blockade. The fact that the blockade is reversible with demethylation suggests that an epigenetic mechanism underlies h*MLH1* gene inactivation and MMR genes deficiency[19]. However, the role of DNA methylation in the loss of *hMSH2* expression has been controversial[20,21]. Aberrant methylation of c-myc can induce over-expression of the gene, and participate in the development of tumors. Hypomethylation of c-myc has been detected in gastric carcinogenesis[22], and it has been reported that folate[23] and MTHFR gene polymorphisms[24] are associated with aberrant methylation of some tumor-related genes.

**Table 5 MTHFR polymorphism and biological characters of patients with gastric cancer (*n*)**

| Biological character | | 677CC | 677CT | 677TT | 1298AA | 1298AC |
|---|---|---|---|---|---|---|
| Degree of infiltration | Muscularis mucosae | 2 | 2 | 1 | 3 | 2 |
| | Muscular layer | 1 | 3 | 2 | 5 | 1 |
| | Serosa | 7 | 9 | 1 | 12 | 5 |
| | Out-serosa | 4 | 5 | 1 | 6 | 4 |
| Lymph node metastasis | Yes | 7 | 11 | 2 | 14 | 6 |
| | No | 7 | 8 | 3 | 12 | 6 |
| Tumor size | < 5 cm | 9 | 7 | 2 | 11 | 7 |
| | ⩾ 5 cm | 5 | 12 | 3 | 15 | 5 |
| TNM classification | Ⅰ | 2 | 2 | 1 | 3 | 2 |
| | Ⅱ | 7 | 12 | 2 | 16 | 5 |
| | Ⅲ | 5 | 5 | 2 | 7 | 5 |

Miao *et al*[25] revealed that the 677TT genotype is associated with an increased risk of gastric cardia cancer. However, our data showed that MTHFR polymorphisms may not be an independent factor affecting initiation and progression of gastric cancers, including antrum and corpus cancers. In addition, due to the limit of sample size, we could not find a significant association between the MTHFR polymorphisms [677 (C→T) and 1298 (A→C)] and the degree of infiltration, lymph node metastasis, tumor size, or clinical stage of gastric cancer. Folate levels but not MTHFR polymorphisms affect the methylation and expression of proto-oncogene or tumor suppressor genes related to human gastric carcinogenesis, although it is unclear how low folate levels lead to c-myc hypomethylation and its down-regulated expression.

In summary, a folate level reduction was observed in gastric cancer tissues. This change, but not methylenetetr ahydrofolate reductase polymorphisms, is associated with upregulation of c-myc expression and hypomethylation of its promoter region.

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## COMMENTS

### Background

Multiple genetic and epigenetic alterations are involved in gastric carcinogenesis. Folate deficiency is thought to be carcinogenic through disruption of DNA methylation and synthesis and impaired DNA repair. However, folate requires metabolic transformations catalyzed by several enzymes including MTHFR, which is highly polymorphic.

### Research frontiers

The present study discussed the relationship between folate concentrations in mucosal tissues, MTHFR gene polymorphisms and expression of tumor-related genes, as well as DNA methylation in human gastric carcinogenesis.

### Innovations and breakthroughs

Gastric cancer originates from epithelial cells, therefore, a study performed in mucosal tissues is more accurate and effective than those performed in blood. The extent to which folate tissue levels and MTHFR polymorphisms interact to affect DNA methylation in gastric carcinogenesis is uncertain.

### Applications

The present study investigated the etiology of gastric carcinogenesis, which may provide the evidence for prevention and treatment of gastric cancer.

### Terminology

Methylenetetrahydrofolate reductase (MTHFR) is a key enzyme in folate metabolism that regulates the intracellular folate pool. The MTHFR gene is highly polymorphic, of which the two most common variants are located at nucleotides 677 (C→T) and 1298 (A→T).

### Peer review

The authors evaluated whether folate level in mucosal tissue and some common MTHFR variants are associated with the risk of gastric cancer through an effect on DNA methylation. The figures are clear. However, the title and conclusion need to be more concise and exact.

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*GASTRIC CANCER*

# Microvessel density is a prognostic marker of human gastric cancer

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## Abstract

**AIM:** To investigate whether microvessel density (MVD) is related with prognosis in gastric cancer patients, and the expression of cyclooxygenase-2 (COX-2) and vessel endothelial growth factor (VEGF) so as to determine the possible role of COX-2 and VEGF in gastric cancer angiogenesis.

**METHODS:** Forty-seven formalin-fixed paraffin-embedded tissue samples of gastric cancer were evaluated for COX-2, VEGF by immunohistochemical staining. To assess tumor angiogenesis, MVD was determined by immunohistochemical staining of endothelial protein factor Ⅷ-related antigen. The relationship among COX-2 and VEGF expression, MVD, and clinicopathologic parameters was analyzed.

**RESULTS:** Among the 67 samples, high MVD was significantly associated with lymph node metastasis and poor survival. Multivariate survival analysis showed that MVD value and lymph node metastasis were independent prognostic factors. The expression rate of COX-2 and VEGF was significantly higher than that of the adjacent tissues. COX-2 and VEGF expression in gastric cancer was significantly correlated with tumor differentiation and depth of invasion, but not with survival. The mean MVD value of COX-2 or VEGF positive tumors was higher than that of COX-2 or VEGF negative tumors. A significant correlation was found between the expressions of COX-2 and VEGF.

**CONCLUSION:** MVD may be one of the important prognostic factors for gastric cancer patients. COX-2 and VEGF may play an important role in tumor progression by stimulating angiogenesis. VEGF might play a main role in the COX-2 angiogenic pathway. The inhibition of angiogenesis or COX-2, VEGF activity may have an important therapeutic benefit in the control of gastric cancer.



**Key words:** Gastric cancer; Angiogenesis; Microvessel density; Vessel endothelial growth factor; Cyclooxygenase; Prognostic marker; Nonsteroidal anti inflammatory drug

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## INTRODUCTION

Gastric cancer is one of the most frequent and lethal malignancies worldwide, especially in Eastern Asia including China, and the 5-year survival rate is only about 20%[1]. A recent research has shown an increasing trend of gastric cancer mortality in China in the past 20 years, especially in rural areas and among aged people[2]. To date, the treatment outcome of this common malignancy is still not satisfactory. One major difficulty in the diagnosis and treatment of gastric cancer is that only a few prognostic indicators can predict its clinical behavior. Recently, angiogenesis has been related to metastasis and poor prognosis in gastric cancer.

Angiogenesis, the process leading to the formation of new blood vessels, plays a central role in cancer cell survival, local tumor growth, and development of distant metastasis[3-5]. The degree of intratumoral microvessel density (MVD) by immunohistochemistry is thought to influence tumor metastasis and consequently prognosis in various human cancers, including gastric cancer[6-10]. The formation of tumor microvessels is dependent on the production of angiogenic growth factors by tumor cells. The formation of tumor microvessels is stimulated

by angiogenic growth factors, including vessel endothelial growth factor (VEGF), platelet derived growth factor (PDGF), and transforming growth factor β1 (TGF-β1). The expression of these factors correlates with tumor angiogenesis, tumor progression and poor prognosis[11-13]. Among the known angiogenic factors, VEGF has emerged as the central regulator of the angiogenic process in cancer. The biological functions of VEGF include selective promotion of mitosis of endothelial cells, stimulation of their proliferation and angiogenesis, an increase in vessel transparency and extra-vasculization of large plasma molecules[14-17].

Cyclooxygenase (COX) is the rate-limiting enzyme in prostaglandin (PG) metabolism. COX has two isoforms: COX-1 and COX-2. COX-1 is constitutively expressed in most normal tissues and is thought to be involved in maintaining physiological function. COX-2 is frequently undetectable in normal tissue, but can be induced in response to growth factors, tumor promoters, hormones and cytokines, thus contributing to the synthesis of prostaglandin in inflamed and malignant tissues[18,19]. Over-expression of COX-2 is detectable in various solid malignancies including gastric cancer, and is thought to be involved in the critical steps in carcinogenesis, as well as a regulator of tumor angiogenesis[20-22]. However, the potential mechanism remains unclear.

To analyze the relationships between MVD, COX-2 and VEGF expression, clinicopathologic parameters and survival time of patients in gastric cancer, 67 specimens were evaluated for COX-2, VEGF and endothelial protein factor Ⅷ-related antigen by immunohistochemical staining of MVD.

## MATERIALS AND METHODS

### *Patients and specimens*

Sixty-seven patients (54 men and 13 women, medium age 56 years) with gastric cancer undergone radical gastrectomy in the Department of Surgery, the First Affiliated Hospital of Anhui Medical University, from October 1997 to October 2000, were enrolled in this study. The eligibility criteria were: histologically proven gastric adenicarcinoma, no previous systemic chemotherapy or radiotherapy before operation, and well documented clinical data. The mean follow-up time was 34 mo (from 16 d to 60 mo).

All tissues were surgically resected. Cancerous tissue and para-cancerous gastric mucosa were all from the same specimens. Each specimen was fixed in 10% phosphate-buffered formalin immediately after resection, embedded in paraffin and cut into 4 μm-thick sections for immunohistochemical study and routine histological examination.

### *Immunohistochemistry*

The sections were dewaxed and rehydrated by sequential immersion in xylene and graded ethanol and water. Endogenous peroxidase activity was blocked by incubation with 3% hydrogen peroxide methanol. Antigen-retrieval treatment was performed in a full pressure cooker for 10-15 min to obtain optimal results. After washed in phosphate-buffered saline (PBS) and exposed to 10% normal horse serum for 10 min to reduce non-specific binding, the sections were incubated with the primary antibody, which reacts specifically with VEGF (polyclonal, L2702, 1:50 dilution, overnight at 4℃; Santa Cruz Biotechnology, Inc.), COX-2 (polyclonal, J1602, 1:50 dilution, overnight at 4℃; Santa Cruz Biotechnology, Inc.), or factor Ⅷ-related antigen (polyclonal, ZA-0111, overnight at 4℃; Santa Cruz Biotechnology, Inc.). All the sections were incubated with biotinylated IgG for 30 min and then with streptoavidin-peroxidase regent for 20 min. Finally, the sections were incubated in PBS containing diaminobenzidine and 1% hydrogen peroxide for 5 min, counterstained with Mayer hematoxylin, and mounted. PBS was substituted for primary antibody as the negative control.

### *Evaluation of immunostaining and microvessel counting*

To evaluate COX-2 and VEGF expression, a score was established corresponding to the sum of a: percentage of positive cells (0 = 0% immunopositive cells, 1 = < 25% positive cells, 2 = 26%-50% positive cells, and 3 = > 50% positive cells), and b: staining intensity (0 = negative, 1 = weak, 2 = moderate, 3 = high). The sum of a + b reached a maximum score at 6. Scores between 0 and 2 were regarded as negative (-), between 3 and 4 as weak (+), and between 5 and 6 as strongly positive (++), respectively.

Intratumoral microvessels were highlighted by immunostaining with anti-factor Ⅷ related antigen polyclonal antibody. Any single brownly stained cells or cluster of endothelial cells clearly separated from adjacent microvessels, tumor cells, and other connective tissue elements were considered as vessels. Branching structures were counted as a single vessel unless there was a discontinuity in the structure. The stained sections were screened at 100-magnification under a light microscope to identify the 5 regions of the section with the highest vascular density. Vessels were counted in the 5 regions at 200-magnification, and the average number of microvessels was recorded. Two observers did the counting, and the mean value was used for analysis.

### *Statistical analysis*

Data were analyzed by SPSS version 10.0 for windows. The correlations between expression of COX-2, VEGF and clinic pathological parameters were assessed by the Chi-square test or the Spearman rank test. The Kaplan-Meier method was used to estimate survival as a function of time, and survival differences were analyzed by the log-rank test. The COX proportional hazard model was used for multivariate analysis of prognostic factors. $P < 0.05$ was considered statistically significant. All $P$ values are represented as two-sided.

## RESULTS

### *Correlation between MVD and clinicopathologic features*

The MVD for 67 tumor specimens ranged from 14 to 58 with a mean MVD of 28.46 ± 8.28. When a mean MVD value of 28 was chosen as the cut-off point

**Table 1 Correlation between MVD, COX-2 and VEGF expression and clinicopathologic parameters of gastric cancer**

| Clinico-pathologic features | *n* | MVD | | | VEGF | | | COX-2 | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Low MVD (*n* = 37) | High MVD (*n* = 30) | *P* | - (*n* = 16) | + ~ + + (*n* = 51) | *P* | - (*n* = 16) | + ~ + + (*n* = 51) | *P* |
| Gender | | | | 1.000 | | | 0.131 | | | 0.431 |
| Male | 54 | 30 | 24 | | 15 | 39 | | 14 | 40 | |
| Female | 13 | 7 | 6 | | 1 | 12 | | 2 | 11 | |
| Age (yr) | | | | 1.000 | | | 0.820 | | | 0.820 |
| < 55 | 31 | 17 | 14 | | 7 | 24 | | 7 | 24 | |
| ⩾ 55 | 36 | 20 | 16 | | 9 | 27 | | 9 | 27 | |
| Size of tumor (cm) | | | | 0.227 | | | 0.636 | | | 0.218 |
| < 5 | 30 | 14 | 16 | | 8 | 22 | | 5 | 25 | |
| ⩾ 5 | 37 | 23 | 14 | | 8 | 29 | | 11 | 26 | |
| Lymph node metastasis | | | | 0.003 | | | 0.528 | | | 0.231 |
| Yes | 33 | 12 | 21 | | 9 | 24 | | 10 | 23 | |
| No | 34 | 25 | 9 | | 7 | 27 | | 6 | 28 | |
| Depth of invasion | | | | 0.280 | | | 0.001 | | | 0.016 |
| Mucosa and submucosa | 9 | 3 | 6 | | 6 | 3 | | 5 | 4 | |
| Muscularis propria | 58 | 34 | 24 | | 10 | 48 | | 11 | 47 | |
| TNM stage | | | | 0.280 | | | 0.342 | | | 0.342 |
| Ⅰ and Ⅱ | 58 | 34 | 24 | | 15 | 43 | | 15 | 43 | |
| Ⅲ and Ⅳ | 9 | 3 | 6 | | 1 | 8 | | 1 | 8 | |

MVD: Microvessel density; VEGF: Vessel endothelial growth factor; COX-2: Cyclooxygenase-2; -: Negative; +~++: Positive to strong positive.

**Table 2 Multivariate analysis of overall survival in gastric cancer**

| Variable | Regression coefficient | Standard error (SE) | Odds ratio (95% CI) | *P* |
|---|---|---|---|---|
| Microvessel density (MVD) | 1.069 | 0.503 | 0.727-0.893 | 0.033 |
| Lymph node metastasis | 1.168 | 0.457 | 1.312-7.882 | 0.011 |

for discrimination of the 67 patients, 37 patients were categorized as low MVD and 30 as high MVD. The correlation between MVD and clinicopathologic features is shown in Table 1. High MVD was significantly associated with lymph node metastasis ($P = 0.003$).

### Multivariate survival analysis

Multivariate survival analysis showed that MVD value and lymph node metastasis were independent prognostic factors (Table 2). No other variables, including COX-2 and VEGF expression, were retained in the model or affected the magnitude of the hazard ratios of variables in the final model. Kaplan Meier curves for patients' survival are shown in Figure 1. A significant difference in the overall survival rate was found between patients according to the MVD value ($P < 0.001$, comparison between low and high MVD).

### Expression of COX-2 and VEGF in gastric cancer tissues

Immunoreactivity of both COX-2 and VEGF proteins was found in the tumor epithelial cells within cytoplasm (Figure 2). However, occasionally normal epithelial cells in adjacent tissues of cancer showed little staining. Among the 67 gastric cancer samples, the positive rates of COX-2 and VEGF expression were 76.1% and 76.1%, significantly higher than those in the adjacent tissues. The expression of VEGF protein in well-differentiated adenocarcinoma was significantly higher than that in

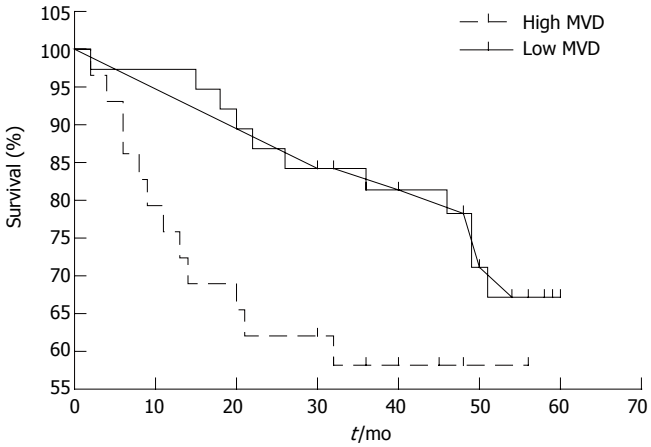


**Figure 1** Kaplan-Meier survival curve correlating disease specific survival with high microvessel density (MVD) or low MVD.

poorly-differentiated adenocarcinoma ($P < 0.05$). There was a statistical difference in the expression of COX-2 protein among well-, moderately- and poorly-differentiated adenocarcinomas ($P < 0.05$).

### Correlation between COX-2 or VEGF expression and MVD

The correlation between COX-2 or VEGF expression and MVD is summarized in Table 3. The mean MVD value of COX-2 or VEGF positive tumors was higher than that of COX-2 or VEGF negative tumors.

### Association between COX-2 and VEGF expression

A significant correlation was found between the expression levels of COX-2 and VEGF (Table 4, $r = 0.425$, $P < 0.001$).

### Correlations of COX-2, VEGF expression with clinicopathologic parameters

The associations between COX-2 and VEGF expressions

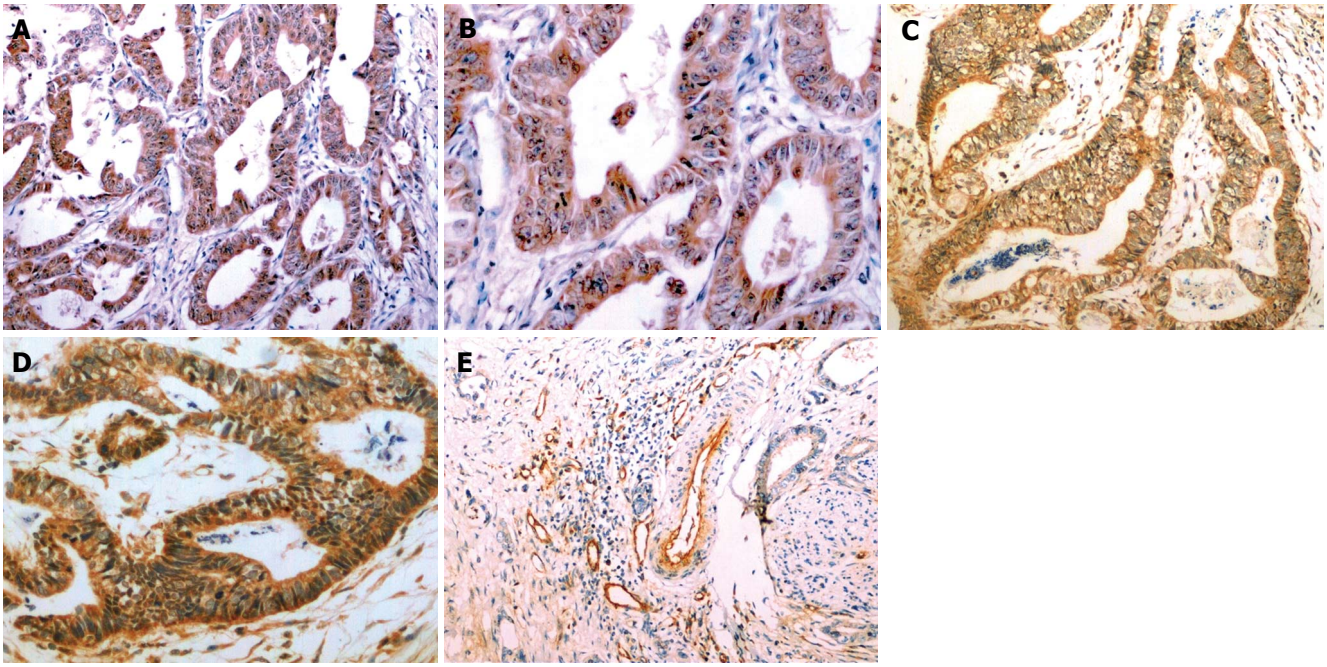


**Figure 2** Immunohistochemical stainings of COX-2 (**A**, **B**), VEGF (**C**, **D**) and microvessels (**E**) in tissue sections obtained from gastric adenocarcinoma. COX-2 was mainly expressed in the cytoplasm of cancer cells (brown staining; **A** × 100, **B** × 200). VEGF expression was restricted to the cytoplasm of cancer cells (brown staining; **C** × 100, **D** × 200). Microvessels were detected in gastric cancer tissues by immunostaining for factor Ⅷ-related antigen (**E** × 100).

**Table 3 Relationship between expressions of VEGF, COX-2 and MVD of gastric cancer (mean ± SD)**

| Group | *n* | MVD | *P* |
|---|---|---|---|
| VEGF | | | 0.008 |
| – | 16 | 20.14 ± 4.52 | |
| +~++ | 51 | 29.46 ± 8.28 | |
| COX-2 | | | 0.005 |
| – | 16 | 22.32 ± 3.80 | |
| +~++ | 51 | 29.88 ± 8.52 | |

VEGF: Vessel endothelial growth factor; COX-2: Cyclooxygenase-2; MVD: Microvessel density; -: Negative; +~++: Positive to strong positive.

**Table 4 Relationship between VEGF and COX-2 expression in gastric cancer**

| VEGF | COX-2 | | *r* | *P* |
|---|---|---|---|---|
| | – | + ~ + + | | |
| – | 9 | 7 | 0.425 | < 0.001 |
| +~++ | 7 | 44 | | |

VEGF: Vessel endothelial growth factor; COX-2: Cyclooxygenase-2; -: Negative; +~++: Positive to strong positive.

and the clinicopathologic parameters are shown in Table 1. The expression of both proteins in gastric cancer was significantly correlated with depth of invasion. There was no significant association between COX-2 and VEGF expression and patient gender, age, tumor size, lymph node metastasis, and TNM stage.

## DISCUSSION

Folkman J and Shing Y[3] initiated a new field of research about tumor angiogenesis in 1971 and found that several factors take part in the process of angiogenesis. Tumor angiogenesis is now believed to be one of the most crucial steps in tumor growth and metastases[3-5]. Moreover, tumor angiogenesis which can be quantified by measurement of MVD is a significant negative prognostic factor[6-10]. In our study, when a mean MVD value was chosen as the cut-off point for discrimination of the study patients, high MVD was significantly associated with lymph node metastasis and poor survival. Multivariate survival analysis showed that MVD value and lymph node metastasis were independent prognostic factors for gastric cancer patients.

Tumor angiogenesis is controlled by a balance between angiogenic and angiostatic regulators involved in multiple pathways that result in endothelial proliferation, differentiation and organization into a functional network of vascular channels[3-5]. Among the reported angiogenic factors[11-14], VEGF (a key factor for induction of tumor angiogenesis) is increased in various human tumors, often correlating with higher MVD[15-17]. In our study, VEGF was over-expressed in gastric cancer tissues. We found that VEGF expression was associated with the histologic types of gastric cancer and depth of invasion. The results suggest that VEGF might be mainly involved in the progression of gastric carcinoma. The mean MVD value of VEGF positive tumors was significantly higher than that of VEGF negative tumors, suggesting that VEGF may facilitate tumor progression by promoting tumor angiogenesis.

Epidemiologic studies indicate that use of aspirin and other non-steroidal anti inflammatory drugs (NSAIDs), with COX being their major target, decreases the incidence

and mortality of colorectal, gastric, and esophageal cancers[23-26]. The expression of COX-2 mRNA and protein is elevated in various human malignancies, which may play a critical role in the development of cancer[27-30]. Our study showed that the positive rate of COX-2 expression in human gastric cancers was significantly higher than that in the matched normal gastric tissue. COX-2 expression was associated with the degree of tumor cell differentiation and depth of invasion, but not with survival. These results suggest that over-expression of COX-2 plays an important role in the development of human gastric cancer, but cannot predicate the outcome in individual cases.

The contributions of COX-2 to tumor angiogenesis include: increasing expression of VEGF, producing of prostaglandin E (PGE) 2 and prostaglandin I (PGI) 2 that can directly stimulate endothelial cell migration and growth factor-induced angiogenesis, and inhibiting endothelial cell apoptosis by stimulation of Bcl-2 or Akt activation[31,32]. In our present study, COX-2 expression was significantly associated with that of VEGF. The mean MVD value of COX-2 or VEGF positive tumors was higher than that of COX-2 or VEGF negative tumors, which is in agreement with previous reports[33,34]. These data strongly suggest that COX-2 and VEGF may be partly responsible for the important process of angiogenesis in the development of human gastric cancer, and VEGF plays the main role in COX-2 stimulated angiogenesis. However, there still exist some other pathways, which also participate in COX-2-induced angiogenesis.

In conclusion, high MVD is significantly associated with lymph node metastasis and poor survival. MVD value and lymph node metastasis are independent prognostic factors for gastric cancer patients. Expression of COX-2 and VEGF is closely correlated to the depth of invasion, and leads to increased angiogenesis, which may be the mechanisms underlying the contribution of COX-2 to the development of gastric cancer. VEGF might play a main role in the COX-2 angiogenic pathway. Inhibition of angiogenesis or COX-2, VEGF activity may have an important therapeutic benefit in the control of gastric cancer.

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## COMMENTS

### Background

Gastric cancer is one of the most frequent and lethal malignancies worldwide, especially in Eastern Asia including China, and the 5-year survival rate is only about 20%. To date, the treatment outcome of this common malignancy is still not satisfactory. One major difficulty in the diagnosis and treatment of gastric cancer is that only a few prognostic indicators can predict its clinical behavior.

### Research frontiers

Tumor angiogenesis plays a central role in cancer cell survival, local tumor growth, and development of distant metastasis, which can be assessed by the MVD. The degree of intratumoral MVD is thought to influence tumor metastasis and consequently prognosis in various human cancers, including gastric cancer. Additionally, tumor MVD is associated with COX-2 and VEGF expression.

### Applications

The present work suggests that high MVD is significantly associated with lymph node metastasis and poor survival. MVD value and lymph node metastasis are two independent prognostic factors for gastric cancer patients. Expression of COX-2 and VEGF is closely correlated to the depth of invasion, and leads to increased angiogenesis, which may be the mechanisms underlying the development of gastric cancer. VEGF might play a main role in the COX-2 angiogenic pathway. Inhibition of angiogenesis or COX-2, VEGF activity may have an important therapeutic benefit in the control of gastric cancer.

### Peer review

This is a well-written and carefully performed study. The title, results and discussion are clear. The abstract and introduction are well-organized.

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*LIVER CANCER*

# New multi protein patterns differentiate liver fibrosis stages and hepatocellular carcinoma in chronic hepatitis C serum samples

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## Abstract

**AIM:** To identify a multi serum protein pattern as well as single protein markers using surface-enhanced laser desorption/ionisation time-of-flight mass spectrometry (SELDI-TOF-MS) for detection and differentiation of liver fibrosis (F1-F2), liver cirrhosis (F4) and hepatocellular carcinoma (HCC) in patients with chronic hepatitis C virus (HCV).

**METHODS:** Serum samples of 39 patients with F1/F2 fibrosis, 44 patients with F4 fibrosis, 34 patients with HCC were applied to CM10 arrays and analyzed using the SELDI-TOF ProteinChip System (PBS-Ⅱc; Ciphergen Biosystems) after anion-exchange fractionation. All patients had chronic hepatitis C and histologically confirmed fibrosis stage/HCC. Data were analyzed for protein patterns by multivariate statistical techniques and artificial neural networks.

**RESULTS:** A 4 peptide/protein multimarker panel (7486, 12843, 44293 and 53598 Da) correctly identified HCCs with a sensitivity of 100% and specificity of 85% in a two way-comparison of HCV-cirrhosis versus HCV-HCC training samples (AUROC 0.943). Sensitivity and specificity for identification of HCC were 68% and 80% for random test samples. Cirrhotic patients could be discriminated against patients with F1 or F2 fibrosis using a 5 peptide/protein multimarker pattern (2873, 6646, 7775, 10525 and 67867 Da) with a specificity of 100% and a sensitivity of 85% in training samples (AUROC 0.976) and a sensitivity and specificity of 80% and 67% for random test samples. Combination of the biomarker classifiers with APRI score and alfa-fetopotein (AFP) improved the diagnostic performance. The 6646 Da marker protein for liver fibrosis was identified as apolipoprotein C-Ⅰ.

**CONCLUSION:** SELDI-TOF-MS technology combined with protein pattern analysis seems a valuable approach for the identification of liver cirrhosis and hepatocellular carcinoma in patients with chronic hepatitis C. Most probably a combination of different serum markers will help to identify liver cirrhosis and early-stage hepatocellular carcinomas in the future.



**Key words:** Hepatocellular carcinoma; Hepatitis C virus; Apolipoprotein C-Ⅰ; Proteomics; Surface-enhanced laser desorption/ionisation

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## INTRODUCTION

Hepatocellular carcinoma (HCC) is the most common primary liver cancer and ranks fifth in frequency of all malignancies in the world[1]. In the last decades incidence and mortality rates of HCC have increased significantly in Western countries[2] with hepatitis C virus (HCV) infection being the most important cause[3]. Liver cirrhosis is the most common complication of chronic HCV infection. Once cirrhosis is established, the risk of developing a HCC is 1%-5% a year[4]. In order to reduce HCC mortality, early identification of liver cirrhosis as well as early detection of hepatocellular carcinoma is needed.

However, prediction of liver cirrhosis and small HCC is often difficult in chronic hepatitis C. Several noninvasive tests like transient elastography (FibroScan®, Echosens, Paris, France), Fibrotest™ (Biopredictive, Paris, France; a serologic marker-based algorithm) and the APRI score (relation of AST to thrombocytes)[5] have been set up for discrimination of liver cirrhosis from lower fibrosis stages. In contrast to liver biopsy, these techniques are noninvasive, simple to use but either need further

evaluation or display insufficient predictive value [6].

Screening for HCC is generally recommended in patients with HCV cirrhosis[7]. Determination of alfa-fetopotein (AFP) and ultrasound every 3 to 6 mo has been proposed for early detection of HCC[7]. However, up to 44% of patients with HCC show normal levels of AFP, particularly during early stages[8]. Elevated serum levels of AFP may also be seen in patients with liver cirrhosis, acute and chronic hepatitis[9]. Low sensitivities of 39%-65% and positive predictive values of 9%-50% limit the use of AFP as a single marker for a cut off of 20 ng/mL[10]. Combination of AFP (cut-off level of 20 μg/L) with abdominal ultrasound improves sensitivity and specificity, but results of ultrasound scanning are influenced by the experience of the operator and the quality of the used equipment. Detection of HCC becomes even more difficult in cirrhotic livers since regenerative nodules may mimic tumors. Sensitivities of ultrasound as a screening tool for the detection of HCC vary from 50% up to 78%[11]. Contrast-enhanced ultrasound may improve sensitivity up to 89%, but specificity still remains rather low[12].

Surface-enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF MS) is one technology for serum protein profiling and identification of biomarkers. This method uses different chromatographic chip surfaces for binding peptides/proteins of biological samples (i.e. serum, urine, cerebrospinal fluid) and has lead to identification of novel biomarkers in prostate, bladder, ovarian, colorectal, or liver cancer[13-17].

The aim of the study was to identify serum protein patterns and single protein markers by SELDI-TOF mass spectrometry in patients with HCV cirrhosis and HCV associated hepatocellular carcinoma.

## MATERIALS AND METHODS

### Samples

A total number of 117 serum samples were collected from patients with chronic hepatitis C between 2001 and 2004. Three groups were enrolled: (1) patients with low grade fibrosis (F1/2, $n$ = 39), (2) patients with cirrhosis but without hepatocellular carcinoma ($n$ = 44) and (3) patients with cirrhosis and HCC ($n$ = 34). All patients were anti-HCV and HCV RNA (bDNA Assay 3.0, Bayer, Leverkusen) positive. Patients with other liver diseases, HIV co-infections, other malignomas or antiviral treatment were excluded. Liver biopsy was available in all patients. Clinical data of the patients are shown in Table 1. Classification of the fibrosis stage was done according to Scheuer *et al*[18] . Blood samples were stored at -80°. No sample had been thawed more than once.

### Protein profiling

Anion-exchange fractionation was used for serum preprocessing, to increase the number of protein peaks. To separate the serum samples into six different fractions (pH9 + flowthrough, pH7, pH5, pH4, pH3, organic elution), an Expression Difference Mapping Kit (Ciphergen Biosystems, Freemont, USA.) was used. Proteins were separated on the basis of their pI-values. Sample preparation and fractionation was done as described in the manufacturer's protocol. Fractions were stored at 4℃ overnight and analyzed the following day. Protein profiling was done using SELDI-TOF mass spectrometry (Ciphergen Biosystems). After testing several chromatographic chip surfaces and binding conditions the CM10 array were chosen for further experiments as they gave the highest number of discriminative peptide/protein peaks. The best results in terms of number of protein peaks and possibility to separate the three groups was found in fraction 1 (flow through + pH9).

**Table 1 Clinical data of patients**

| | Fibrosis F1/F2 ($n$ = 39) | Cirrhosis F4 ($n$ = 44) | HCC ($n$ = 34) |
|---|---|---|---|
| Age | 44 ± 11 | 62 ± 8 | 67 ± 8 |
| Gender (male/female) | 29/10 | 22/22 | 25/9 |
| ALT | 57 ± 100 | 62 ± 43 | 55 ± 47 |
| AST | 28 ± 30 | 46 ± 30 | 52 ± 34 |
| Bilirubin (mg/dL) | 0.8 ± 0.5 | 1.2 ± 1.0 | 2.0 ± 4.0 |
| Quick (%) | 105 ± 7 | 85 ± 16 | 85 ± 17 |
| AFP (μg/mL)[1] | 3.4 | 6.2 | 39.95 |
| Platelets (1000/μL) | 214 ± 61 | 131 ± 62 | 145 ± 73 |
| Albumin (g/dL) | 4.5 ± 0.3 | 3.9 ± 0.6 | 3.5 ± 0.6 |
| Child Pugh (A/B/C) (%) | NA | 35/7/2<br>80/16/4 | 20/11/3<br>59/32/9 |
| Okuda | NA | NA | 18/2/4 |

ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; AFP: Alfa-fetoprotein; NA: Not available. [1]Median values are given.

The CM10 arrays were equilibrated twice with 150 μL of low stringency binding buffer (0.1 mol/L sodium acetate; pH4.0; Ciphergen Biosystems). Ten microliters of fraction 1 and 90 μL of binding buffer were applied to each spot of the ProteinChip Array and incubated for 30 min at room temperature. Spots were washed three times with binding buffer and rinsed twice with de-ionized water. Arrays were allowed to air dry and 1 μL of sinapinic acid in 50% acetonitrile (ACN) and 0.5% trifluoroacetic acid (TFA) were applied twice to each spot. Afterwards the protein chip arrays were analyzed using the SELDI ProteinChip Reader (PBS-Ⅱc; Ciphergen Biosystems).

### Purification and identification of candidate biomarker

Anion Exchange Fractionation Selected sera (100 μL) containing biomarkers of interest were further fractionated with Q HyperD® F spin columns (Ciphergen Biosystems, Inc.). Each fraction was collected and analyzed again on a CM10 array with low stringency buffer. The candidate biomarkers were seen in the flow through and pH3 fraction. Both fractions were purified first with hydrophobic chromatography resin (BioSepra Q HyperD® F resins (BioSepra, Sergy Saint Christophe, France) then with RPC Poly-Bio beads (BioSepra).

### Hydrophobic chromatography

Fifty microliters of RPC Poly-Bio beads (BioSepra) were equilibrated with 500 μL of 10% ACN/0.1% TFA. The full sample fraction was adjusted to a final concentration of 10% ACN/0.5% TFA and mixed with 50 μL of the

bead-material for 30 min at room temperature. Afterwards, the probe was centrifuged for 1 min at 5000 r/min. The supernatant was removed. Increasing concentrations of ACN (10%-60%) in 0.1% TFA were added in each step, mixed for 5 min and centrifuged for 1 min at 5000 rpm. Proteins in the eluted fractions were detected by profiling 1 μL of each fraction combined with 1 μL of SPA on a NP20 array.

### Gel electrophoresis and passive elution

Fractions containing the candidate biomarkers eluted from the hydrophobic bead material were completely dried in a Speed-Vac, resolubilised in SDS-PAGE sample buffer and loaded onto different SDS-PAGE gels (Invitrogen, Carlsbad, USA). An 18% Tris-Glycine gel was used containing TrisGly SDS running buffer for the 6.6 kDa-marker. The gel was fixed and stained using Invitrogen Staining NuPage® Novex protocols. Bands of interest were excised from the gels and placed in tubes. Destaining was achieved by addition of 150 μL 50% ACN/50 mmol/L ammonium bicarbonate (3 times, 5 min at room temperature) and 200 μL of 50% ACN/100 mmol/L ammonium bicarbonate once. The gel pieces were then covered with 100 μL of 100% ACN for 10 min and dried in a Speed Vac.

For passive elution 100 μL of 45% formic acid, 30% ACN and 10% isopropanol was added. The tubes were sonicated for 30 min in a water bath at room temperature and incubated at room temperature for 4 h. One microliter of each sample was analysed on a NP20 array. Remainder of passive elution was incubated overnight and sonicated the next morning. Each supernatant was transferred to a new tube and dried in a Speed-Vac.

Sequencing grade modified porcine trypsin (Promega, Charbonnières-les-bains, France) with a concentration of 20 ng/μL in 25 mmol/L ammonium bicarbonate was added to each gel piece and incubated at 37℃ for 4 h. One microliter of the peptide digests was analysed on a NP20 array adding 1 μL of 20% CHCA (alpha-cyano-4-hydroxy-cinnamic acid) in 50% ACN/0.5% TFA. External calibration was performed using the All-In-One-Peptide Standard (Ciphergen Biosystems, Inc.). Peptides of the resulting tryptic digest were submitted to a database search with the Mascot search engine (http://www.matrixscience.com) using the Swiss-Prot and NCBI databases.

Additionally sequencing of the most important peptides in the tryptic digest was done using a Micromass Q quattro 2Q-TOF tandem quadruple TOF mass spectrometer equipped with a SELDI-TOF MS ProteinChip Interface PCI1000. The MS/MS data were exported as Sequest files and investigated with the Mascot search engine. Determination of Apo C- Ⅰ was furthermore confirmed by an immunoassay on protein A beads (Biosepra) using a specific rabbit anti-human apolipoprotein C- Ⅰ antibody (Academy Bio-Medical Company, Cambridge, UK) and a non-specific rabbit IgG control antibody. After loading of the beads with the antibody, subsequent washing with PBS (2 ×), followed by an incubation for 1 h with the flow through fraction of one serum sample, the beads were washed again with PBS (3 ×) and deionized water (1 ×). Finally, the captured proteins were eluted with 15 μL 100 mmol/L acetic acid and profiled on NP20 ProteinChip Arrays.

### Statistical analysis

Data were analyzed using the ProteinChip Software package version 3.1 (Ciphergen Biosystems). For acquiring best results, two different protocols were established. The optimization range was set between 2.5 and 50 kDa for the first, 10 and 80 kDa for the second protocol. Mass spectra were generated using laser intensities of 167 and 190, detector sensitivities of 6 and 9, respectively. A total of 130 laser shots for each spot were collected. The protein masses were calibrated externally using the All-In-One Protein Standard (Ciphergen Biosystems.). All mass spectra were normalized to total ion current (TIC normalization), baseline was subtracted.

Protein peaks of all sample spectra were clustered with the Ciphergen Express software, version 3.0.1. The following clustering settings were used: auto-detect peaks to cluster; first pass 5.0 S/N (signal to noise) 3.0 valley depth; minimal peak threshold: 20% of all spectra. Cluster mass window: 0.2 peak width; second pass: 3.0 S/N; 3.0 valley depth; add estimated peaks to complete cluster; mass to charge ratio (M/z) range from 2.5 to 75 kDa. This cluster list was used to perform a decision tree classification with the Biomarker Patterns software, version 5.0.2 (Ciphergen Biosystems.). Initially, a training set of all serum samples was used for generating a decision tree model. The sample set was in addition taken for internal cross-validation (test set). The classification tree split the data into two nodes using one rule at a time in the form of peak intensity. The splitting decisions in this case were based on the normalized intensity levels of peaks from SELDI protein expression profiles. The process of splitting was continued until terminal nodes were created.

## RESULTS

### Biomarker pattern

The comparison between low fibrotic and cirrhotic patients revealed 22 significant peaks. For the differentiation of cirrhotic and HCC-patients 17 significant protein peaks with *P*-values less than 0.05 were found.

For differentiation between low fibrosis and cirrhosis a decision tree using 5 biomarkers with mass values of 2873, 6646, 7775, 10525 and 67867 Da was established (Figure 1). The algorithm correctly assigned 33 of 39 fibrotic (85%) and 44 of 44 (100%) cirrhotic patients in the training set. The ROC analysis gave an AUROC of 0.976. The algorithm for discrimination of cirrhosis versus low fibrosis based on the test data gave a sensitivity of 80%, a specificity of 67%, a positive predictive value (ppv) of 73% and a negative predictive value (npv) of 74% for liver cirrhosis. APRI score (cut-off 1.5) allowed determination of cirrhosis with a sensitivity of 75%, a specificity of 87%, a ppv of 86% and a npv of 76%. The combination of APRI-score with three biomarkers (2873, 6646 and 10525 kDa) resulted in a sensitivity of 93%, a specificity of 95%, a ppv of 95% and a npv of 93%. AUROC increased to 0.955 for the combination of APRI and biomarkers

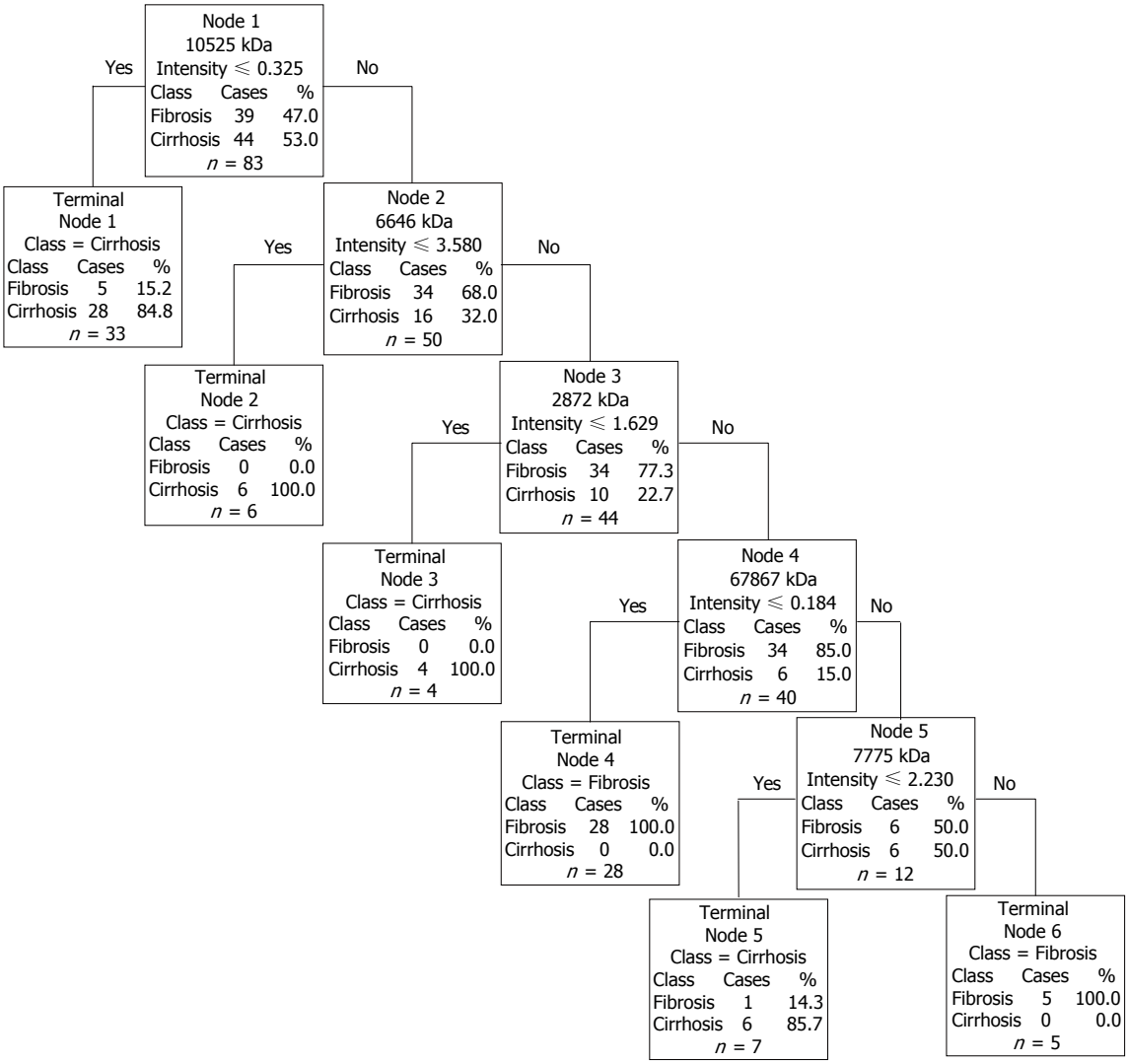



**Figure 1** Decision tree for the differentiation of fibrosis versus cirrhosis**.** The root nodes contain the mass of the selected peak ("node") which is followed by the intensity value. Samples with intensities lower or equal to the intensity value go to the left terminal node, samples with higher intensities go to the next right descendant nodes.

compared APRI alone with an AUROC of 0.811.

Cirrhotic patients could be differentiated from HCC patients by creating a decision tree with 4 biomarkers with mean mass values of 7486, 12843, 44293 and 53598 Da (Figure 2). The training set allowed a correct classification of HCC in 34 of 34 (100%) and cirrhosis in 37 of 44 (84%) patients. The AUROC in the ROC plot was 0.943. The algorithm for discrimination of HCC versus cirrhosis in the test set revealed a sensitivity of 68% for HCC, a specificity of 80%, a ppv of 72% and a npv of 76%. AFP alone (cut-off 18 μg/L) achieved a sensitivity of 76%, a specificity of 82%, ppv of 76% and a npv of 82%. The AUROC was 0.791. Combination of AFP and the 12843 Da biomarker resulted in a sensitivity of 88%, a specificity of 82%, a ppv of 78% and a npv of 90%. The AUROC mounted to 0.861.

### *Identification of novel biomarkers*

A biomarker of 6.6 kDa, used in the first decision tree, could be further characterized. Representative spectra views of the chosen protein are shown in Figure 3. SELDI analysis results of one sample expressing the 6.6 kDa marker after fractionation, purification and overnight passive elution as well as the separation within the stained gel are shown in Figure 4. The peptide mass fingerprinting of the 6.6 kDa biomarker after trypsin digestion is shown in Figure 5. By database search the tryptic digested 6.6 kDa protein was identified as apolipoprotein C-Ⅰ. Apolipoprotein C-Ⅰ was down-regulated in patients with liver cirrhosis (Figure 3) compared to patients with lower stages of liver fibrosis. Confirmation of the apolipoprotein C-Ⅰ identity was performed by an immunoassay using a rabbit apolipoprotein C-Ⅰ polyclonal antibody (Figure 6). The AUROC of apolipoprotein C-Ⅰ for differentiation of fibrosis from cirrhosis was 0.68.

## DISCUSSION

Despite an increasing number of noninvasive tests and imaging techniques, detection of liver cirrhosis and hepatocellular cancer is often difficult in chronic hepatitis C patients. The present study therefore aimed at the identification of serum protein patterns and single protein markers by SELDI-TOF mass spectrometry to predict liver cirrhosis and hepatocellular carcinoma.

Using a weak cationic array (CM10) a 5 biomarker

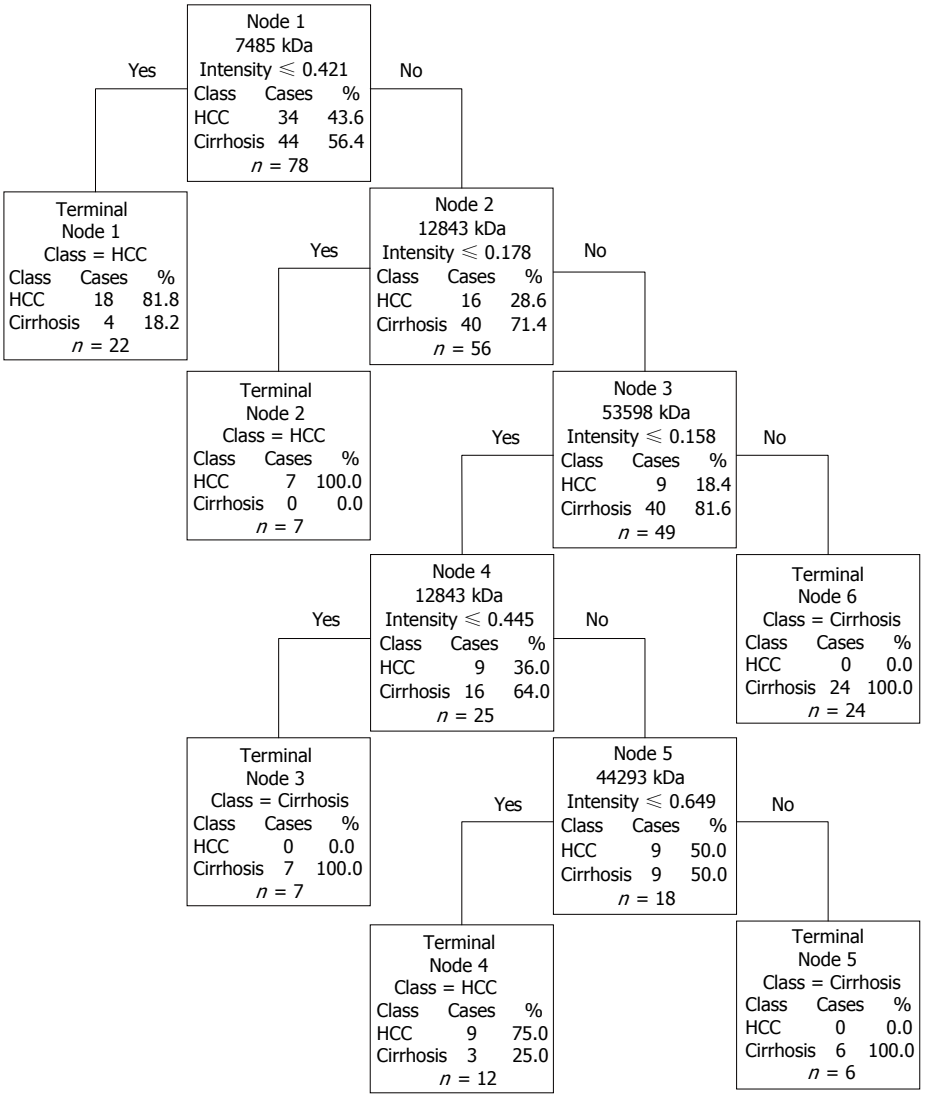



**Figure 2** Decision tree for the differentiation of HCC versus cirrhosis.

pattern was identified, that allowed discrimination of liver cirrhosis from low fibrosis stages with a sensitivity of 80%, a specificity of 67% and a positive predictive value of 73%. APRI score in patients with hepatitis C gave, depending on the chosen cut-off, sensitivities of 57%-89%, specificities of 75%-93% and a ppv of 38%-57% for the identification of patients with cirrhosis compared to non-cirrhotics[5]. FibroTest is another non-invasive test, using a mathematical algorithm of 5 parameters (α2-macroglobulin, haptoglobin, γ-glutamyl transpeptidase, apolipoprotein A-Ⅰ, total bilirubin), to predict liver cirrhosis. Sensitivities and specificities vary significantly dependent on the chosen cut-off[19]. Fibroscan®, which determines liver stiffness by transient elastography, achieves a sensitivity of 87%, a specificity of 91% and a ppv of 77% using 12.5 kPa as the cutoff[6]. Correct prediction of an underlying HCC was possible from the same sample preparation using a 4 biomarker pattern with a sensitivity of 68%, a specificity of 80% and a ppv of 72% in patients with proven liver cirrhosis. Alfa-fetoprotein, which is the most widely used serum marker for diagnosis and surveillance of HCC, achieves sensitivities of 39%-65% and ppv of 9%-50%[7,10,20] according to literature but performed better in the present data set. Although proteomic patterns might not be superior to the mentioned noninvasive tests, they carry the advantage of being operator independent (compared to transient elastography) and allow both the discrimination of liver cirrhosis and hepatocellular carcinoma with one sample preparation.

Previous studies using SELDI-TOF mass spectrometry including only patients with hepatitis C liver cirrhosis have provided evidence that proteomic pattern can be used to discriminate cirrhosis from HCC yielding sensitivities of 85% to 94% and specificities of 86% to 91%[17,21]. The differences to the present study may be explained by the use of a different protein array (CM10 *vs* IMAC) and data analysis with different neuronal networks. A relative complex neuronal network was elaborated by Ward *et al*[21], using a majority vote of six committee models, which included between 4 and 17 protein peaks. Interpretation of the present results with the results of other studies testing proteomic patterns in patients with HCC were hampered by the fact that different HCC etiologies[22] or non-relevant controls like healthy volunteers were included in the other studies[23].

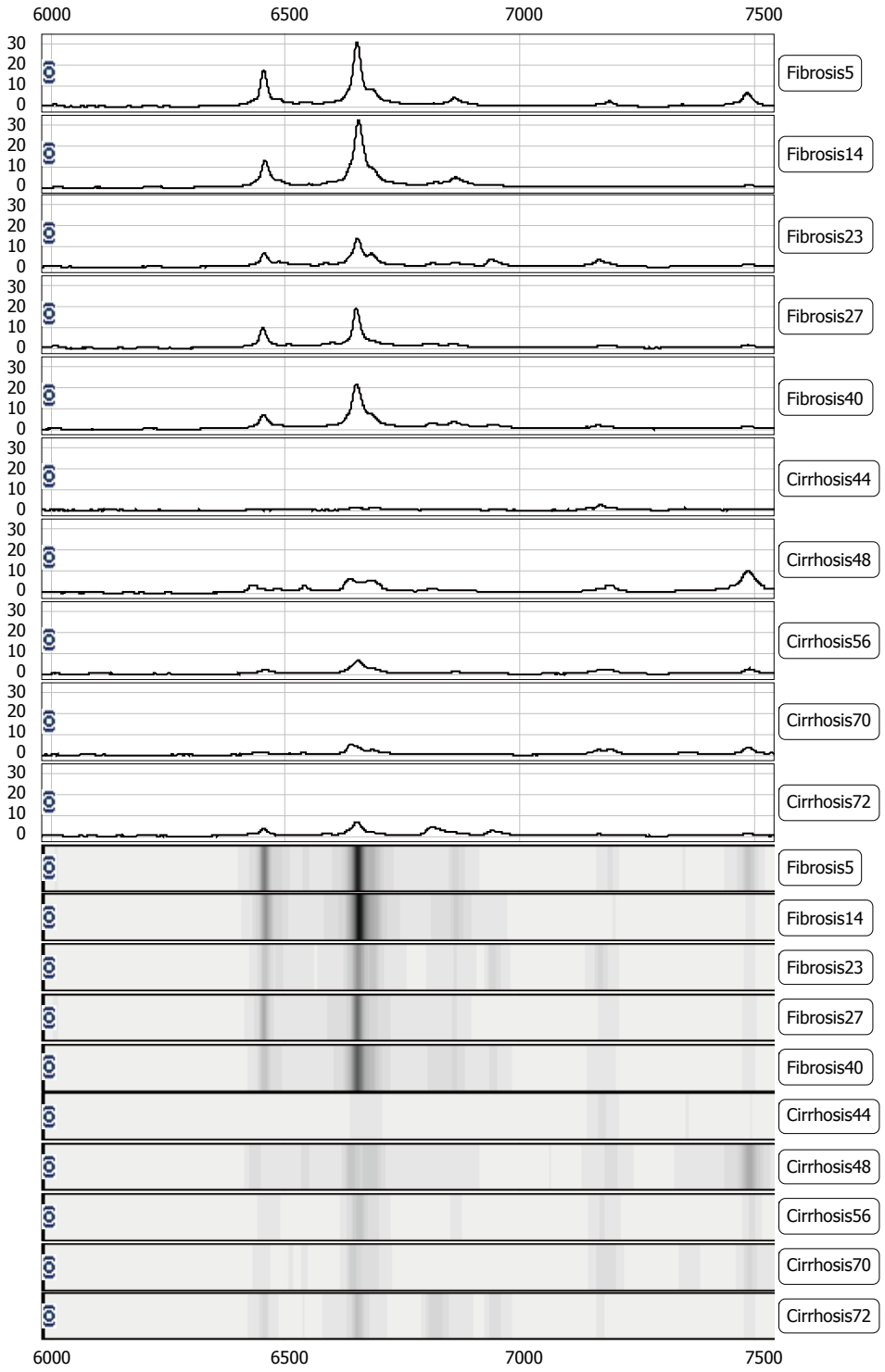



**Figure 3** Representative protein spectra of the 6.6 kDa peak and gel view (below) for the differentiation of fibrosis versus cirrhosis patients. Numbering of patients is according to internally used data.

Apolipoprotein C-Ⅰ was identified as a marker for differentiation of liver fibrosis and cirrhosis. A role for apolipoproteins in liver fibrosis has been recognized earlier. Apolipoprotein A-Ⅰ is one of the parameters used for prediction of liver fibrosis within the Fibrotest-algorithm, in addition an index of prothrombin time γ-glutamyl transpeptidase and apolipoprotein A-Ⅰ has been proposed for identification of severe alcoholic liver disease[24]. However, apolipoproteins have not only been identified as a serum discriminator of fibrosis but also as a marker in different types of cancer[25]. Apolipoprotein C-Ⅰ down-regulation was detected to reliably distinguish colorectal cancer patients from healthy controls[26].

Apolipoprotein C-Ⅰ is primarily synthesized in the liver and only to a lesser degree in the small intestine. It is originally formed as a pro-peptide of 9.3 kDa which generates the mature protein upon cleavage during translation[27]. The observed decrease in serum levels of apolipoprotein C-Ⅰ in cirrhosis and HCC might be due to decreased synthesis rate or due to degradation by activated

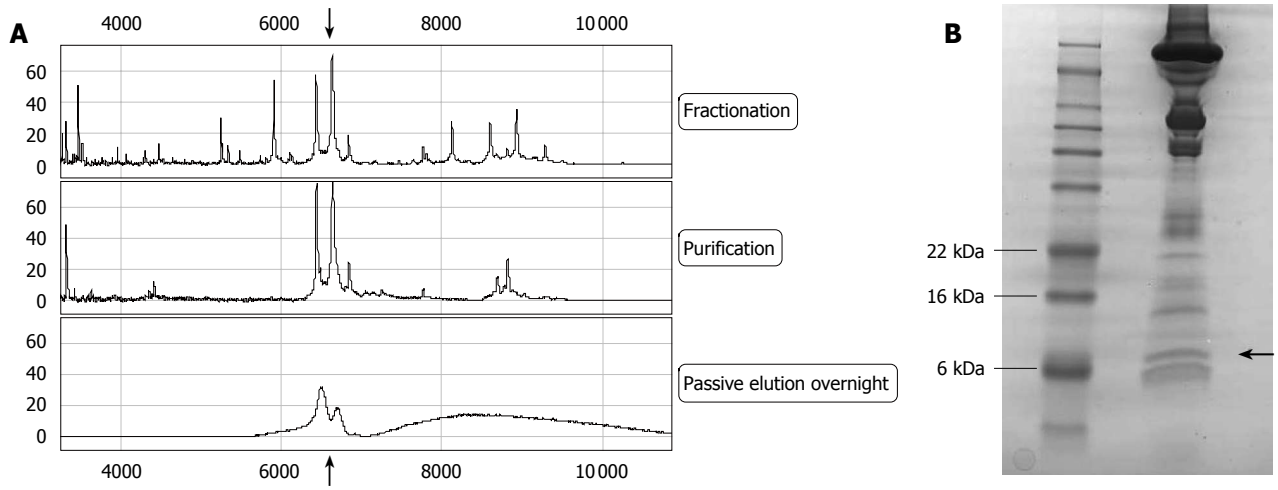
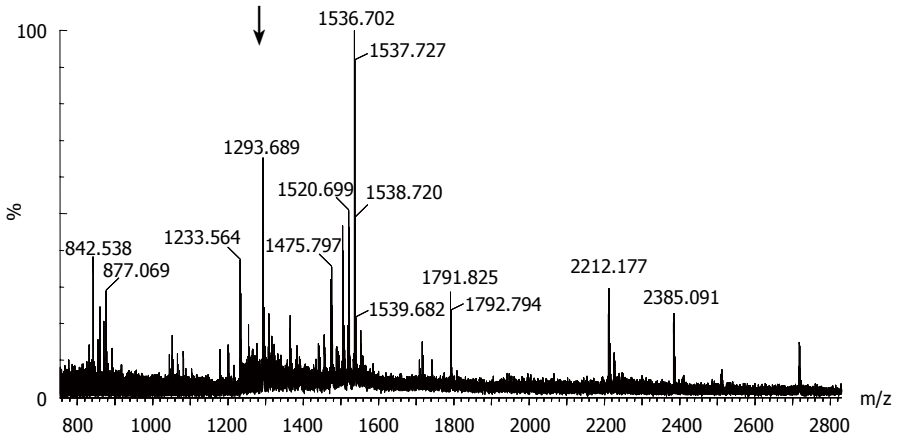



**Figure 4** Isolation of the 6.6 kDa peak. First line shows spectrum after fractionation of the sample with Q Ceramic HyperD®F spin columns, the second line shows the spectra after purification using reverse phase chromatography. The last line spectra represent the purified biomarker on a NP20 array after SDS-gel purification and passive elution. The arrow indicates the 6.6 kDa peak (**A**); One-dimensional SDS polyacrylamide gel electrophoresis after staining of enriched sample (second lane). First lane: molecular marker proteins (See Blue® Plus2, Invitrogen, Carlsbad, USA). The arrow indicates the 6.6 kDa protein (**B**).




Mascot mapping result of marked peptide: Apolipoprotein C- I

| Start-End | Observed | Mr (expct) | Mr (calc) | Delta | Score | Sequence |
|---|---|---|---|---|---|---|
| 37-47 | 1293.68 | 1292.67 | 1292.66 | 0.01 | 86 | LKEFGNTLEDK |

**Figure 5** Peptide mass fingerprint spectra of the 6.6 kDa biomarker after trypsin digestion using a Micomass Q quattro 2Q-TOF tandem quadruple TOF mass spectrometer equipped with a SELDI-TOF MS ProteinChip Interface PCI1000. The arrow indicates the peptide with the highest score in the database search. Mascot search results of the same peptide are shown below including start and end position of the found peptide sequence, the observed, experimental and calculated mass from the matched peptide sequence, the mass difference (delta), achieved ion score and the peptide sequence.

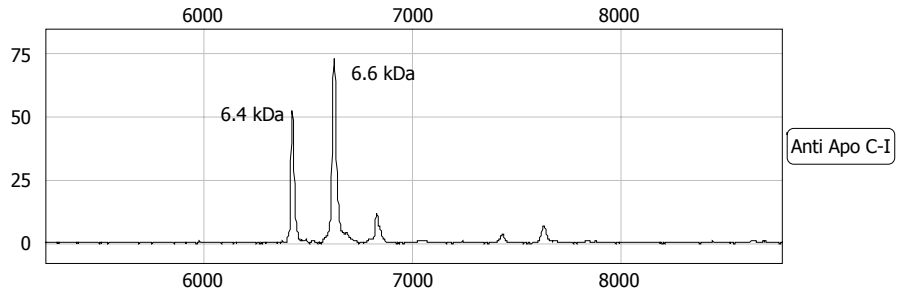



**Figure 6** MS-spectrum of the immunoassay capture using a rabbit anti-human apolipoprotein C-I antibody on a NP20 array. Same peaks as seen in the passive elution are shown. The 6.6 kDa represents Apo C-I, the 6.4 kDa peak is Apolipoprotein C-I missing two N-terminal amino acids.

proteases. However, it has been previously demonstrated that multiple cellular genes involved in lipid metabolism are differentially regulated in chronic hepatitis C[28]. There is considerable evidence that cholesterol and fatty-acid-biosynthesis pathways play a role in HCV replication and infection. Liver steatosis is a typical feature of HCV infection[29], HCV core and NS5A proteins associated with lipid droplets and apolipoproteins A-Ⅰ and A-Ⅱ[30,31]. Interference with lipoprotein metabolism by the use of a HMG-CoA inhibitor pravasin, has been shown to improve survival in patients with hepatitis C associated hepatocellular carcinoma[32] and lovastatin inhibited HCV replication in the replicon model[33]. Thus, besides the function of apolipoprotein C-Ⅰ in lipid metabolism[34] an additional pathogenic role in liver fibrosis and cancerogenesis appears possible.

A number of other protein markers have been identified by mass spectrometry or 2D-gel electrophoresis

in HCV associated hepatocellular carcinoma. Among these are ferritin light chain[35-37], vitronectin[17], apolipoprotein E, chloride intracellular channel 1[37], liver aldolase, tropomyosin β-chain, ketohexokinase, enoyl-CoA hydratase, albumin, smoothelin, arginase-1[36], complement C3a[38] and brain derived neurotrophic factor (BDNF)[39]. Differences to the present study might result from the available samples (e.g. serum *vs* tissue), sample preparation (e.g. fractionation), applied methods (e.g. electrophoresis *vs* SELDI-TOF MS) and patient or control characteristics.

The present study indicates that SELDI-TOF MS is a suitable technique for identification of serum markers in HCV associated liver cirrhosis and hepatocellular carcinoma. Apolipoprotein C- Ⅰ appears to be a valuable marker, however larger studies will be needed to define exactly the role of the biomarker patterns and the single protein markers. Most probably a combination of different serum markers will help to identify liver cirrhosis and early-stage hepatocellular carcinomas in the future.

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# Antitumor activity of an hTERT promoter-regulated tumor-selective oncolytic adenovirus in human hepatocellular carcinoma

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## Abstract

**AIM:** To construct a tumor-selective replication-competent adenovirus (RCAd), SG300, using a modified promoter of human telomerase reverse transcriptase (hTERT).

**METHODS:** The antitumor efficacy of SG300 in hepatocellular carcinoma was assessed *in vitro* and *in vivo*. *In vitro* cell viability by MTT assay was used to assess the tumor-selective oncolysis and safety features of SG300, and *in vivo* antitumor activity of SG300 was assessed in established hepatocellular carcinoma models in nude mice.

**RESULTS:** SG300 could lyse hepatocellular carcinoma cells at a low multiplicity of infection (MOI), but could not affect growth of normal cells even at a high MOI. Both in Hep3B and SMMC-7721 xenograft models of hepatocellular carcinoma, SG300 had an obvious antitumor effect, resulting in a decrease in tumor volume. Its selective oncolysis to tumor cells and safety to normal cells was also superior to that of ONYX-015. Pathological examination of tumor specimens showed that SG300 replicated selectively in cancer cells and resulted in apoptosis and necrosis of cancer cells.

**CONCLUSION:** hTERT promoter-regulated replicative adenovirus SG300 has a better cancer-selective replication-competent ability, and can specifically kill a wide range of cancer cells with positive telomerase activity, and thus has better potential for targeting therapy of hepatocellular carcinoma.



**Key words:** Virotherapy; Oncolytic adenovirus; Human telomerase reverse transcriptase; Hepatocellular carcinoma; Animal tumor model

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## INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the malignant diseases with a high incidence and mortality in China. Today, malignant tumors have already become the first death cause in Chinese city residents, and the second in country residents. Among various malignant tumors, HCC is the second cause of cancer death, next only to lung cancer. Based on recent incidence and mortality data available, the International Arctic Research Center (IARC) estimated that there were about 564 000 new cases of HCC in 2000 worldwide, in which 306 000 people were in China. About 300 000 people died of HCC annually in China[1]. The mortality of HCC is more than 40/100 000, especially in the high-risk area of Qidong, Jiangsu Province. HCC severely threatens people's life partially because of lack of effective therapeutic methods in clinics, and most strategies of gene therapy are not quite satisfactory. Therefore, to develop a therapeutic approach mainly targeting at HCC and synchronously at other tumors is an urgent task.

In cancer gene therapy, human adenoviruses have been used extensively[2], because they can infect most types of human cells, carry a large fragment of foreign sequence, and be grown in cultures to high-titered stable stocks[3]. Most adenoviral vectors used in gene transfer are replication-deficient because the adenoviral E1 region is deleted and replaced by antitumor therapeutic genes[4]. The

first generated replication-deficient adenovirus (RDAd) lacks only one (*E1*) or two (*E1* and *E3*) early genes. The second and third ones also contain *E2* and/or *E4* deletions[5,6]. Although the clinical protocols employing RDAd have been large in number[6], all of them have major problems. Since RDAd cannot replicate, they express foreign therapeutic genes at low levels only in cells that are initially infected. As a result, the antitumor effect is easily lost rapidly and the bystander effect on proximal cancer cells is limited. Another problem of RDAd is lack of specificity targeting cancer cells[7], which may further decrease the therapeutic effect on cancer cells and result in toxicity to normal cells. Therefore, development of novel effective tumor-targeting adenoviral vectors to enhance the efficiency and specificity of transgene expression is needed.

Recently, cancer-selective replication-competent adenovirus (RCAd) has attracted more attention in the field of cancer gene therapy. RCAds can specifically replicate in cancer cells, induce cell death and release progeny virions. The progeny virions will continue to infect neighboring cancer cells, thus magnifying the oncolytic effect[8]. In this approach of virotherapy, the specificity of the viral agent to selectively kill cancer cells, while relatively spare non-cancer cells, is achieved by controlling viral replication[9]. Abnormal biological features shared by various cancers have been used to restrict replication of adenoviruses to cancer cells. Recently the promoter of human telomerase reverse transcriptase (hTERT) was used to restrict adenoviral replication to telomerase-positive cancer cells through controlling the adenoviral *E1a* gene or *E4* gene[10,11]. The results suggested that the tight regulation of the adenoviral *E1a* gene is more crucial for replication of viruses. Compared with other promoters, the hTERT promoter is highly active in more than 85% of different human cancers, but inactive in most normal somatic cells, and thus this mechanism can be applied to a wide range of cancers.

We constructed an hTERT promoter-regulated RCAd, designated SG300, in which the adenoviral E1a gene was driven by the hTERT promoter core sequence (-212 bp to +46 bp) with additional 3 E-boxes (CACGTG). It can selectively replicate in a broad array of human cancer cells with positive telomerase activity, but not in normal cells[12]. However, its effect in treating HCC *in vitro* and *in vivo* is unknown. In this study, we further tested the antitumor activity of the tumor-selective RCAd, SG300, and confirmed its highly efficient antitumor activity *in vitro* in several types of cancer cell lines and *in vivo* in xenograft models of HCC.

## MATERIALS AND METHODS

### Cell culture

Human primary HCC cell lines Hep3B and HepG2, and human normal fibroblast cell lines MRC-5 and IMR-90 were purchased from American Type Culture Collection (ATCC, Manassas, VA). Human HCC cell lines BEL-7401 and SMMC-7721, and human normal hepatocellular line L02 were obtained from the Institute of Cell Biology, Chinese Academy of Sciences, Shanghai, China. All cell lines were cultured in media recommended by the providers: MRC-5, and IMR-90 in modified Eagle's medium (Gibco BRL, Gaithersburg, MD), and all other cell lines in Dulbecco's modified Eagle's medium (GibcoBRL) in a 5% $CO_2$ atmosphere at 37℃. All media were supplemented with 10% heat-inactivated fetal bovine serum (FBS) containing 100 U/mL penicillin and 100 μg/mL streptomycin. Telomerase activity of every cell line was measured by the telomeric repeat amplification protocol (TRAP) and ELISA assay with TRAP-PCR-ELISA telomerase detection kit (Chemicon International, Temecula, CA) according to the protocols.

### Construction of SG300

hTERT promoter core sequence (-212 bp to +46 bp) containing three E-box (CACGTG) motifs was synthesized and cloned into plasmid pMD18-T (TaKaRa Biotech. Co., Ltd., Ohtsu, Japan), and plasmid pCAG166TP was generated. After sequencing, the fragment containing hTERT promoter released from pCAG166TP was inserted into upstream of *E1a* gene of the adenoviral vector plasmid pXC1 (Microbix Biosystem Inc.), which has type 5 adenovirus sequence from 22 bp to 5790 bp containing *E1* gene. The obtained novel adenoviral vector plasmid, named pXC20-TP, was co-transfected with pBHGE3 (Microbix Biosystem Inc.), a plasmid of type 5 adenoviral right arm, into HEK293 cells using the Effectene Transfection Reagent (QIAGEN Inc.) to construct the recombinant adenovirus. About 9 to 14 d after transfection, single white plagues emerged in HEK293 cells. After plague purification three times, we obtained a recombinant replicative adenovirus designated SG300, in which *E1a* gene expression was driven by the hTERT core promoter. SG300 was amplified in HEK293 cells, extracted using QIAamp DNA Blood Mini Kit (QIAGEN Inc.), and purified by ultra-centrifugation on cesium chloride (CsCl) gradients.

### Cell viability assay

Cell viability was measured by MTT assay using Cell Proliferation Kit I (Roche Molecular Biochemicals, Indianapolis, IN). Serial concentrations of cells were diluted from $2 \times 10^4$ to $2 \times 10^5$ cells/mL, and seeded in 96-well plates at 100 μL per well. Every concentration was installed in 8 wells. After cultured for 24 h, 100 μL medium per well without FBS was added and cells were continuously cultured for another 7 d. After removal of culture medium from the plates, 100 μL 0.1 mol/L PBS and 10 μL MTT labeling reagent per well were added. The plates were then placed in an incubator for 4 h. After 100 μL solubilization solution was added per well and cultured overnight, the plates were examined with Microplate Reader Model 550 (BIO-RAD Laboratories, Tokyo, Japan) at 570 nm with a reference of 655 nm. Cell viability curves were drawn, on which the most suitable cell concentrations or densities were defined. To test the multiplicity of infection (MOI) of cell viability, every cell line was plated at the density defined above in 96-well plates and infected 24 h later with SG300, ONYX-015 (a control replicative adenovirus, kindly given by Berk AJ, University of California-Los Angeles, Los Angeles, CA), and wild-type

adenovirus 5 (WAd5) at a wide range of MOIs from 0.0001 to 500 pfu/cell. Seven days after infection, MTT assays were performed as described above.

### Virus replication

Cells were plated in 6-well plates at $10^6$ cells per well and infected with the replicative adenovirus SG300 or ONYX-015 at an MOI of 1 pfu/cell synchronously with MTT assay. At 48 h after infection, the cells and supernatants were harvested, and their viral titers were examined with TCID50 method. The titer data were normalized to that at the beginning of infection and expressed as replicative times.

### Animal experiments

BALB/c nude mice aged 6 to 8 wk were purchased from Shanghai Experimental Animal Center, Chinese Academy of Sciences, Shanghai, China. Human HCC cell lines, SMMC-7721 and Hep3B, in log phase ($10^7$ cells in 100 μL medium) were subcutaneously injected into the right flanks of mice. When tumors were induced, mice were divided randomly into four groups as SMMC-7721 models (SG300, ONYX-015, WAd5 and control groups, $n$ = 10 mice/group), and three groups as Hep3B models (SG300, ONYX-015 and control groups, $n$ = 10 mice/group), and given intratumoral injection of $2 \times 10^8$ pfu viruses in a volume of 100 μL viral preservation solution (10 mmol/L Tris-HCl pH 8.0, 2 mmol/L $MgCl_2$, 4% sucrose), once every other day with total injections of five times and total dosage of $10^9$ pfu per mouse in the SG300, ONYX-015 and WAd5 groups, respectively. In the control group, mice received injections of the same volume of viral preservation solution five times. Tumor sizes were measured regularly using calipers. Tumor volumes were estimated with the following formula: $a \times b^2 \times 0.5$, in which $a$ and $b$ represent the maximal and minimal diameters, respectively.

Mice were killed on d 42 after treatment in SMMC-7721 model group and on d 56 in Hep3B model group by cervical dislocation. Specimens from the tumor, liver, and lung were collected, fixed in 10% neutral formaldehyde for 6 h and paraffin-embedded, and 5 μm-thick consecutive sections were sliced for pathological examination. The expression of adenoviral capsid protein, hexon, was located using mouse anti-adenoviral hexon antibody (Biodesign International, MA, USA). To demonstrate apoptotic cell death of tumor tissues on paraffin-embedded sections, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay was performed using In Situ Cell Death Detection Kit (Roche Diagnostics, Basel, Switzerland) according to the manufacturer's instructions. Positive index (PI) was counted from five randomly selected high-power fields under light microscope, and expressed as a percentage of total cells counted.

### Statistical analysis

Experiments were performed three times and the data were presented as mean ± SD. Student's $t$-test was carried out to assess the statistical difference. $P < 0.05$ was considered to be significant.

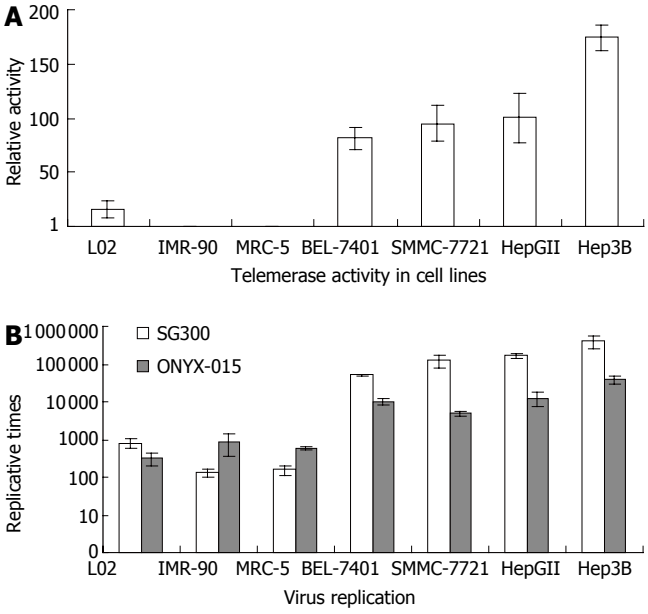


**Figure 1** Relationship between telomerase activity of the cell lines and replicative capability of SG300. By telomeric repeat amplification protocol (TRAP) and ELISA assay (**A**), every cancer cell line was positive for telomerase activity, but the normal fibroblast cell lines were not, whereas the hepatocellular line L02 showed weak telomerase activity. By TCID50 method (**B**), SG300 replicated selectively in cancer cells but rarely in normal cells ($P$ = 0.0453). The replicative capability of SG300 was coincident with the telomerase activity in various cell lines.

## RESULTS

### Telomerase activity of cell lines

By TRAP-ELISA assay, telomerase activity of cultured cell lines was measured. It showed that all cancer cell lines were positive for telomerase activity; however, the two normal fibroblast cell lines were not. The normal hepatocellular line L02 showed weak telomerase activity (Figure 1A).

### In vitro virus selective replication

By TCID50 method, we measured virus titers on d 4 after infected with SG300 and ONYX-015, respectively. The replicative capability of SG300 was markedly enhanced in different cancer cell lines with increases of virus yields by 50 793 to 406 250 times. In normal fibroblast cell lines, however, SG300 had a lower replicative capability. Its replicative times were below 160 ($P$ = 0.0453). It showed a certain replicative activity in human hepatocellular cell line L02, with replicative times of 796 (Figure 1B). ONYX-015 replicated more slowly than SG300 in cancer cells ($P$ = 0.0348).

### In vitro cell viability

To quantify the viability of cells, MTT assay was used. First, a suitable cell concentration or density for each cell line was determined, at which cells were seeded in 96-well plates and would not induce any cytopathic effect (CPE) within 7 d. This was to make sure that the killing effect that might appear in the following experiments was due to adenoviral replication. Second, we investigated the viability of cells, including HCC cell lines and normal cell lines, respectively infected with SG300, ONYX-015 and

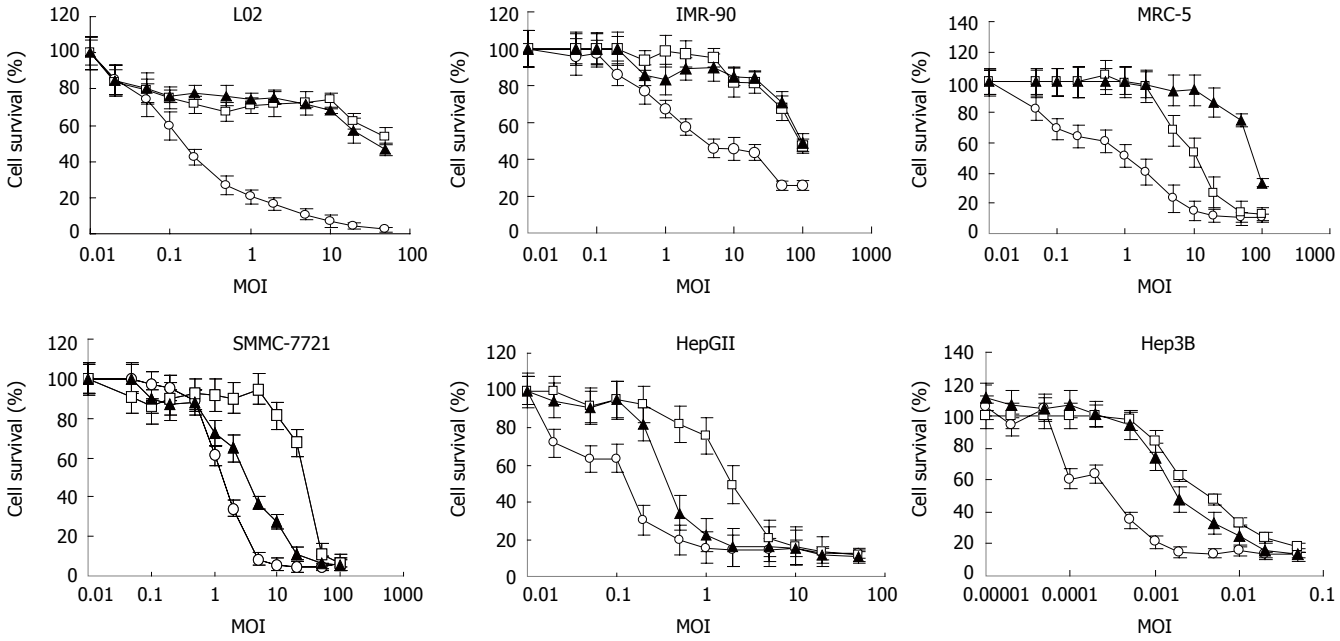


**Figure 2** Selective oncolytic effect of SG300 on HCC cell lines. At MOI = 10, the cell viability was < 30% in HCC cells, but > 68% in normal cells when infected with SG300. The oncolytic effect of SG300 was stronger on HCC cells and weaker on normal cells than that of ONYX-015, being close to that of WAd5. The killing effect of SG300 and ONYX-015 on normal cells became apparent when MOI = 100. —▲—SG300, —□—ONYX-015, —◇—WAd5.

WAd5, which reflected the selectivity and safety of SG300. The results showed that SG300 could lyse HCC cells at a very low MOI (Figure 2). Seven days after infection of SG300 at an MOI of 10 pfu/cell, the viability of each HCC cell line was below 30% (15.3%, 27.6% in HepGII, SMMC-7721, respectively); however, at the same MOI, the viability of each normal cell line was higher (68.0%, 84.8%, 94.0% in L02, IMR-90, MRC-5, respectively). The IC50s (MOI values of 50% viability) of SG300 in cancer cells were markedly lower than that in normal cells ($P$ = 0.0111) (Table 1). Hep3B was very sensitive to SG300, and cells were killed obviously and cell viability decreased to 13.2% at an MOI of 0.05 pfu/cell. ONYX-015 also had a killing effect on cancer cells, but it required a higher MOI than SG300 to reach the same effective level as SG300. The viability of HepGII or SMMC-7721 infected with ONYX-015 was 16.3% or 81.1% at an MOI of 10 pfu/cell, and of Hep3B was 17.8% at an MOI of 0.05 pfu/cell. There was no difference between the IC50s of WAd5 in cancer cells and normal cells ($P$ = 0.4081). MTT assay directly showed that the killing effect of SG300 was several times or even more than that of ONYX-015 on HCC cells, but weaker than that of ONYX-015 on normal cells.

**Table 1 IC50 values of adenoviruses in different cells**

| Cells | SG300 | ONYX-015 | WAd5 |
|---|---|---|---|
| L02 | 41.5658 ± 2.2122 | 58.0351 ± 8.9114 | 0.1520 ± 0.0099 |
| IMR-90 | 94.5783 ± 5.2813 | 90.4534 ± 8.2359 | 2.1342 ± 0.0897 |
| MRC-5 | 82.5674 ± 8.5549 | 14.2622 ± 1.7781 | 1.1525 ± 0.0336 |
| SMMC-7721 | 2.8954 ± 0.0641[a] | 25.5323 ± 6.2351 | 1.3426 ± 0.0625 |
| HepGII | 0.3232 ± 0.0085[a] | 1.9845 ± 0.0046[a] | 0.1123 ± 0.0030 |
| Hep3B | 0.0017 ± 0.0003[a] | 0.0042 ± 0.0005[a] | 0.0013 ± 0.0002 |

[a]$P$ < 0.05 *vs* normal cells in the same group.

### *In vivo antitumor activity of SG300*

Hep3B and SMMC-7721 cells were implanted into BALB/c nude mice to induce subcutaneous tumors. Tumors were seen 20 d later after implantation of SMMC-7721 cells, and about 30 d later after implantation of Hep3B cells. The adenoviruses and the control buffer (viral preservation solution) were injected intralesionally. Forty-two days later, half mice (5/10) died in WAd5 group of SMMC-7721 models. The groups injected with adenoviruses both in these two models of HCC showed antitumor effects to some extent when compared with the control group ($P$ = 0.0001, 0.0364 for SG300 and ONYX-015, respectively, in SMMC-7721 models; $P$ = 0.0005, 0.0019 for SG300 and ONYX-015, respectively, in Hep3B models), and the effects of SG300, being close to that of WAd5, were better than that of ONYX-015 ($P$ = 0.0029, 0.0066 in SMMC-7721 and Hep3B models, respectively) (Figure 3). Mice of the control group in Hep3B models were sacrificed ahead of time on d 42 after treatment by cervical dislocation due to overloading of tumors.

### *Pathological examination*

Pathological examination showed that there were many areas of necrosis in tumor tissues of the SG300-treated group and ONYX-015-treated group. In the control group, however, cancer cells grew luxuriantly with only a few, small focal areas of necrosis (Figure 4A and B). Immunohistochemistry demonstrated that most cancer cells positive for hexon were distributed mainly around the necrotic areas (Figure 4C and D), and that more apoptotic cancer cells were positive for TUNEL labeling in tumor tissues of the SG300-treated group, compared

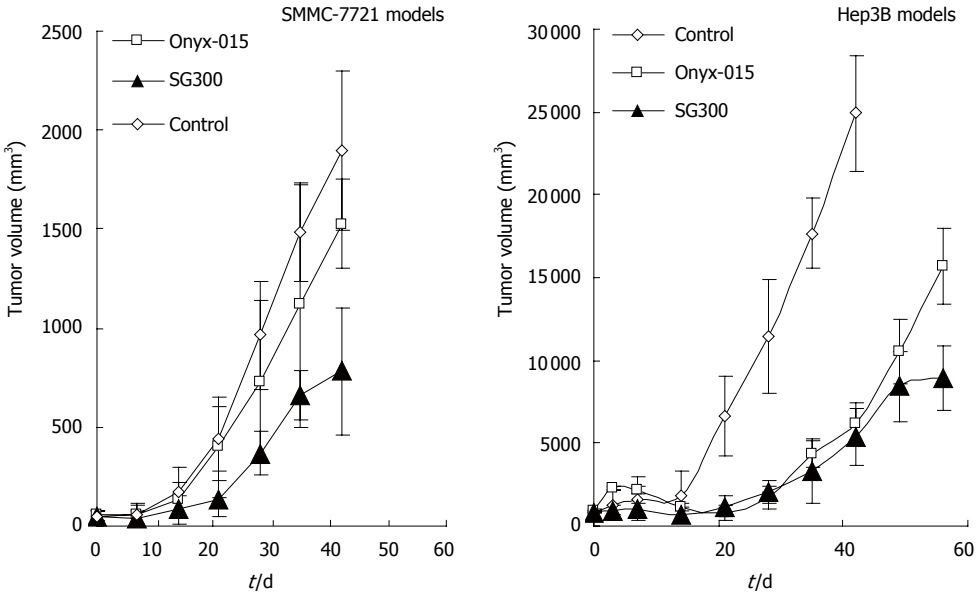


**Figure 3** Antitumor activity of SG300 on HCC xenografts in nude mice. In SMMC-7721 and Hep3B models, efficient antitumor activities were found in groups treated with SG300 when compared with control group on d 42 ($P < 0.05$). The antitumor effect of SG300 was better than that of ONYX-015. WAd5 group in SMMC-7721 models also showed efficient antitumor efficacy, however, 5 of 10 mice died during the period of observation.

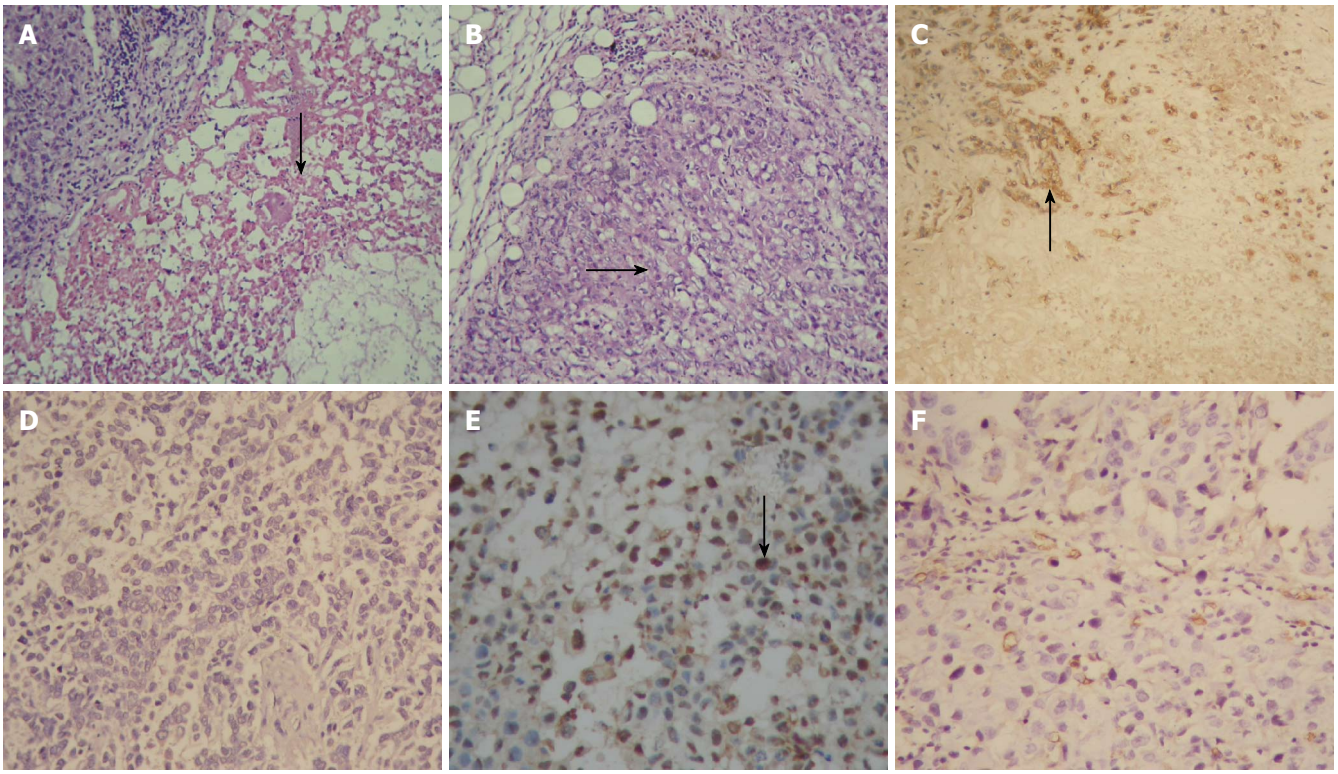


**Figure 4** Pathological examination of tumor specimens (× 200). HE staining showed wide areas of necrosis (arrow) in tumor tissues of the SG300-treated group (**A**), but cancer cells grew luxuriantly (arrow) in the control group (**B**). Immunohistochemistry demonstrated that most cancer cells around the necrotic area were positive for adenoviral capsid protein hexon (arrow) in the SG300-treated group (**C**), but cancer cells were negative for hexon in the control group (**D**). More cancer cells were positive for TUNEL labeling (arrow) in tumor tissues of the SG300-treated group (**E**), whereas only a few cancer cells were positive for TUNEL labeling in the control group (**F**).

with the control group (Figure 4E and F). PIs in SG300, ONYX-015 and control groups were 59.4 ± 21.1, 42.3 ± 18.2, 17.5 ± 8.9 for TUNEL labeling, respectively. There was a difference between TUNEL labeling PI in the control group and in any other replicative adenovirus-treated group ($P = 0.0007$ for SG300, $P = 0.0032$ for ONYX-015). The liver and lung tissues of mice were negative for hexon in SG300-treated group as well as ONYX-015 and control groups.

## DISCUSSION

Virotherapy employing tumor-selective replication-competent adenovirus (RCAd) is a promising strategy in cancer treatment. An alternative approach to restrict adenoviral replication to targeting cancer cells and achieve RCAd is to modify adenovirus by partial deletion of viral genes that are essential for replication in normal cells but not in tumor cells[13-15]. The representative RCAd of

this type is ONYX-015[16]. It has a deletion of *E1b-55K* gene and replicates specifically in cancer cells lacking wide-type *p53*[17,18]. An additional approach to achieve RCAd is to place viral replicative genes under the control of cancer-specific promoters that are activated only in cancer cells[19]. Several cancer-specific promoters that are more active in particular cancer cells but inactive or only weakly active in cancer-originating somatic cells, have been identified and explored to construct RCAds, such as the carcinoembryonic antigen (CEA) promoter targeting colorectal and lung cancer[20], the α-fetoprotein promoter targeting hepatocellular cancer[21,22], the prostate-specific antigen (PSA) promoter targeting prostate cancer[2], the MUC1 antigen promoter targeting breast cancer[7], the E2F transcriptional factor promoter targeting cancers with a defective pRb/E2F/p16 pathway[23-25], the surfactant protein B (SPB) promoter targeting lung cancer[3], and the L-plastin promoter targeting ovarian and bladder cancers[26]. These cancer-specific promoters can provide RCAds, the selective replication in corresponding cancer cells and demonstrate antitumor activity in preclinical models and clinical trials. However, most of them are limited, targeting a narrow range of cancers expressing the corresponding tumor antigen, thus attenuating their efficacy in cancer therapy[27].

A number of gene therapeutic approaches have been proposed to kill cancer cells or inhibit growth of caners by targeting telomerase[28]. Most of them utilize the hTERT promoter to drive antitumor gene expression selectively in cancer cells with positive telomerase activity, without affecting normal cells negative for telomerase[29,30]. It indicates the potential therapeutic application of the strategy targeting telomerase for a wide range of various cancers. Recently the hTERT promoter has been tested for targeting cancer gene therapy with replication-competent viral vectors. It was used to control the adenoviral early gene expression and produce a series of RCAds, but experimental results were inconsistent. Some studies showed that the hTERT promoter provided a sufficient selection for viral replication in cancer cells and did not in normal cells when it was used to construct CRAds by controlling *E1a* gene[10,31,32]. Other studies demonstrated that the hTERT promoter failed to block viral replication in telomerase-negative cells when it was used to control *E4* gene expression[11]. Because the wild-type hTERT promoter is incapable of mediating a high level of RCAd replication in cancer cells, it is necessary to develop a modified form of the promoter with improved transcriptional activity and cancer specificity. Wirth and coworkers reported that the transcriptional activity of the hTERT promoter could be enhanced significantly in a majority of tumor cell lines by inserting the E1a TATA box upstream of the transcription start site of its controlled gene[33]. This modification in an RCAd, named hTERT-Ad, did not alter the tumor-selective specificity of the promoter. Kim *et al*[34] constructed a modified hTERT promoter-controlled RCAd, Ad-mTERT-Δ19, in which a fragment containing one copy of c-Myc binding site and five copies of SP1 binding sites was incorporated into the downstream region of the wild-type hTERT promoter. The experimental results strongly suggested that the transcriptional activity of the modified hTERT promoter was up-regulated in cancer cells but not in normal cells, and the replicative capability and cytolytic effect of Ad-mTERT-Δ19 were enhanced only in cancer cells compared with wild-type hTERT promoter-controlled RCAd, Ad-TERT-Δ19. To further increase the tumor-selective specificity of RCAd controlled by the hTERT promoter and decrease the adenoviral toxicity to normal cells, we previously used a modified hTERT promoter with three additional copies of E-boxes (CACGTG) to construct an RCAd, named SG300. The results suggested that by this modification, the specificity of RCAd targeting cancer cells could be improved and its toxicity to normal cells could be reduced[12].

Our experiments *in vitro* showed that SG300 lysed or destroyed cancer cells at a low MOI, including human HCC cell lines, Hep3B, HepGII, BEL-7401 and SMMC-7721, but did not affect growth of normal cells such as human normal fibroblast cell lines, MRC-5 and IMR-90, even at relative high MOIs. The oncolytic ability of CRAds varied in different types of HCC cells. Both SG300 and ONYX-015 had strong effects on Hep3B. The selective cytolytic ability of SG300 was more powerful than that of ONYX-015 in HCC cells. The effect of SG300 on normal cells was less than that of ONYX-015. ONYX-015 is an oncolytic adenovirus that shows different mechanisms and replicates preferably in p53-deficient tumor cells. Because the positive rate of telomerase activity was higher than the incidence rate of p53-deficiency in human cancers, ONYX-015 targets a narrower range of cancer types than SG300. Furthermore, our previous studies and the current data proved that the replicative capacity of ONYX-015 in tumor cells was weaker than that of SG300 series[12]. Probably that is why the selective cytolytic ability of ONYX-015 was weaker than that of SG300 in HCC cells. All these results, combined with the data of telomerase activity and viral replicative capability in cultured cell lines, demonstrated that SG300 has a better cancer-selective replication-competent ability, and can specifically kill a wide range of cancer cells with positive telomerase activity. The killing effect of SG300 was not only consistent with its replicative capacity in different cell lines but also with the telomerase activity of these cells. L02 cells showed a weak telomerase activity, and SG300 could replicate in this cell line to some extent and resulted in weak cytolysis.

BALB/c nude mice were subcutaneously injected with Hep3B and SMMC-7721 cells, respectively. When the tumors were induced, the replicative adenoviruses were injected intralesionally with a total dosage of $10^9$ pfu per mouse. The results showed that SG300 yielded an obvious antitumor effect on HCC xenografts. Tumor growth was inhibited with a decrease in tumor volume. The antitumor effect of SG300 was better than that of ONYX-015. During the period of observation, half mice of WAd5-infected group in SMMC-7721 models died of virus toxicity, but no mice died in SG300-treated groups either in SMMC-7721 or in Hep3B models, demonstrating that SG300 had no toxicity to normal tissues or organs and therefore, was safe in cancer therapy. Pathological examinations showed that SG300 replicated selectively in

HCC cells and resulted in apoptosis and necrosis of cancer cells. In brief, hTERT promoter-regulated replicative adenovirus SG300 has a selective replicative ability and a specific cytolytic ability targeting HCC cells and other cancer cells that are positive for telomerase activity. It shows superior selective replication and antitumor effect when compared with ONYX-015, and thus has better potential for HCC therapy.

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## COMMENTS

### Background

Recently, a number of gene therapeutic approaches by cancer-selective replication-competent adenovirus (RCAd) have been proposed in the field of cancer gene therapy, and attracted much attention. However, in vitro experimental results are inconsistent. The objective of this study was to further increase the tumor-selective specificity of RCAd and decrease the adenoviral toxicity to normal cells.

### Research frontiers

In this study we constructed SG300 by use of a modified hTERT promoter. It could lyse hepatocellular carcinoma cells at a low multiplicity of infection (MOI), but did not affect growth of normal cells even at a high MOI. Its selective oncolytic ability was stronger than that of ONYX-015, which is the representative selective replicative adenovirus, and its influence on normal cells was less than that of ONYX-015.

### Innovations and breakthroughs

To further increase the tumor-selective specificity of RCAd controlled by the hTERT promoter and decrease the adenoviral toxicity to normal cells, we constructed an RCAd, SG300, by use of a modified hTERT promoter with three additional copies of E-box (CACGTG). Its antitumor activity in vitro and in vivo was shown to be excellent. Both in Hep3B and SMMC-7721 xenograft models of hepatocellular carcinomas, SG300 had an obvious antitumor effect, resulting in a decrease in tumor volume due to its selective replication in cancer cells and its specific oncolysis.

### Applications

hTERT promoter-regulated replicative adenovirus SG300 has a selective replicative ability and a specific cytolytic ability targeting hepatocellular carcinoma cells and other cancer cells that are positive for telomerase activity. It displays superior selective replication and antitumor effect when compared with ONYX-015, and thus has better potential for HCC therapy.

### Terminology

RCAd: replication-competent adenovirus; hTERT: human telomerase reverse transcriptase

### Peer review

hTERT promoter-regulated replicative adenovirus SG300 has a better cancer-selective replication-competent ability, and can specifically kill a wide range of cancer cells with positive telomerase activity, and thus has better potential for targeting therapy of hepatocellular carcinoma.

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# Bone morphogenetic protein-2 is a negative regulator of hepatocyte proliferation downregulated in the regenerating liver

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## Abstract

**AIM:** To characterize the expression and dynamic changes of bone morphogenetic protein (BMP)-2 in hepatocytes in the regenerating liver in rats after partial hepatectomy (PH), and examine the effects of BMP-2 on proliferation of human Huh7 hepatoma cells.

**METHODS:** Fifty-four adult male Wistar rats were randomly divided into three groups: A normal control (NC) group, a partial hepatectomized (PH) group and a sham operated (SO) group. To study the effect of liver regeneration on BMP-2 expression, rats were sacrificed before and at different time points after PH or the sham intervention (6, 12, 24 and 48 h). For each time point, six rats were used in parallel. Expression and distribution of BMP-2 protein were determined in regenerating liver tissue by Western blot analysis and immunohistochemistry. Effects of BMP-2 on cell proliferation of human Huh7 hepatoma cell line were assessed using an MTT assay.

**RESULTS:** In the normal liver strong BMP-2 expression was observed around the central and portal veins. The expression of BMP-2 decreased rapidly as measured by both immunohistochemistry and Western blot analysis. This decrease was at a maximum of 3.22 fold after 12 h and returned to normal levels at 48 h after PH. No significant changes in BMP-2 immunoreactivity were observed in the SO group. BMP-2 inhibited serum induced Huh7 cell proliferation.

**CONCLUSION:** BMP-2 is expressed in normal adult rat liver and negatively regulates hepatocyte proliferation. The observed down regulation of BMP-2 following partial hepatectomy suggests that such down regulation may be necessary for hepatocyte proliferation.



**Key words:** Bone morphogenetic protein-2; Partial hepatectomy; Liver regeneration; Hepatocellular carcinoma

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## INTRODUCTION

Bone morphogenetic proteins (BMPs) were first identified in the 1960s[1]. BMPs are multi-functional growth factors that belong to the transforming growth factor beta (TGF-β) superfamily[2]. Mature BMPs are 30-38 kDa proteins that utilize BMP receptors and intracellular SMADs to transduce their signals to regulate cell proliferation, differentiation, morphogenesis and apoptosis. The role of BMPs in embryonic development and in postnatal and adult animals has been extensively studied in recent years. In addition to their well recognized role in bone physiology, BMPs are known to regulate the development and homeostasis of other organs including the liver[3]. Previous research showed that a receptor for BMP-9 is expressed in the HepG2 liver tumor cells. HepG2 cells bind BMP-9 and undergo a proliferative response[4]. Northern blotting analysis demonstrated the presence of BMP-6 in non-parenchymal liver cells and a role for BMP-6 in the regeneration of liver tissue was proposed[5]. BMP signaling plays a critical role in the regulation of liver development. BMP signaling from the septum transversum mesenchyme is necessary to induce liver genes in the endoderm and the morphogenetic growth of the hepatic endoderm into a liver bud[6,7]. Similar to TGF-β signaling, BMP signaling has been implicated in the development of hepatic fibrosis as BMPs have been shown to stimulate activation of hepatic stellate cells, which results in their

transdifferentiation to an α-smooth muscle antigen positive myofibroblast-like phenotype[8,9].

Among BMPs, BMP-2 has gained more attention because it is the predominant form in natural bone morphogenetic protein extracts[10], and it is widely expressed during mouse development[11]. Researchers find that high affinity receptors for BMP-2 are present not only on osteoblastic cells but also on a large variety of non-hematopoietic cell types[12]. Preclinical and clinical studies have suggested that recombinant BMP-2 may have therapeutic potential in bone repair[13].

A hallmark of developmental biology is that similar pathways are involved in multiple different systems. It is clear that the role of BMP signaling is not restricted to the bone. The liver, just as the bone, has a remarkable regenerative potential, which involves tightly regulated molecular mechanisms that control hepatic proliferation, differentiation and morphogenesis after the loss of hepatic tissue[14]. However, the role of BMP signaling in the regenerating liver remains unclear. Here we focus on the expression and dynamic changes of BMP-2 in hepatocytes in regenerating liver of adult rats.

## MATERIALS AND METHODS

### Experimental animals and grouping

Fifty-four adult and healthy male Wistar rats weighing 180-220 g, obtained from the Animal Center of Shanxi Medical University, were employed in the present study. All rats received humane care during the study under a protocol that was in accordance with institutional guidelines for animal research and was approved by the Ethics and Research Committee of Shanxi Medical University. Experimental rats were randomly divided into three groups: Normal control (NC, $n = 6$) group, partial hepatectomized (PH, $n = 24$) group and sham operation (SO, $n = 24$) group. Rats were sacrificed at 6, 12, 24 and 48 h after partial hepatectomy or sham operation. For each time point indicated, six rats ($n = 6$) were used in parallel.

### Animal model and sample preparation

Rats were maintained on a 12/12 h light-dark cycle. The surgery was performed between 8 and 10 AM. Rats were fasted 12 h before surgery and anesthetized with pentobarbital sodium (30 mg/kg) intra-abdominally, then the abdominal skin was shaved and sterilized with an iodine solution. Two-thirds hepatectomy was performed as described by Higgins and Anderson. In the sham operated rats the liver was manipulated but not resected. Rats were anesthetized with pentobarbital sodium (20 mg/kg) intra-abdominally and killed at 6, 12, 24 or 48 h after partial hepatectomy or the sham procedure. The remnant livers were removed, parts of which were fixed 24 h in 10% buffered neutral formalin. The fixed livers were dehydrated through increasing concentrations of ethanol and in xylene and embedded in paraffin. Liver tissues embedded in paraffin were sectioned at 4 μm for immunohistochemistry. Part of the livers was snap frozen in liquid nitrogen for the preparation of protein lysates for Western blotting.

### Immunohistochemistry

Immunohistochemistry was performed as described in detail below. Paraffin sections (4 μm) were dewaxed and dehydrated in graded alcohols. Endogenous peroxidase activity was quenched with 1.5% $H_2O_2$ in PBS for 30 min at room temperature. Antigen retrieval was performed by heating for 10 min at 95℃ in 0.01 mol/L sodium citrate, and non-specific staining was blocked with TENG-T (10 mmol/L Tris, 5 mmol/L EDTA, 0.15 mol/L NaCl, 0.25% gelatin, 0.05% [vol/vol] Tween-20, pH 8.0) for 30 min at room temperature. Endogenous avidin binding activity due to biotin was overcome by successive 20 min incubations of the tissue sections in 0.1% avidin and 0.01% biotin (DAKO Biotin Blocking System). After a washing with PBS (3 × 5 min), BMP-2 primary antibody (mouse monoclonal BMP-2, MAB355, 1:500, R&D) was applied in PBS containing 1% bovine serum albumin and 0.1% Triton and incubated overnight at 4℃. The following day, for BMP-2 staining, sections were incubated with biotinylated goat-anti-mouse IgG (DAKO 1:200) for 60 min in PBS with 10% human serum, then washed in PBS (3 × 10 min) and incubated for 60 min with streptavidin-biotin-horse-radish peroxidase (DAKO) for 1 h, and washed again 3 × 5 min in PBS. And peroxidase activity was detected with DAB (Sigma), resulting in the formation of a brown reaction product. Finally, sections were briefly counterstained with hematoxylin, then dehydrated, cleared and mounted in neutral gum under cover slips. For controls, the primary and secondary antibodies were substituted and an appropriate IgG control (mouse IgG2b was applied at 1:50) was used to perform negative control staining. A known positive staining specimen (bone tissue of rat) was used as a positive control.

### Immunoblotting

Rat liver tissue was homogenized in lysis buffer. Protein concentrations were measured using the Bradford method. Lysates were diluted as per 300 μL protein sample buffer was added with 600 μg extract in 2 × protein sample buffer and 30 μL of each sample of homogenates was loaded per lane on an SDS-PAGE gel. Equal protein loading was confirmed using β-actin (Santa Cruz) antibodies on the same blots after stripping off the old antibodies in stripping buffer. After protein separation the proteins were blotted onto a PVDF membrane (Millipore, Bedford, MA). The membranes were blocked with 2% protein (Nutricia, The Netherlands) in PBS supplemented with 0.1% Tween-20 for 1 h at room temperature. After a brief wash in washing buffer (0.2% protifar: 0.1% Tween-20), membranes were incubated overnight at 4℃ with primary antibody (mouse monoclonal BMP-2, MAB 355, 1:1000, R&D systems) in 2% blocking buffer. The following day, membranes were washed three times for 5 min, and subsequently incubated with a secondary horseradish peroxidase (HRP)-conjugated antibody in wash buffer (0.2% low fat milk powder) at 1:1000 dilution. After enhanced chemoluminescence using Lumilight + substrate (Roche, Mannheim, Germany), antibody binding was visualized using a Lumi-Image-Pro Plus 5.0.

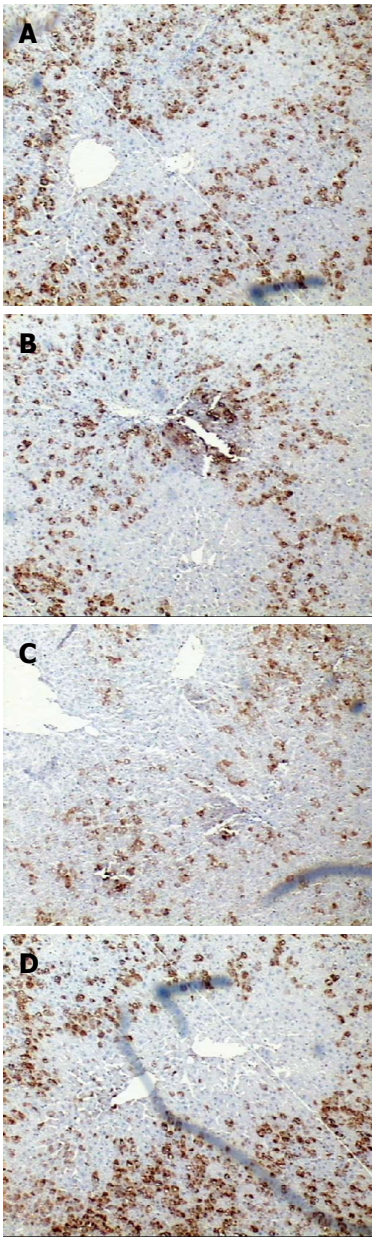


**Figure 1** By immunohistochemistry, BMP-2 is expressed predominantly in hepatocytes around the central vein and portal triad. Strong BMP-2 staining is seen localized to the cytoplasm of NC group (**A**). The strong BMP-2 staining seen in normal tissue is lost at 6,12 h group (**B**,**C**) and resumes at 48 h group (**D**) following PH. There was no significant change in SO group compared with NC group in BMP-2 immunoreactivity (data not shown). Control stainings were performed by omitting the primary antibody and using a control IgG2b that showed no staining. A known positive staining specimen was used as a positive control (data not shown). (**A**, **B**, **C**, and **D** x 200).

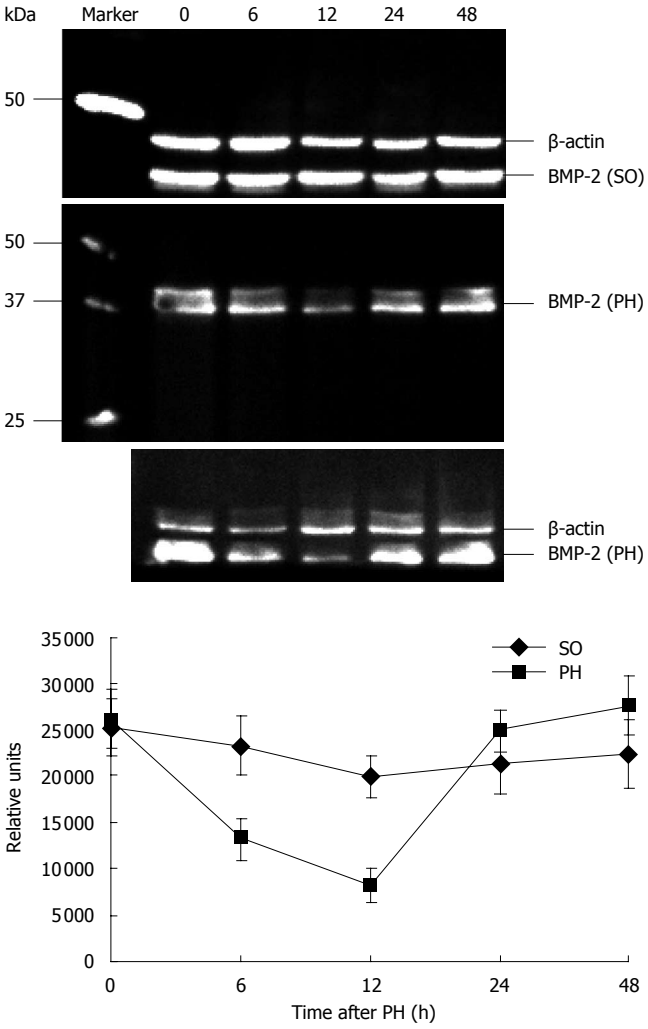


**Figure 2** Immunoblot for BMP-2 in rat liver of NC, SO and PH groups. At all the time points (note: 0 h = NC), rat liver extracts tested showed a band at approximately 37 kDa on the immunoblots. Bio-Rad Precision Protein Standards were used. Equal protein loading was confirmed using β-actin antibodies on the same blots by striping the old antibodies in strip buffer. The calculated molecular weights are shown. Quantification of 12 h immunoblots revealed 3.22 fold lower ($P < 0.01$) BMP-2 protein expression levels in PH group as compared with NC group. BMP-2 protein levels increased progressively to that of NC group at 48 h after PH.

### *Cell culture and proliferation assay*

The Huh7 human hepatoma cell line[15] was obtained from the ATCC (American Type Culture Collection) and cultured in Dulbecco's Modified Eagle's Medium (DMEM)(Gibco, Paisley, Scotland) with 4.5 g/L glucose and 1% L-glutamine. This was supplemented with penicillin (50 U/mL), streptomycin (50 μg/mL) and 10% FBS (Gibco). Cells were grown in monolayers in a humidified atmosphere containing 5% $CO_2$. Eighty to ninety percent confluent monolayers of Huh7 cells were trypsinized and taken up in medium with 10% FBS. Cells at $5 \times 10^4$ per well were seeded in triplicate in flat-bottomed tissue culture of 24 well plates (Falcon) overnight in DMEM medium containing 10% FBS in the absence or presence of recombinant human BMP-2 (rhBMP-2 355-BM, R&D systems) at indicated concentrations for 72 h. MTT [3-(4,5-methylthiazol-2-yl)-2,5-dipheyl-tetrazolium bromide] reagent was added to all wells for 30 min. The medium was removed from the cells, and cells were lysed in acidic isopropanol and absorbance was measured at 550 nm with an enzyme-linked immunosorbent assay plate reader (Molecular Devices, Ther/vio max microplate reader).

### *Statistical analysis*

Values are expressed as mean ± SE. Results were evaluated using analysis of variance and correlated by SPSS11.0 software. $P < 0.05$ was considered significant.

## RESULTS

### *Localization of BMP-2 expression in regenerating rat liver*

To determine the expression and localization of BMP-2 in the regenerating rat liver, immunostaining was carried out using a specific monoclonal anti-BMP-2 antibody. As evident from Figure 1, clear and strong BMP-2 immunoreactivity was present in the cytoplasm of hepatocytes around the central vein (CV) and portal triad (PT) in normal liver. In contrast, immunoreactivity of BMP-2 declined significantly at 12 h after PH. Thereafter

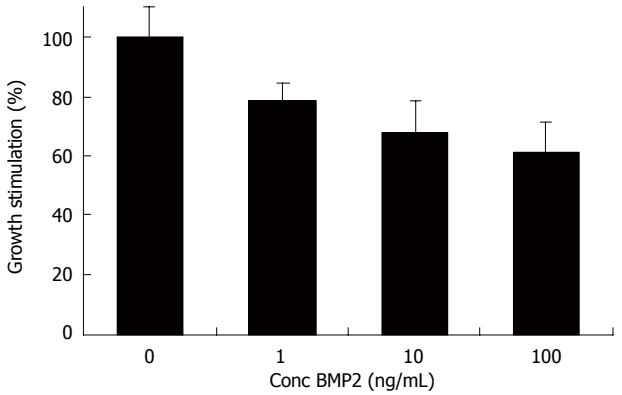


**Figure 3** MTT assay of human hepatoma Huh7 cell line treated for 72 h with various concentrations of BMP2. Values obtained with no BMP2 were set at 100. Error bars represent the standard error of the mean. BMP-2 inhibited the growth of Huh7 cells, maximal effects occurring at 100 ng/mL after 72 h incubation. BMP-2 significantly inhibited the growth of Huh7 cells by 38.8% ± 0.4% ($P < 0.01$).

the immunoreactivity of BMP-2 increased progressively and was back to normal levels at 48 h after PH. No significant change in BMP2 expression in SO group compared to normal controls was observed (data not shown).

### *Levels of BMP-2 in regenerating rat liver*

To confirm the specificity of the observed changes in BMP-2 expression in the regenerating rat liver, levels of BMP-2 expression were determined by Western blot analysis. As shown in Figure 2, using the antibody a band at approximately 37 kDa was detected on the immunoblots, which corresponds to BMP-2 precursor protein. Quantification of BMP-2 expression showed a 3.22 fold reduction ($P < 0.01$) of BMP-2 protein expression compared to controls at 12 h after PH. Similar to the results obtained by immunohistochemistry, BMP-2 protein levels returned to control levels at 48 h after PH. There were no significant changes in BMP-2 expression in the SO group compared to normal controls.

### *Effects of BMP-2 on human hepatoma Huh7 cell line proliferation*

Because BMP-2 expression was down regulated during liver regeneration, we next attempted to determine whether BMP-2 was capable of modulating Huh7 cell proliferation. To this end, Huh7 cells were treated with increasing concentrations of rhBMP-2. MTT assay demonstrated that proliferation of Huh7 cells was dose dependently inhibited by rhBMP-2. A maximal inhibition of 38.8% ± 0.4% ($P < 0.01$) was observed at 100 ng/mL (Figure 3).

## DISCUSSION

Following partial hepatectomy, there is a rapid and highly orchestrated series of biochemical events that regulate hepatic regeneration. This is a complex process, which allows for a short period of rapid cellular proliferation but is subsequently followed by cell cycle arrest and cellular differentiation[14]. It has been shown that BMP signaling plays a critical role in hepatogenesis during endodermal patterning[6]. However, the role of BMPs in hepatic regeneration in the adult has not been studied.

The present study analyzed the expression of BMP-2 in normal rat liver. We studied the changes in BMP-2 expression during liver regeneration in the two-thirds hepatectomy model in rats. Immunohistochemical analysis showed that strong BMP-2 immunoreactivity was present in the cytoplasm of normal rat hepatocytes surrounding CV and PT. Both immunohistochemistry and Western blot analysis showed that BMP-2 declined significantly at 12 h after partial hepatectomy and returned to normal at 48 h. We showed that BMP-2 suppressed growth of Huh7 hepatoma cells *in vitro,* suggesting that BMP signaling negatively regulates hepatocyte proliferation. However, this *in vitro* observation needs further confirmation *in vivo* in a model of hepatocyte regeneration.

Our data remain descriptive but suggest that the role of signaling by BMP-2 may be distinct from that of TGF-$\beta_1$, another TGF-β family member that signals through a different receptor complex. TGF-$\beta_1$ is also an inhibitor of hepatocyte proliferation[16]; however, the normal liver expresses very low levels of TGF-$\beta_1$. Levels of TGF-$\beta_1$ expression increase rapidly after partial hepatectomy and peak at 12 h[17] exactly when the expression of BMP-2 is at its lowest. In mice with a liver specific deletion of TGF-β receptor type II, which is required for TGF-β signaling, increased hepatocyte proliferation and liver mass in response to partial hepatectomy was resulted[18], indicating that TGF-β signaling acts as a negative feedback loop in hepatic regeneration that keeps the mitogenic response in check. The dynamic expression of BMP-2 suggests a distinct role for this pathway. BMP-2 is readily detected in the normal liver and its expression rapidly declines after partial hepatectomy. This may suggest that BMP-2 signaling does not act in a negative feedback loop in hepatocyte regeneration but that instead, its down regulation may be necessary for the initiation of hepatocyte proliferation. Hepatocytes are the first to proliferate after partial hepatectomy. Hepatocyte proliferation started exactly in the area around the CV where BMP-2 expression was lost well before the onset of proliferation which peaked at 24 h[19].

In conclusion, both the localization and timing of expression of BMP-2 suggest that BMP-2 may be a negative regulator of hepatocyte proliferation that needs to be down regulated in order to allow the initiation of hepatocyte proliferation around the CV. Our data remain descriptive and interventional studies need to be performed to test this hypothesis.

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## COMMENTS

### *Background*

The role of BMP signaling is not restricted to the bone. The liver, just as the bone, has remarkable regenerative potential which involves tightly regulated molecular mechanisms that control hepatic proliferation, differentiation and morphogenesis after the loss of hepatic tissue.

### *Research frontiers*

A hallmark of developmental biology is that similar pathways are involved in multiple different systems.

### *Innovations and breakthroughs*

We aimed to summarize and emphasize the differences, from other related or similar articles so that readers may catch up the major points of the article easily.

### *Peer review*

This study examines the role of BMP-2 in hepatocyte proliferation following PH and *in vitro* in a human hepatoma cell line. BMP-2 has been shown to play an important role in bone development and may also be important in liver development. The authors proposed that this cytokine may act as a growth inhibitor during liver regeneration. The study is overall well designed and potentially important.

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*VIRAL HEPATITIS*

# Expression patterns and action analysis of genes associated with hepatitis virus infection during rat liver regeneration

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## Abstract

**AIM:** To study the action of hepatitis virus infection-associated genes at transcription level during liver regeneration (LR).

**METHODS:** Hepatitis virus infection-associated genes were obtained by collecting the data from databases and retrieving the correlated articles, and their expression changes in the regenerating rat liver were detected with the rat genome 230 2.0 array.

**RESULTS:** Eighty-eight genes were found to be associated with liver regeneration. The number of genes initially and totally expressed during initial LR [0.5-4 h after partial hepatectomy (PH)], transition from G0 to G1 (4-6 h after PH), cell proliferation (6-66 h after PH), cell differentiation and reorganization of structure-function (66-168 h after PH) was 37, 8, 48, 3 and 37, 26, 80, 57, respectively, indicating that the genes were mainly triggered at the early stage of LR (0.5-4 h after PH), and worked at different phases. These genes were classified into 5 types according to their expression similarity, namely 37 up-regulated, 9 predominantly up-regulated, 34 down-regulated, 6 predominantly down-regulated and 2 up/down-regulated genes. Their total up- and down-regulation frequencies were 359 and 149 during LR, indicating that the expression of most genes was enhanced, while the expression of a small number of genes was attenuated during LR. According to time relevance, they were classified into 12 groups (0.5 and 1 h, 2 and 4 h, 6 h, 8 and 12 h, 16 and 96 h, 18 and 24 h, 30 and 42 h, 36 and 48 h, 54 and 60 h, 66 and 72 h, 120 and 144 h, 168 h), demonstrating that the cellular physiological and biochemical activities during LR were fluctuated. According to expression changes of the genes, their expression patterns were classified into 23 types, suggesting that the cellular physiological and biochemical activities during LR were diverse and complicated.

**CONCLUSION:** The anti-virus infection capacity of regenerating liver can be enhanced and 88 genes play an important role in LR.



**Key words:** Partial hepatectomy; Rat genome 230 2.0 array; Hepatitis virus infection; Genes associated with liver regeneration

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## INTRODUCTION

The liver can regenerate and precisely regulate its size. Hepatocytes maintain the ability to proliferate in response to hepatectomy, liver damage caused by viruses or chemicals, liver cell death, *etc*[1,2]. The remaining liver may compensate for the lost hepatic tissue in which the growth situation of regenerating liver can be accurately detected, and liver regeneration (LR) may stop at a proper time point[3,4]. The regeneration process is usually categorized based on hepatic physiological biochemical activities into four stages: initiation [0.5-4 h after partial hepatectomy (PH)], transition from G0 to G1 (0.5-4 h after PH), cell proliferation (6-66 h after PH), cell redifferentiation and reorganization of the architecture-function (66-168 h after PH)[5]. In this process, the cellular physiological and biochemical activities and the gene expression situations have a very sweeping change.

Hepatitis viruses causing hepatic injury and liver diseases[6] include hepatitis A virus (HAV), hepatitis B virus (HBV), hepatitis C virus (HCV), hepatitis D virus (HDV) and hepatitis E virus (HEV)[7]. HAV and HEV do not result in chronic hepatic inflammation because of their self-limited infection, whereas HBV, HCV and HDV cause both acute and chronic hepatic inflammation, and finally

result in chronic hepatitis, cirrhosis or hepatoma[8-10]. In infection, viruses first bind specifically to receptors, then complete their replication cycle by many processes such as adsorbing, penetrating, shelling, biosynthesis, assembling and release. Viruses interact with cells, and alter the gene expression of host cells. The process during which the viruses are cleaned by the host immune system results in changes in physiology and pathology of the host[11,12]. It has been reported that liver tissues with hepatitis may appear inflammation, necrosis or hyperplasia, with which over 200 genes are associated[9,13].

Since hepatitis virus infection-associated genes have a wide variety of kinds and complicated functions, it is almost impossible to give insights into their action during liver regeneration at transcriptional level unless high-throughput gene expression profile analysis is performed[14-16]. Therefore, we used the rat genome 230 2.0 array containing 193 genes associated with hepatitis virus infection to detect the gene expression changes in the regenerating liver after 2/3 hepatectomy. A total of 88 genes were found to be associated with LR, and their expression changes, patterns and actions during liver regeneration were primarily analyzed.

## MATERIALS AND METHODS

### Regenerating liver preparation

Healthy SD rats weighing 200-250 g were obtained from the Animal Center of Henan Normal University. The rats were divided into groups at random, 6 rats in each group (Male: Female = 1:1). Partial hepatectomy (PH) was performed as previously described[17], the left and middle lobes of liver were removed. The rats were killed by cervical vertebra dislocation at 0.5, 1, 2, 4, 6, 8, 12, 16, 18, 24, 30, 36, 42, 48, 54, 60, 66, 72, 96, 120, 144 and 168 h after PH and the regenerating livers were observed at corresponding time points. The livers were rinsed three times in PBS at 4℃, then 100-200 mg liver tissue was cut from the middle of right lobe. Six samples were taken from each group and mixed into 1-2 g (0.1-0.2 g × 6) liver tissue, then stored at -80℃. The sham-operation (SO) groups underwent the same PH without removal of the liver lobes. The animal protection laws in China were strictly followed.

### RNA isolation and purification

Total RNA was isolated from frozen livers according to the manual of Trizol kit (Invittrogen)[18] and then purified based on the guide of RNeasy mini kit (Qiagen)[19]. Total RNA sample was checked to exhibit a 2:1 ratio of 28S to 18S rRNA intensities by agarose electrophoresis (180V, 0.5 h). Total RNA concentration and purity were estimated by optical density measurements at 260/280 nm[20].

### cDNA, cRNA synthesis and purification

As a template, 1-8 μg total RNA was used for cDNA synthesis. cDNA and cRNA synthesis was carried out as previously described[21]. cRNA labeled with biotin was synthesized using 12 μL synthesized cDNA as a template, cDNA and cRNA were purifid[21]. Measurement of concentration, purity and quality of cDNA and cRNA was performed as previously reported[20].

### RNA fragmentation and microarray detection

Fifteen microliters (1 μg/μL) cRNA incubated with 5 × fragmentation buffer at 94℃ for 35 min was digested into 35-200 bp fragments. Rat genome 230 2.0 microarry produced by Affymetrix was prehybridized, then the hybridization buffer was centrifuged at 60 r/min for 16 h at 45℃. The microarray was washed and stained with GeneChip fluidics station 450 (Affymetrix Inc., USA). The chips were scanned by GeneChip scan 3000 (Affymetrix Inc., USA), and the signal values of gene expression were observed[22].

### Microarray data analysis

The normalized signal values, signal detections (P, A, M) and experiment/control (Ri) were obtained by quantifing and normalizing the signal values using GCOS1.2[22].

### Normalisation of microarray data

To minimize errors in the microarray analysis, each analysis was performed three times by rat genome 230 2.0 microarray. Results with a maximal total ratio ($R^m$) and the average of three housekeeping genes (β-actin, hexokinase and glyseraldehyde-3-phosphate dehydrogenase) approached to 1.0 ($R^h$) were taken as a reference. Modified data were generated by applying a correction factor ($R^m/R^h$) multiplying the ratio of every gene in $R^h$ at each time point. To remove spurious gene expression changes resulting from errors in the microarray analysis, the gene expression profiles at 0-4 h, 6-12 h and 12-24 h after PH were reorganized by normalization analysis program (NAP) software according to the cell cycle progression of regenerating hepatocytes. Data statistics and cluster analysis were done using GeneMath, GeneSpring, Microsoft Excel softwares[22-24].

### Identification of genes associated with liver regeneration

Firstly, the nomenclature of structure and activity of hepatitis virus (e.g. hepatitis B virus) were adopted from the NCBI (http://www.ncbi.nlm.nih.gov/) and GENEONTOLOGY database (www.geneontology.org/), and input into the databases at AmiGO (http://www.godatabase.org/), NCBI (www.ncbi.nlm.nih.gov/), RGD (rgd.mcw.edu/), MGI (http://www.informatics.jax.org/), UniProt (http://www.pir.uniprot.org/) to identify the rat, mouse and human genes associated with hepatitis virus infection. Then the genes associated with hepatitis virus infection were collated. The results of this analysis were codified, and compared with the results obtained for mouse and human searches in order to identify human genes which are different from those of rats. Compared these genes with the analysis output of the Rat Genome 230 2.0 array, those genes which showed a greater than twofold change in expression level, observed as meaningful expression changes[25], were referred to as rat homologous genes or rat specific genes associated with hepatitis virus infection. Genes, which displayed reproducible results with three independent analyses with the chip and showed a greater than twofold change in expression level in at least one time point during liver regeneration with significant difference ($0.01 \leqslant P < 0.05$) or extremely significant difference ($P$

**Table 1 Expression abundance of 88 hepatitis virus infection-associated genes during LR**

| Gene Abbr. | Involved in others | Fold difference | Gene Abbr. | Involved in others | Fold difference | Gene Abbr. | Involved in others | Fold difference | Gene Abbr. | Involved in others | Fold difference |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 Virus life cycle and virion | | | Ifng | | 6.5 | Bcl2 | | 0.3 | Rgd1306332 | | 0.3 |
| Abce1 | | 2.7 | Il1b | 3 | 0.4 | Casp8 | | 10.6 | Serpinf2 | | 0.2 |
| Ccl2 | 3 | 128.0 | Il1r1 | | 0.5 | Ccl2 | | 128.0 | Snrpd1 | | 4.3 |
| Ccl4 | | 0.2, 3.0 | Il4 | | 2.6 | Cyp17a1 | | 0.3 | Tap1 | | 2.2 |
| Ctbp2 | | 1.4 | Ilf3 | | 4.0 | Cyp2d6 | | 0.3 | Tgfb1 | | 4.0 |
| Ctse | | 2.0 | Mapk8 | | 19.7 | Ddx3x | | 0.4 | Tlr2 | | 10.6 |
| Gfi1 | | 0.2, 2.4 | Mbl2 | | 0.2 | Dffa | | 0.3, 2.8 | Tnf | 2 | 3.2 |
| Hbxip | 2 | 2.0 | Mmp9 | | 9.5 | E2f1 | | 21.2 | Tp53 | 2 | 2.9 |
| Hipk2 | | 0.2, 2.8 | Nfkb1 | | 2.3 | Eif4a1 | | 4.1 | Vapa | | 28.8 |
| Hrmt1l2 | | 0.5, 2.3 | Nr4a1 | | 7.5 | Ephx1 | | 0.4, 2.8 | Vipr1 | | 2.3 |
| Npap60 | | 1.3 | Nrf1 | | 2.4 | Hm13 | | 0.1 | Wnt1 | | 0.5 |
| Oprk1 | | 1.6 | Pcna | | 10.6 | Ifna1 | | 13.0 | Ywhaz | | 2.3 |
| Ppia | | 2.6 | Ptgs2 | 3 | 2.1 | Ikbkb | | 0.3 | 4 Involved in other hepatitis | | |
| Tnip1 | | 0.5 | Ptk2b | | 3.6 | Il1b | 2 | 0.4 | virus | | |
| Ubp1 | | 0.5 | Rfx1 | | 7.9 | Irf1 | | 0.3 | Adarb1 | | 0.4 |
| Wwp1 | | 3.9 | Serpinb3 | | 0.1 | Ltbr | | 0.4 | Ambp | | 5.1 |
| 2 Hepatitis B virus | | | Shc1 | | 0.5 | Mapk1 | | 2.7 | Btbd1 | | 0.5 |
| Creb1 | | 0.5 | Tcf1 | | 6.8 | Mbp | | 0.4 | Copg | | 0.1 |
| Ddb2 | | 2.8 | Timp1 | | 8.6 | Nfkbib | | 11.8 | Copg2 | | 2.8 |
| Egr2 | | 6.8 | Timp3 | | 0.5 | Nolc1 | | 3.7 | Dap3 | | 5.5 |
| Esr1 | | 6.1 | Tnf | 3 | 3.2 | Pitx1 | | 4.6 | Havcr1 | | 16.0 |
| Foxa2 | | 0.4 | Tp53 | 3 | 2.9 | Ptbp2 | | 2.2 | Nd1 | | 0.4 |
| Hbxip | | 0.5 | Xpo1 | | 3.2 | Pten | | 0.5 | Snrpn | | 7.3 |
| Hnf4a | | 4.5 | 3 Hepatitis C virus | | | Ptgs2 | 2 | 0.1, 2.1 | Sos1 | | 0.4, 14.9 |
| Hspa5 | | 0.1 | Apoe | | 0.1 | Rb1 | | 2.6 | | | |

Involved in others: associated with other type of hepatitis virus infection liver diseases.

$\leqslant$ 0.01) between PH and SO, were referred to as associated with liver regeneration.

## RESULTS

### *Expression changes of hepatitis virus infection-associated genes during LR*

According to the data from AmiGO, NCBI, RGD, MGI and UniProt databases, 204 genes were associated with hepatitis virus infection, and 193 of the above 204 genes were contained in rat genome 230 2.0 array. Among them, 88 genes revealed meaningful changes in expression at least at one time point after partial hepatectomy (PH), and showed significant or extreme significant differences in expression when compared with sham operation (SO), and were reproducible in three detections by rat genome 230 2.0 array, suggesting that the genes were associated with LR (Table 1). The analysis indicated that 37 genes were up regulated, 34 genes down-regulated, and 17 genes up/down-regulated during LR. Total expression frequencies of up- and down-regulated genes were 359 and 149, respectively (Figure 1A). The expression patterns varied with the phases in regenerating liver. At the initiation stage of LR (0.5-4 h after PH), 20 genes displayed up-regulation, 14 genes down-, 2 genes up/down-regulation; at the transition phase from G0 to G1 (4-6 h after PH), 15 genes revealed up-, 11 genes down-regulation; at cell proliferation phase (6-66 h after PH), 40 genes showed up-, 26 genes down-regulation, 12 genes up/down-regulation; at cell differentiation and structure-function reorganization stage (66-168 h after PH), 35 genes displayed up-, 17 genes down-, 5 genes up/down-regulation (Figure 1B).

### *Initial and total number of hepatitis virus infection-associated genes in expression at each time point of LR*

At each time point of LR, the number of initially up-, down-regulated and totally up-, down-regulated genes was 13 and 5 at 0.5 h; 6, 5 and 15, 7 at 1 h; 3, 2 and 13, 3 at 2 h; 0, 3 and 13, 6 at 4 h; 2, 3 and 13, 7 at 6 h; 0, 0 and 12, 4 at 8 h; 2, 1 and 16, 3 at 12 h; 8, 5 and 21, 7 at 16 h; 5, 9 and 24, 12 at 18 h; 0, 2 and 23, 11 at 24 h; 3, 0 and 13, 7 at 30 h; 0, 0 and 17, 9 at 36 h; 0, 0 and 14, 5 at 42 h; 3, 3 and 23, 11 at 48 h; 1, 1 and 20, 10 at 54 h; 0, 0 and 19, 7 at 60 h; 0, 0 and 21, 4 at 66 h; 0, 0 and 13, 5 at 72 h; 0, 2 and 13, 10 at 96 h; 1, 0 and 15, 3 at 120 h; 0, 0 and 14, 6 at 144 h; 0, 0 and 14, 7 at 168 h (Figure 2).

### *Expression similarity and time relevance of hepatitis virus infection-associated genes during LR*

A total of 88 genes during LR could be divided into as following: 37 up-regulated, 9 predominantly up-regulated, 34 down-regulated, 6 predominantly down-regulated, and 2 up/down-regulated genes based on their similarity in expression (Figure 3). These 88 genes during liver regeneration could also be classified based on time relevance into 12 groups (0.5 and 1 h, 2 and 4 h, 6 h, 8 and 12 h, 16 and 96 h, 18 and 24 h, 30 and 42 h, 36 and 48 h, 54 and 60 h, 66 and 72 h, 120 and 144 h, 168 h) in which the number

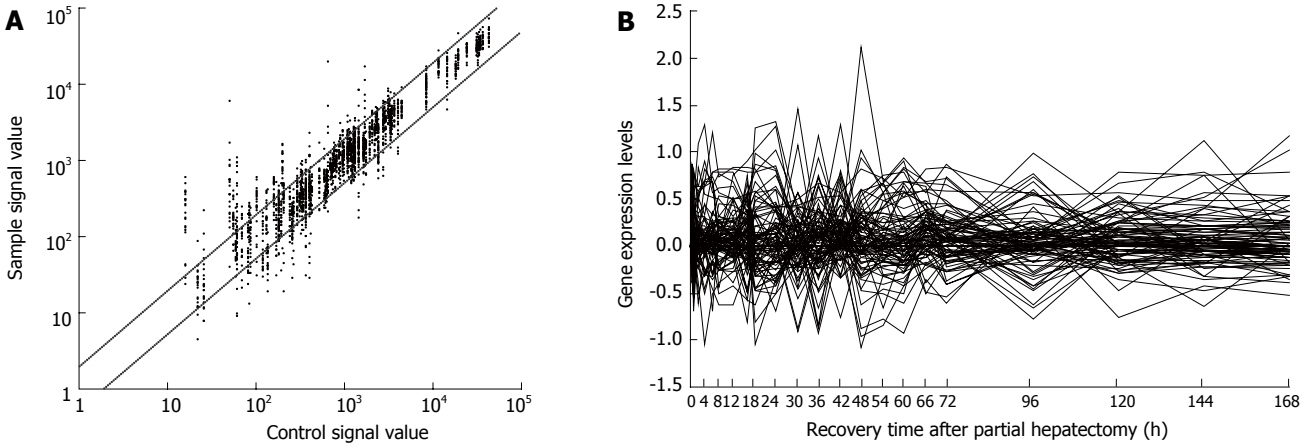


**Figure 1** Expression files of 88 hepatitis virus infection-associated genes in rat liver regeneration. **A**: The abundance and frequency of gene expression, each point represents the signal value of one gene at the corresponding time point. The dots above bias indicate that the genes are more than two-fold up-expressed, the dots under bias indicate that the genes are more than two-fold down-expressed, and the dots between biases indicate that the genes have no alteration in expression. The more far genes are from the bias, the longer the time the greater the gene change; **B**: Expression changes in genes associated with LR.

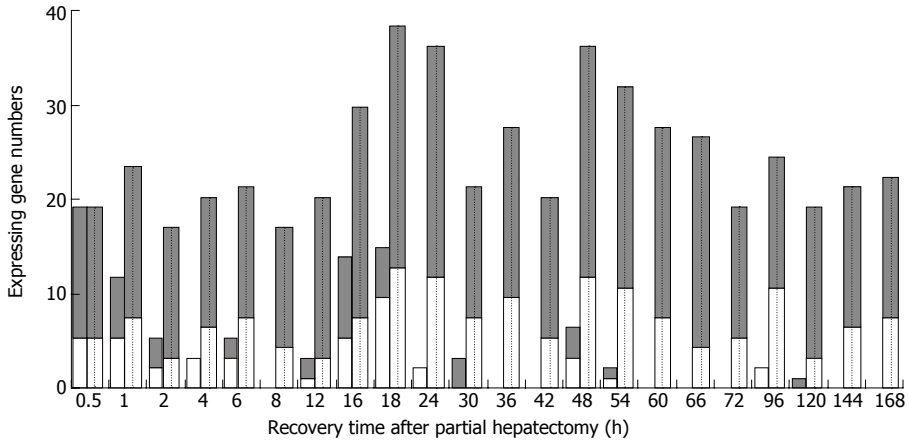


**Figure 2** The initial and total expression profiles of 88 hepatitis virus infection-associated genes at each time point of liver regeneration. Blank bars: the number of initially expressed genes; Dotted bars: the number of totally expressed genes; Grey bars: up-regulated genes; White bars: down-regulated genes.

of up- and down-regulated genes was 28 and 12, 26 and 9, 13 and 7, 28 and 7, 34 and 17, 47 and 23, 27 and 12, 40 and 20, 39 and 17, 34 and 9, 29 and 9, 14 and 7 (Figure 3).

### *Expression patterns of hepatitis virus infection-associated genes during LR*

The expression patterns of 88 genes during liver regeneration might be categorized into 23 types according to the expression changes: (1) up-regulated expression at one time point, i.e. 30, 48, 120 h after PH (Figure 4A-C and E) with 4 genes involved; (2) up-regulated expression at two time points, i.e. 30 and 42 h, 16 and 42 h, 16 and 96 h, 18 and 54 h (Figure 4A and B) with 4 genes involved; (3) up-regulated expression at multiple time points (Figure 4A and B) with 5 genes involved; (4) up-regulated expression at one phase, i.e. 1-168 h, 16-96 h (Figure 4B and E) with 2 genes involved; (5) up-regulation expression at two phases, i.e. 16-24 and 42-48 h (Figure 4B) with 1 gene involved; (6) up-regulated expression at multiple phases (Figure 4E) with 1 gene involved; (7) up-regulated expression at one time point/one phase, i.e. 54 and 144-168 h (Figure 4C) with 1 gene involved; (8) up-regulated expression at one time point/two phases (Figure 4B-D) with 4 genes involved; (9) up-regulated expression at one time point/multiple phases (Figure 4E and J) with 2 genes involved; (10) up-regulated expression at two time points/one phase (Figure 4B, D and E) with 3 genes involved; (11) up-regulated expression at two time points/two phases (Figure 4B and D) with 2 genes involved; (12) up-regulated expression at two time points/multiple phases (Figure 4B, D and E) with 3 genes involved; (13) up-regulated expression at multiple time points/one phase (Figure 4A and C) with 2 genes involved; (14) up-regulated expression at multiple time points/two phases (Figure 4C, D and E) with 3 genes involved; (15) down-regulated expression at one time point, i.e. 2, 4, 6, 16, 18, 24, 48 , 96h (Figure 4F, G and H) with 14 genes involved; (16) down-regulated expression at two time points, i.e. 0.5 and 4 h, 16 and 96 h, 18 and 54 h (Figure 4G, H and I) with 3 genes involved; (17) down-regulated expression at multiple time points (Figure 4G, H and I) with 6 genes involved; (18) down-regulated expression at one phase, i.e. 4-6 h, 18-24 h, 54-60 h (Figure 4G and H) with 3 genes involved; (19) down-regulated expression at one time point and one phase, i.e. 1 and 144-168 h, 48 and 18-24 h, 30 and 2-8 h (Figure 4H, I and J) with 3 genes involved; (20) down-regulated expression at one time point/two phases (Figure 4I) with 1 gene involved; (21) down-regulated expression at two time points/one phase

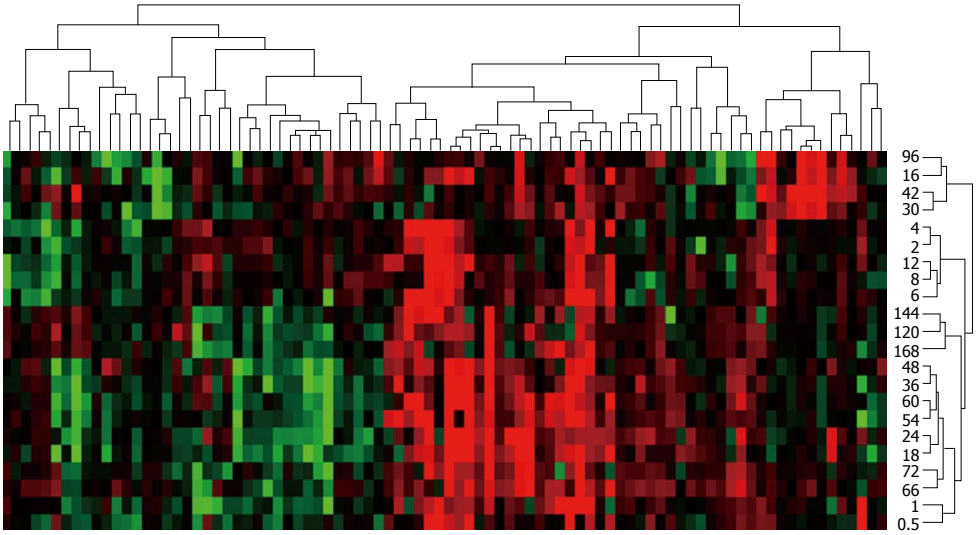


**Figure 3** Expression and time series clusters of 88 hepatitis virus infection-associated genes during liver regeneration. Detection data of rat genome 230 2.0 array were analyzed by H-clustering. Red: up-regulated genes; Green: down-regulated genes; Black: no-sense genes in expression; Upper and right trees showing expression and time series clusters, respectively.

(Figure 4I) with 1 gene involved; (22) down-regulated expression at two time points/two phases (Figure 4I) with 3 genes involved; (23) up/down-regulated expression (Figure 4B, H and J) with 17 genes involved.

## DISCUSSION

The role of 193 genes associated with hepatitis virus infection in liver regeneration was studied in this study. Among the 45 genes associated with hepatitis virus life cycle and virion, peptidylprolyl isomerase A (PPIA), nuclear pore associated protein (NPAP60) and opioid receptor kappa 1 (OPRκ1) are involved in viral infection[26]. Eight genes, including chemokine ligand 2 (CCL2), WW domain containing E3 ubiquitin protein ligase 1 (WWP1), chemokine ligand 3 (CCL3), C-terminal binding protein 2 (CTBP2), cathepsin E (CTSE), TNFAIP3 interacting protein 1 (TNIP1), upstream binding protein 1 (UBP1) and heterogeneous nuclear ribonucleoproteins methyltransferase-like 2 (HRMT1L2), participate in the viral genome replication and transcription[27,28]. Hbx interacting protein (HBXIP) suppresses HBV X protein activity[29]. ATP-binding cassette sub-family E member 1 (ABCE1) plays a part in viral capsid assembly[30]. Homeodomain interacting protein kinase 2 (HIPK2) relates to induction of apoptosis[31]. Growth factor independent 1 (GFI1) controls the differentiation of dendritic cells into macrophages[32]. The above genes tend to have a same or similar expression at some time points, but a different expression at other time points which may enhance inflammatory reaction and immunity of regenerating liver.

Of the 65 genes associated with hepatitis B virus infection, forkhead box A2 (FOXA2), interleukin 4 (IL4) and transcription factor 1 (TCF1) may restrain HBV gene replication and RNA transcription[33,34], whereas regulatory factor X 1 (RFX1) promotes RNA transcription by binding to HBV enhancer[35]. Tissue inhibitor of metalloproteinases 1, 3 (TIMP1, TIMP3) exhibits low level expression after HBV transinfection[36], but matrix metallopeptidase 9 (MMP9) displays high activity after HBV transinfection[36]. Interferon gamma (IFNγ) supresses hepatic cell proliferation[37]. CAMP responsive element binding protein 1 (CREB1), nuclear respiratory factor 1 (NRF1) and hepatocyte nuclear factor 4 alpha (HNF4α) activate HBV promoter[34,38,39]. Nine genes, such as early growth response 2 (EGR2), proliferating cell nuclear antigen (PCNA), tumor suppressor p53 (TP53), serpin peptidase inhibitor clade B member 3 (SERPINB3), prostaglandin-endoperoxide synthase 2 (PTGS2), SHC transforming protein 1 (SHC1), interleukin enhancer binding factor 3 (ILF3), nuclear factor of kappa light chain gene enhancer in B-cells 1 (NFκB1) and mitogen-activated protein kinase 8 (MAPK8), accelerate cell proliferation[40-45]. Tumor necrosis factor (TNF) and estrogen receptor 1 (ESR1) increase cell susceptibility to HBV infection[46,47]. 70kDa heat shock protein 5 (HSPA5) plays a role in anti-apoptosis[48]. Protein tyrosine kinase 2 beta (PTK2β) promotes migration and invasion of glioblastoma[49]. Exportin 1 (XPO1) takes part in HBV-induced aberrant centriole replication and abnormal mitotic spindles[50]. Mannose binding lectin 2 (MBL2) participates in HBV infection[51]. Interleukin 1 beta (IL1β) may enhance resistance to chronic diseases[52]. Interleukin 1 receptor type I (IL1R1) elevates blood-brain barrier[53]. Damage specific DNA binding protein 2 (DDB2) promotes turnover of HBV X protein[54]. Nuclear receptor subfamily 4 group A member 1 (NR4A1) enhances role of the HBx-induced Fas/FasL signaling pathway[55]. The above genes may have same or similar expression changes at some time points, but different expression changes at other time points, suggesting that they promote inflammation recovery and strengthen anti-hepatitis B virus infection ability.

Among the 90 genes associated with hepatitis C virus infection, Three genes concluding polypyrimidine-tract binding protein 2 (PTBP2), mitogen-activated protein kinase 1 (MAPK1) and interferon alpha 1 (IFNα1) play a role in suppression of hepatitis C virus replication[56-58]. Nucleolar and coiled-body phosphoprotein 1 (NOLC1), transporter 1 ATP-binding cassette sub-family B (TAP1) and eukaryotic translation initiation factor 4A1 (EIF4A1) promote protein biosynthesis[59]. Six genes including toll-like receptor (TLR2), transforming growth factor beta 1 (TGFβ1), tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta (YWHAζ),

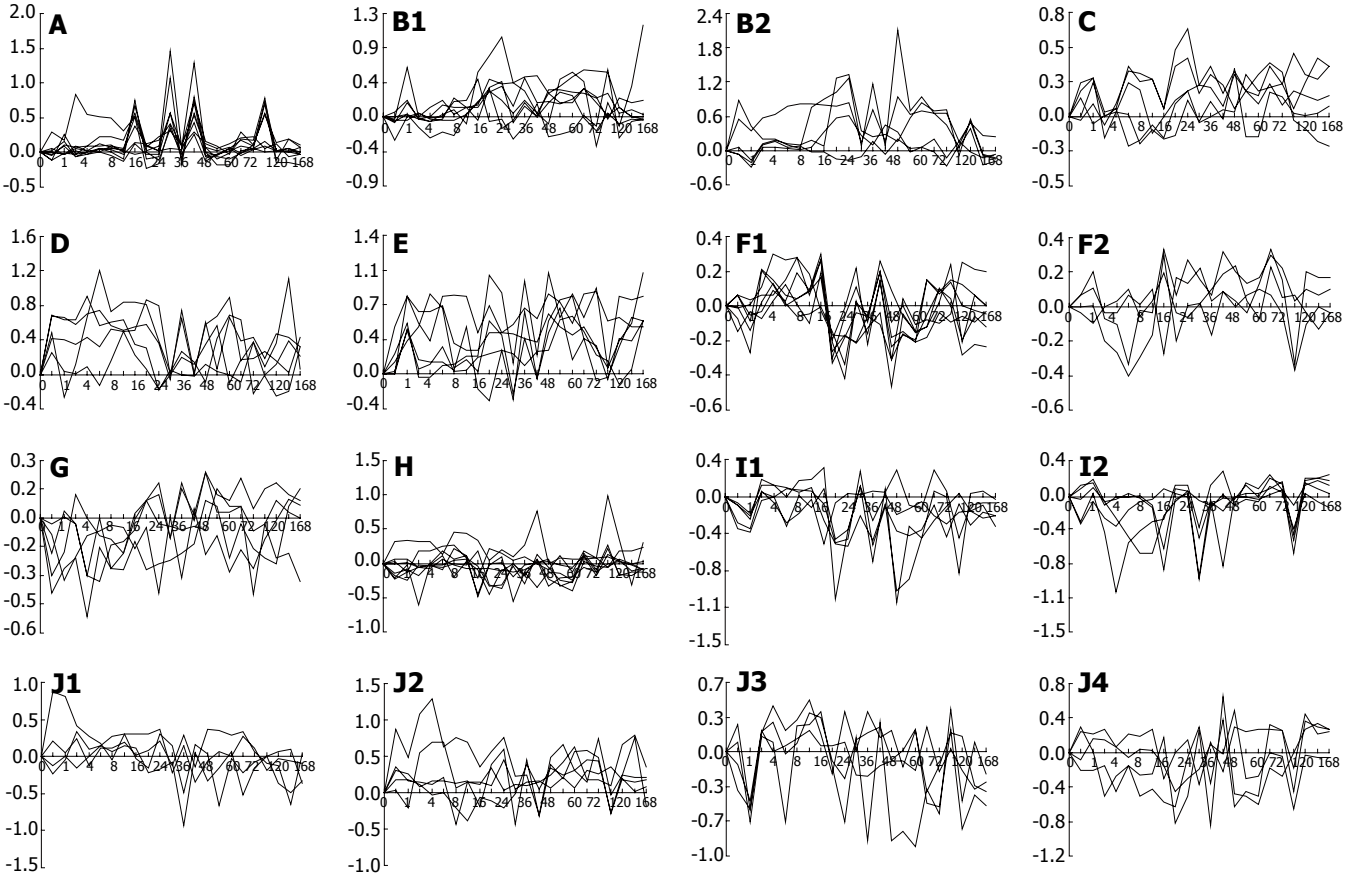


**Figure 4** Expression patterns of 88 hepatitis virus infection-associated genes during liver regeneration. Expression patterns of these genes exhibit 23 types. **A-E**: up-regulated expression; **F-I**: down-regulated expression; **J**: up/down-regulated expression. X-axis represents recovery time after PH (h), Y-axis shows logarithm ratio of the signal values of genes at each time point to control.

vasoactive intestinal peptide receptor 1 (VIPR1), cytochrome P450 family 2 subfamily D polypeptide 6 (CYP2D6) and lymphotoxin B receptor (LTBR), are related to immune reponse[60,61]. VAMP-associated protein A (VAPA) accelerates HCV replication[62]. Nuclear factor of kappa light chain gene enhancer in B-cell inhibitor beta (NFκBIβ) and E2F transcription factor 1 (E2F1) have transcriptional activator activity[63]. Inhibitor of kappaB kinase beta (IKκBKβ) inhibits activation of NFκB. Small nuclear ribonucleoprotein D1 (SNRPD1) and serine peptidase inhibitor clade F member 2 (SERPINF2) can repair liver damage[64,65]. Paired-like homeodomain transcription factor 1 (PITX1) modulates interferon expression[66]. Genetic polymorphisms of cytochrome P450 family 17 subfamily alpha polypeptide 1 (CYP17α1) and epoxide hydrolase 1 (EPHX1) are closely associated with liver disease[67,68]. Four genes containing interferon regulatory factor 1 (IRF1), phosphatase and tensin homolog (PTEN), myelin basic protein (MBP) and DEAD box polypeptide 3 X-linked (DDX3X) are associated with regulation of liver disease[69-72]. Apolipoprotein E (APOE) has a role in liver protection[73]. Retinoblastoma 1 (RB1) and caspase 8 (CASP8) inhibit cell growth[74,75]. Wingless-type MMTV integration site family member 1 (WNT1) can inhibit cell apoptosis[76]. B-cell leukemia/lymphoma 2 (BCL2) is associated with infectious vasculitis[77]. Histocompatibility antigen 13 (HM13) is responsible for immunological recognition[78]. HM13 is highly expressed in human liver HL-7702 cells as HCV NS3-transactivated protein 1 (RGD1306332)[79]. DNA fragmentation factor alpha subunit (DFFα) plays a role in suppressing tumors[80]. The above genes may have the same or similar expression changes at some time points, different expression changes at other time points, thus promoting inflammatory reaction and anti-infection ability of regenerating liver.

In summary, the expression changes of hepatitis virus infection-associated genes after rat partial hepatectomy can be analyzed with high-throughput gene expression profiling. Immunological competence, abilities of anti-inflammation and anti-infection are increased during liver regeneration. Rat genome 230 2.0 array is a useful tool for analyzing hepatitis virus infection and liver disease at transcriptional level. However, DNA→ mRNA→protein is affected by many factors including protein interaction. Further study is needed to confirm the results at cell level.

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*BASIC RESEARCH*

# Sorbitol-based osmotic diarrhea: Possible causes and mechanism of prevention investigated in rats

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## Abstract

**AIM:** To study the possible causes of sorbitol (S)-based diarrhea and its mechanism of reduction by rice gruel (RG) in cecectomized rats.

**METHODS:** S was dissolved either in distilled water or in RG (50 g/L) and ingested as a single oral dose (1.2 g/kg body mass, containing 0.5 g/L phenol red as a recovery marker) by S (control) and S + RG groups ($n$ = 7), respectively. This dose is over the laxative dose for humans. Animals were sacrificed exactly 1 h after dose ingestion, without any access to drinking water. The whole gastro-intestinal tract was divided into seven segments and sampled to analyze the S and marker remaining in its contents.

**RESULTS:** Gastric-emptying and intestinal transit were comparatively slower in the S + RG group. Also, the S absorption index in the 3rd and last quarter of the small intestine (24.85 ± 18.88% *vs* 0.0 ± 0.0% and 39.09 ± 32.75% *vs* 0.0 ± 0.0%, respectively, $P < 0.05$) was significantly higher in the S + RG group than in the control group. The S absorption index and the intestinal fluid volume are inversely related to each other.

**CONCLUSION:** The intestinal mal-absorption of S is the main reason for S-based osmotic diarrhea. Where RG enhanced the absorption of S through passive diffusion, the degree of diarrhea was reduced in cecectomized rats.



**Key words:** Cecectomized rat; Sorbitol; Diarrhea; Rice gruel; Phenol red

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## INTRODUCTION

Sugar alcohols are widely used as sugar-substitute sweeteners in the food industry, especially in candy, chewing gum, dietetics and diabetic foods, for their lower calorific value and various beneficial effects on health. Among the many sugar alcohols, sorbitol (S) is chosen most often because of its functional, nutritional, and disease-preventative effects impacting positively on health. However, higher dose or over-intake of S causes bloating, flatulence, cramping, abdominal pain and diarrhea both in adults[1] and in children[2]. As the laxative threshold of S varies from person to person, the actual cause of S-based gastrointestinal complications is still unclear. Several possible and dissimilar causes have been previously indicated by a number of investigators[3-7], although most of them reported that diarrhea was caused by increasing colonic osmolality when higher doses were consumed. Soergel reported that indigestible carbohydrate-induced diarrhea occurs when the amount of carbohydrate entering the colon exceeds its fermentation capacity[3]. In another report, it was stated that intestinal mal-absorption is the main reason for diarrhea[4]. Read *et al*[5] reported that the tendency for diarrhea in response to a meal containing un-absorbable carbohydrate depends more on the lack of colonic accommodation than on the small intestinal transit. Short chain fatty acids (SCFAs), the fermentation product of sugar alcohol, were reported to decrease intestinal or colonic water absorption, resulting in diarrhea[6]. Some other investigators found higher lactic and succinic acid levels during diarrhea in the colon of cecectomized rats[7] and in pig feces[8], and thought that the above fermentation products may increase the colonic osmolality to cause diarrhea. Although higher colonic osmolality and lower fluid absorption from the colon are indicated in the above studies as the main reasons for indigestible or fermentable material-based diarrhea, the actual cause of the induction of large gut osmolality is a matter of controversy.

The major difference between the digestive physiology of the rat and humans is that the rat has a large voluminous cecum, whereas humans have no distinct cecum. The cecum in rat acts as an important reservoir and fermentation site especially for indigestible and fermentable materials. Recently, we reported that the digestive physiology of cecectomized rats is more analogous to humans than to normal rats[7]. It was also proposed that the cecectomized rat could be a useful experimental tool to study the physiological effects of indigestible or fermentable materials in humans. In

another study[9], we found that rice gruel (RG), a source of rice starch, significantly reduced the degree of S-based diarrhea in cecectomized rats. In the report of that study, we hypothesized that in the presence of the extra glucose from rice gruel, the influx of S across the luminal membrane increased significantly[10]. This may enhance the small intestinal absorption of S in the presence of the extra glucose released from RG, and only a small amount of S is transported to the colon from the small intestine for fermentation[9]. By the above proposed mechanisms, the colonic organic acid concentration as well as the osmolality was decreased and hard feces were formed in the mid to distal colon of the cecectomized rats. As a result, the degree of diarrhea was reduced in these rats[9].

The present study was undertaken to further examine our above hypothesis that small intestinal mal-absorption, or perhaps rather inadequate fermentation of S or mal-absorption of S fermentation products, is responsible for increasing the colonic osmolality as well as the occurring osmotic diarrhea, and to examine the mechanisms involved in reducing the degree of S-based diarrhea by RG in cecectomized rats.

## MATERIALS AND METHODS

### Animals

Three week-old male juvenile Wistar rats (50.3 ± 3.55 g body mass, Nippon SLC, Hamamatsu, Japan) were housed identically as reported in our previous study[7]. Animals were allowed free access to a diet of commercial pellets (Labo MR Stock, Nihon Nosan Kogyo K.K. Ltd., Tsukuba, Japan) and drinking water for the initial 3 wk, in their acclimatization period. After this period, when the mean body mass of the animals was 184.23 ± 12.85 g, cecectomy of all animals was performed according to our previous report[7]. After a 2 wk recovery period with free access to the commercial pellets and drinking water, animals were randomly divided into two subgroups of 7 animals each as the S (control) and S + RG groups with a similar mean body mass in each group (314.6 ± 8.94 g). They were fasted overnight (16 h) with free access to drinking water only, and then experimental diets were provided as a single bolus by gastric intubations. Animals were maintained according to the rules and regulations of the animal experiments committee of Okayama University.

### Rice gruel preparation

Rice gruel (50 g/L) was prepared according to the procedures described in our previous report[9]. Briefly, 100 g *japonica* rice (*Oryza sativa* L.) was soaked in 200-250 mL of distilled water for about 1 h, and blended until reaching a dense milky appearance. The volume of blended rice was made up to 2.2 L with distilled water and boiled over a gas burner with continuous stirring until the final volume was 2 L.

### Feeding and sampling

For a single oral dose, animals were lightly anesthetized with diethyl ether then S (90 g/L) (Sorbitol, Nacalai Tesque Inc., Kyoto, Japan; Lot no. M3G8055) was dissolved either in distilled water or in RG and ingested as a single oral dose (1.2 g/kg b.w., containing 0.5 g/L phenol red as a recovery marker) to the S (control) and S + RG groups ($n$ = 7) respectively. This dose is over the laxative dose for humans. Animals were sacrificed by diethyl ether anesthesia exactly 1 h after dose administration, without any access to drinking water[11]. The whole gastro-intestinal tract was removed as quickly as possible and frozen in dry-ice-acetone to prevent the movement of gastro-intestinal contents, and immediately preserved at -30℃ for subsequent analysis.

### Analytical methods

The frozen gastrointestinal tract was defrosted over the ice bag, weighed and subdivided into the stomach, small intestine (1st, 2nd, 3rd and last quarter), the proximal half and the distal half of the colon. The contents of each segment were separately collected, homogenized and centrifuged according to the procedures described by Soontornchai *et al*[12]. The mass of the contents of each gastrointestinal segment was calculated from the mass of tissue with and without contents. The collected supernatants were analyzed colorimetrically (UV 1200, Shimadzu Corporation, Kyoto, Japan) for phenol red with bile acid correction[13]. For S analysis, supernatants were deproteinized, derivatized and analyzed by HPLC (Stainless steel column: Inertsil ODS-80A 150 mm × 4.6 mm i.d., Dectector: Shimadzu SPD-10A UV-VIS, Shimadzu Corporation, Kyoto, Japan) methods as described by Miwa *et al*[14]. For maximum recovery of phenol red from gut segments, the tissue of each segment was thoroughly homogenized in 5 mL physiological saline with a rotary stainless steel homogenizer in a centrifuge tube, and centrifuged at 45000 × $g$ at 4℃ for 15 min (Himac-GX series: Himac CS 100GX micro ultra centrifuge, Hitachi Ltd., Tokyo, Japan). The collected supernatants were treated similarly for measurement of phenol red.

### Calculations

The gastric emptying, intestinal transit, and S absorption indexes for each segment of intestine were calculated by the following equations, which were slightly modified from those of Reynell and Spray[11].

Gastric emptying (%) =

$$\frac{\textit{Total phenol red recovered from the int estinal tract}}{\textit{Total phenol red recovered from whole gastro int estinal tract}} \times 100$$

Intestinal transit from the 1st to the last quarter of the small intestine and the proximal colon of the large intestine was calculated by the following formula.

Digesta transit in a certain quarter or part of the intestine (%) = $\frac{a}{b} \times 100$

Where, "a" is the amount of phenol red recovered from the 1st quarter of the small intestine to the distal colon excluding the amount of phenol red recovered from that respective quarter or the part of colon, and "b" is the total amount of recovered phenol red from that respective quarter to distal colon.

The S absorption index in a certain quarter of the small intestine and in the proximal and distal colon was calculated by the following formula.

Sorbitol absorption index (%) = $\left(1 - \frac{a/b}{c/d}\right) \times 100$

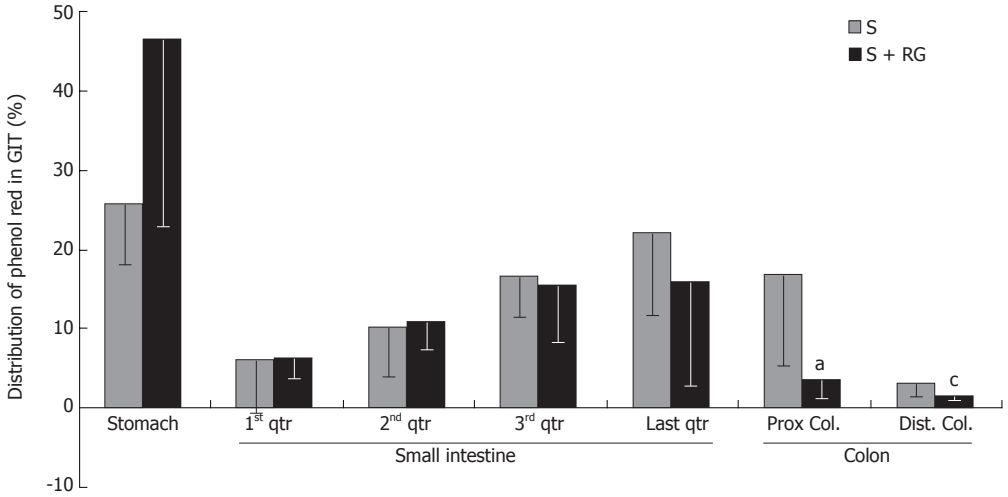


**Figure 1** Distribution of marker (phenol red) in the different segments of the gastrointestinal tract 1 h after the dose ingestion. $^{a}P = 0.035$, $^{c}P = 0.045$ *vs* S group.

**Table 1 Transit of the marker (phenol red) in the different segments of the intestinal tract during 1 h after the dose ingestion (mean ± SD)**

| Group | Small intestine | | | | Colon |
|---|---|---|---|---|---|
| | 1st quarter | 2nd quarter | 3rd quarter | Last quarter | Proximal |
| Sorbitol (%) | 91.94 ± 8.74 | 84.56 ± 10.2 | 69.66 ± 15.42 | 44.22 ± 19.68 | 2.48 ± 2.09 |
| S + RG (%) | 87.40 ± 9.05 | 76.39 ± 10.4 | 56.97 ± 16.04 | 36.48 ± 17.96 | 3.07 ± 2.27 |

Values indicate the amount of marker (phenol red) passing into the following segments, as a percentage of the total marker that entered that particular segment.

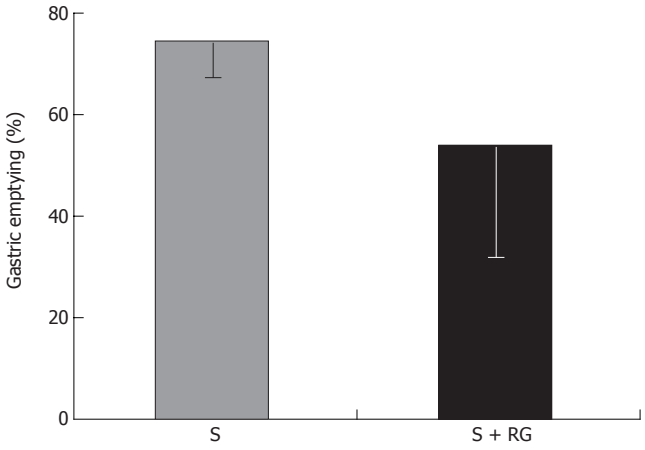


**Figure 2** Gastric emptying 1 h after the dose ingestion.

Where, "a" is the total amount of S recovered from that respective segment and "b" is the total amount of phenol recovered from the same segment of the small intestine; "c" and "d" are the amount of S and phenol red injected into corresponding animal, respectively. Put simply, the value of the S absorption index (%) for a certain segment means the percentage of total S passing through that segment that was absorbed.

### *Statistical analysis*

Data are presented as means and standard deviations for 7 animals. Statistical analyses were performed between control (S) and S + RG groups by the Tukey-Kramer's test using statistical software (Statview, Version 5.0, SAS Institute Inc., USA). Significance of difference was considered to be at $P < 0.05$.

## RESULTS

Phenol red was used as a recovery marker to measure the gastric emptying, intestinal transit, and intestinal S absorption index in this experiment. The mean recovery of the phenol red from the gastrointestinal tract was 93.8% ± 1.56% of the given amount. A very small amount of phenol red was detected in the distal colon (Figure 1) and no feces were defecated in either group during the 1 h period after the dose. Although the marker concentration did not vary significantly (data not shown) between the groups for different volumes of intestinal fluid, the distribution of marker from the stomach to the different segments of the intestine showed that the marker concentration was relatively lower in the distal part of the small intestine, and was significantly lower in the proximal (3.54% ± 2.47% *vs* 16.77% ± 11.48%, $P = 0.035$) and distal (1.38% ± 0.38% *vs* 3.07% ± 1.55%, $P = 0.045$) colon for the S + RG group relative to the S group (Figure 1). The concentration of marker in the stomach of the S + RG group was comparatively higher (46.29% ± 23.33% *vs* 25.69% ± 7.54%, $P = 0.097$) than for the S group (Figure 1) due to slower (53.78% ± 22.02% *vs* 74.3% ± 7.12%, $P = 0.098$) gastric emptying (Figure 2). As a result of the slower gastric emptying, a smaller amount of marker was delivered to the small intestine and onward at a given time in the S + RG fed group compared to the S group. For the same reason, digesta transit in the different segments of the gastrointestinal tract was relatively slower in the S + RG group than in the S-only group (Table 1).

The index for S absorption in the distal part (3rd and last quarters) of the gastrointestinal tract was greatly modified by the addition of RG to the S (Figure 3). Although the S absorption index was relatively lower in the 1st quarter of the small intestine for the S + RG group, it gradually increased from the 3rd to the last quarter of small intestine,

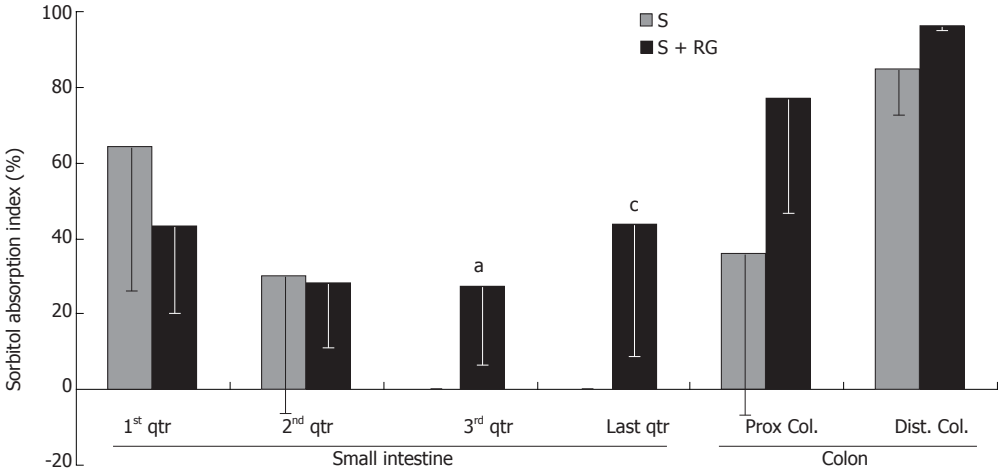


**Figure 3** Sorbitol absorption index in the different segments of the intestinal tract during the 1 h period after dose ingestion. $^{a}P = 0.016$, $^{c}P = 0.023$ *vs* S group.

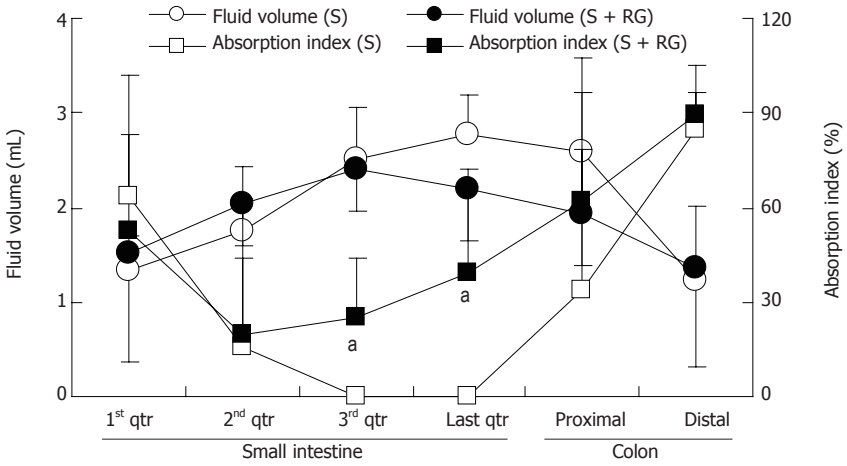


**Figure 4** Relationship between the intestinal fluid volume and sorbitol absorption index of S and S + RG ingestion groups. One gram of intestinal contents was considered as 1 mL for the calculation of the volume of intestinal fluid. $^{a}P < 0.05$ *vs* S group.

and to the distal part of the colon. Significant differences were found at the 3rd and last quarters of the small intestine (24.85% ± 18.88% *vs* 0.0 ± 0.0% and 39.09% ± 32.75% *vs* 0.0 ± 0.0%, respectively, $P < 0.05$) when compared to the S group. It was noted that the absorption index in these two segments for the S group was zero.

The relationship between the intestinal fluid volume and the S absorption index was inversely proportional for both the S and S + RG groups (Figure 4). In other words, the lower the fluid volume, the higher the absorption index of S, in both groups. The linear correlations between the S absorption index and the intestinal fluid volume for the different segments of the small intestine and colon are presented in Figure 5. From these correlations, although the S absorption index was significantly ($P = 0.006$) higher for the S group than the S + RG group in the first quarter of the small intestine, significant but inverse results were observed from the 2nd and last quarters of the small intestine and in the proximal colon of the large intestine ($P = 0.056$, 0.0085, 0.025, respectively). In addition, the overall S absorption index in the whole intestine was significantly ($P < 0.0001$) higher for the S + RG group than for the S group (Figure 5).

## DISCUSSION

When a moderate amount of S (up to 30 g) is taken by a human, a very small amount of it is slowly absorbed in the small intestine through passive diffusion, while most enters into the colon for bacterial fermentation to produce various short chain fatty acids (SCFAs), and less than 10% of the ingested amount is excreted in the feces[15,16]. Higher concentrations of SCFAs, especially succinic and lactic acid, in the colon are responsible for increased colonic osmolality and cause osmotic diarrhea in cecectomized rats[7,9] and pigs[8]. It was reported that ingestion of 30-50 g of S (500-800 mg/kg b.w.) caused diarrhea in adult humans[17,18]. In this study, a non-physiological high and osmotically-active single oral dose of S (1200 mg/kg b.w.) was ingested to study the effects of RG on intestinal absorption of S. This is also more than the laxative threshold dose of S for humans (500-800 mg/kg).

A non-absorbable water-soluble recovery marker (phenol red) was used in this study to precisely measure the gastric emptying, intestinal transit and intestinal absorption indexes of S. It has been reported that there may be some binding of phenol red by the stomach tissue, even if the rat is killed within seconds of intubation[11]. Therefore, the phenol red was extracted from both the gastrointestinal contents and their respective tissues for measurement in this experiment, and a substantial amount of marker was recovered from the gut tissues (13.86% ± 4.62% of the total recovery).

The total recovery of the marker was 93.8% ± 1.56% of the given amount. Urine was not sampled for determination of the concentration of either S or for phenol red,

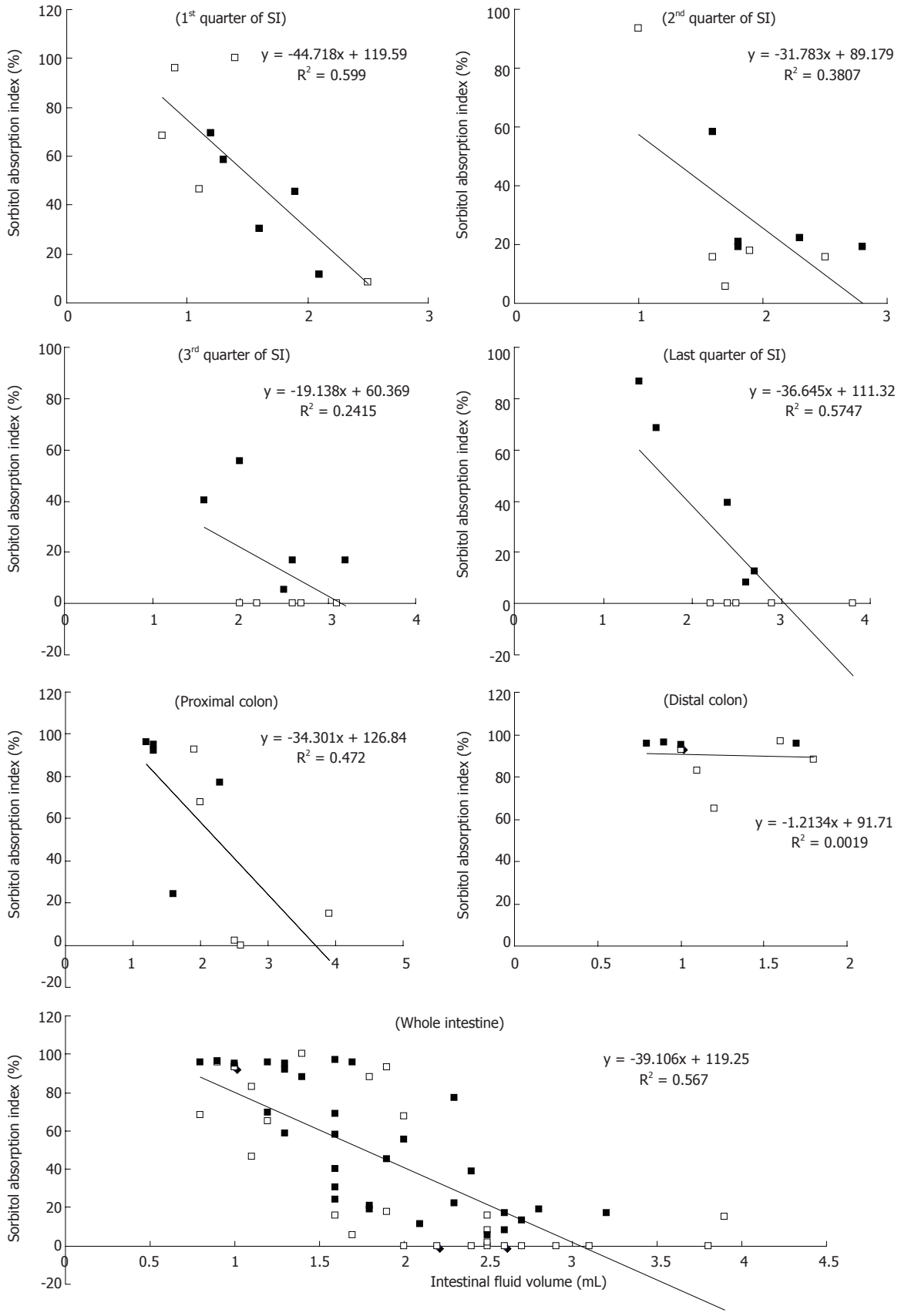


**Figure 5** Correlation between the sorbitol absorption index (Y axis) and intestinal fluid volume (X axis) of S (□) and S + RG (■) groups in the different segments of the small intestine (SI), colon, and whole intestine. The *P* values indicate significant differences in the 1st quarter ($P = 0.0064$), 2nd quarter ($P = 0.056$), last quarter ($P = 0.0085$) of the small intestine; proximal colon of large intestine ($P = 0.0259$); and for the whole intestine ($P < 0.0001$) when S and S + RG groups are compared.

as it is a non-absorbable marker and a very trace amount only can be detected in the urine[11]. The excretion of S in the urine is also only of trace amounts, either with a low or high single oral dose[19].

The determination of the concentration of S along with a non-absorbable marker in the different parts of the digestive tract after a single bolus dose with or without RG is an important investigation that may clarify the effects

of RG on S absorption in the small intestine. In a recent study, Islam *et al*[9] reported that intestinal transit and mean retention time of S are relatively longer when S is fed with RG rather than alone. From the present experiment, it was confirmed that slower gastric emptying (Figure 2) in the S + RG group compared to the S group caused slower digesta transit in the different segments of the gastrointestinal tract.

Rice starch (RS) contained in RG is a bulk aggregation ($n$ = 20-60) of smaller granules (3-8 μm in diameter) than in corn starch (5-15 μm in diameter), and is larger in size (0.99 kg/L) and denser in structure than corn starch (0.63 kg/L)[20]; it was found to be degraded slowly by pancreatic amylase and gradually released oligosaccharides and glucose at a slower rate in the intestinal brush border than corn starch[21]. Therefore, the rate of intestinal transit of S was relatively slower when ingested with RG, rather than alone. After consumption of S, a shorter orocecal transit time has been reported as a cause of S intolerance in a human-based study[22]. The slower gastric emptying and slower intestinal transit may result in maintenance of a constant normal osmotic environment in the inner intestinal lumen and facilitation of S absorption to prevent osmotic diarrhea in cecectomized rats[9]. In addition, slower gastric emptying and slower intestinal transit increases small intestinal mineral absorption in rats[23]. The energy value of sugar alcohols also depends on the extent to which they are absorbed along the small intestine[24]. *In vivo* and *in vitro* studies showed that longer transit times in the large intestine can have profound effects on bacterial physiology and metabolism, leading to breakdown of protein and fermentation of amino acids making an increased contribution to colonic SCFA pools[25-28]. Through this mechanism, RG increases the nutritional value of S not only by increasing the energy value through small intestinal absorption, but also by preventing the possible risk of osmotic diarrhea.

The S absorption index in the 3$^{rd}$ to the last quarter of the small intestine was significantly increased in this experiment when ingested with RG. In contrast, no S was absorbed from the above region during a 1 h period after the ingestion of S only. It was found that the absorption index of S in the small intestine was increased by the addition of RG, but the actual location(s) of increased absorption was not clear from the results of the single oral dose study. However, from the existing data it can be assumed that the highest amount of S was absorbed in the upper half of the small intestine, for both groups. Further study using a continuous feeding method could confirm the quantitative information on S absorption from the small intestinal tract. However, it was previously reported that RG releases an increased amount of glucose at the luminal surface as well as over a large segment of the small intestine[21]. In the presence of extra glucose, the influx of S across the luminal membrane increased significantly[10]. Moreover, slower gastric emptying and slower transit time caused by RG lengthened the contact time between S and intestinal mucosa, and facilitated the absorption of S by passive diffusion[29]. Although RG releases an increased amount of glucose, it avoids creating additional osmolality at the luminal surface, because the release of glucose occurs slowly from the polysaccharide and is over a large segment of the intestine[21]. The higher absorption of S in the presence of extra glucose prevented hyper-osmolality in the intestinal lumen, which prevented osmotic diarrhea in cecectomized rats.

Transit and volume of the intestinal fluid are important determinants of the degree of digestion and absorption in the small intestine. Both parameters, when they are increased excessively, have the potential to reduce degradation and absorption by limiting substrate contact with the mucosal surface[30,31]. The relatively slower transit and lower intestinal fluid volume in the S + RG group than in the S group enhanced the absorption of S from distal parts of the small intestine and colon. A linear correlation between the S absorption index and fluid volume was found in this study (Figure 5).

From the results of our present study it can be summarized that the possible causes of indigestible but fermentable material-based diarrhea are: (1) faster intestinal transit; (2) small intestinal mal-absorption; (3) incomplete colonic fermentation of S, and (4) slower absorption of some organic acids (e.g., lactic, succinic) from the colon.

The above summarized points are interrelated, with a sequential tendency, and are supported by some previously reported proposals of Rambaud *et al*[4], Ammon *et al*[6], and Islam *et al*[7].

In summary, our study shows that RG plays an important role in stimulating the small intestinal absorption of S. As a result, a smaller amount of S entered into the colon for fermentation. Moreover, due to a slower transit time, the S that entered the colon was completely fermented to rapidly absorbable SCFAs and this prevented the possible increment of osmolality by some organic acids (e.g., lactic, succinic). These may be the mechanisms by which hard feces were formed in the mid to distal colon of the cecectomized rats.

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*BASIC RESEARCH*

# Effective regularity in modulation on gastric motility induced by different acupoint stimulation

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## Abstract

**AIM:** To investigate whether manual acupuncture at representative acupoints in different parts of the body can modulate responses of gastric motility in rats and regular effects in different acupoint stimulation.

**METHODS:** The gastric motor activity of rats was recorded by the intrapyloric balloon. The changes of gastric motility induced by the stimulation were compared with the background activity in intragastric pressure and/or waves of gastric contraction recorded before any stimulation. Morphological study was also conducted by observing the Evans dye extravasation in the skin after mustard oil injection into the intragastric mucous membrane to certify cutaneous innervations of blue dots related to gastric segmental innervations.

**RESULTS:** In all six rats that received mustard oil injections into intragastric mucosa, small blue dots appeared in the skin over the whole abdomen, but mainly in peri-midline upper- and middle- abdomen and middle-back, a few in thigh and groin. It may speculate that cutaneous innervations of blue dots have the same distribution as gastric segmental innervations. Acu-stimulation in acupoints of head-neck, four limbs, upper chest-dorsum and lower-dorsum induced markedly augmentation of gastric motility (acupoints on head-neck such as St-2: $n$ = 16, 105.19 ± 1.36 *vs* 112.25 ± 2.02 and St-3: $n$ = 14, 101.5 ± 1.75 *vs* 109.36 ± 1.8; acupoints on limbs such as Sp-6: $n$ = 19, 100.74 ± 1.54 *vs* 110.26 ± 3.88; St-32: $n$ = 17, 103.59 ± 1.64 *vs* 108.24 ± 2.41; St-36: $n$ = 16, 104.81 ± 1.72 *vs* 110.81 ± 2.74 and Li-11: $n$ = 17, 106.47 ± 2.61 *vs* 114.77 ± 3.77, $P$ < 0.05-0.001). Vigorous inhibitory regulations of gastric motility induced by acu-stimulation applied in acupoints on whole abdomen and middle-dorsum were significantly different as compared with the controls before acu-stimulation (abdomen acupoints such as Cv-12: $n$ = 11, 109.36 ± 2.09 *vs* 101 ± 2.21; Cv-6: $n$ = 18, 104.39 ± 1.42 *vs* 91.83 ± 3.22 and St-21: $n$ = 12, 107 ± 2.97 *vs* 98.58 ± 2.81; acupoints on middle-dorsum such as Bl-17: $n$ = 19, 100.63 ± 1.4 *vs* 92.21 ± 2.07 and Bl-21: $n$ = 19, 103.84 ± 1.48 *vs* 97.58 ± 2.16, $P$ < 0.05-0.001).

**CONCLUSION:** Regular regulatory effects of facilitation and inhibition on gastric motility appear to be somatotopically organized in the acupoints of whole body, and the effective regularity of site-special acupoints on gastric motility is involved in segmental innervations between stomach and acupoints.



**Key words:** Gastric motility; Acu-stimulation; Effective regularity; Segmental innervation

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## INTRODUCTION

Acupuncture therapy, originated and prospering in China and prevailing in Asia, has been used empirically in clinical practice for several millennia. It has long been accepted that acupuncture at certain points on the body, can have analgesic and therapeutic effects in the treatment of various diseases[1]. This therapy has drawn the attention of many investigators and become a research subject of international interest around the world. Although a large number of previous clinical studies support the efficacy of acupuncture for treating gastrointestinal symptoms and/or diseases[2-4], little is known about the underlying mechanism(s). Motility of the digestive tract encompasses the phenomena of myoelectrical activity, contractile activity, tone, compliance, and transit. In the functional gastrointestinal disorders, various types of dysmotility have been documented repeatedly, and most likely reflect

dysfunction at one or more levels of the brain-gut axis. It has been demonstrated that the somatosensory inputs from the skin and/or muscle are involved in the control of various autonomic functions[5-7]. A series of investigations regarding somato-autonomic reflexes has also been carried out focusing on gastrointestinal function. In some of those investigations, there is good evidence indicating the importance of cutaneo-sensory inputs in the autonomic control of gastrointestinal motility. In anesthetized rats, for instance, it has been shown that the cutaneo-gastric reflexes mediate the inhibition and the stimulation of gastric motility *via* sympathetic and parasympathetic efferents, respectively[7-9]. It was shown that the cutaneo-sensory stimulation induced by pinching abdominal skin of rats inhibits gastric motility by increasing sympathetic activity. On the other hand, cutaneo-sensory stimulation induced by pinching the hindlimb enhances gastric motility by increasing vagal activity[8].

It is generally believed that acupuncture at different acupoints show different effects. Previous studies suggest the site-specific inhibitory or stimulatory effects of acupuncture on gastric motility[8,10,11]. The Pc-6 (neiguan) at wrist and St-36 (zusanli) at hindlimb are the common acupoints used for treating gastric symptoms such as nausea and vomiting[12,13], suggesting that acupuncture at these acupoints may stimulate gastric motility. In contrast, acupuncture on the abdomen has been used for treating abdominal pain[2-4], suggesting that acupuncture at this point may inhibit gastric motility and/or reduce gastrospasm. Acupuncture at St-36 and Pc-6 has been shown to enhance the gastric migrating motor complex in conscious dogs[14] and in anesthetized rats[10]. It has been also shown that manual acupuncture on the abdomen inhibits gastric motility[10] and induced gastric relaxations *via* the somato-sympathetic pathway[15] in anesthetized rats. Meanwhile, it was also existent in inter-contradictive results[16-19]. Recent investigations have suggested that the modulations of acupuncture on visceral functional activities can demonstrate totally different facilitative/inhibitory effects on gastric motility in diverse site-acupoints given. Therefore, the present studies were to investigate whether manual acupuncture at representative meridian-acupoints in different parts of the body can modulate responses of gastric motility, and what are regular effects in different acupoint stimulation given.

## MATERIALS AND METHODS

### *Animal preparation and recording procedures*

All animal experiments were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee of China Academy of Chinese Medical Sciences. Experiments were performed on 48 adult male Sprague-Dawley rats weighing between 250 and 300 g. The animals were maintained on a 12:12-h light-dark cycle (8:00-20:00) with free access to food and water. The rats were fasted overnight with free access to water in proxima luce and anesthetized with an intraperitoneal injection of urethane (1.0-1.2 g/kg). The trachea was cannulated but not immobilized only to keep respiratory tract unobstructed and a catheter was inserted into one of the jugular veins for infusion of the necessary solutions or anesthetics. The abdomen was opened by midline section, and a small longitudinal incision was made in the duodenum about 2-3 cm from the pylorus. A small balloon made of flexible condom rubber was inserted via incision of the duodenum into the pyloric area of rat and kept in position by tying the connecting catheter to the duodenum, and another catheter (inner diameter of 1 mm) was also inserted into the same hole by incision in order to drain digestive juices secreted from stomach. Then the cut skin of the abdomen was closed by suture before experiments. The balloon was filled with warm-water about 0.2-0.5 mL, which gave about 80-150 mm$H_2O$ pressures. Pressure in the balloon was measured by a transducer (TP-400T, Nihon Kohden) for low pressure through a thin polyethylene tube (1.5 mm in outer diameter) and then input on a polygraph (RM-6000, Nihon Kohden) amplifier and led into a data acquisition system (Power-Lab) for further analysis. Demi-fasting gastric motor activity was recorded as a control for at least 1 h before either the acupuncture or somatic stimulation.

The changes of gastric motility induced by the stimulation were compared with the background activity in intragastric pressure and/or waves of gastric contraction recorded before any stimulation. If the changes of gastric motility during stimulation were 20% more or less than the background activity, the response was then considered to have an excitatory or inhibitory regulation, respectively. Systemic blood pressure was continuously monitored from a common carotid artery and heart rate was continuously monitored, rectal temperature kept constantly around 37℃ by means of a feedback-controlled heating blanket.

### *Skin stimulus and acupuncture*

Cutaneous stimulation was obtained by pinching a localized skin area of about 5 $mm^2$ with a forceps, pinching area distributed in forelimb, lateral abdomen, dorsum and hindlimb. And an additional hot water bath (50℃) on the tail was given. Manual acupuncture with a needle of 0.3 mm in diameter was inserted into the skin and underlying muscles at different acupoints of the body, and it was rotated clockwise and anti-clockwise at 2 Hz for 30 s. Based on the comparative anatomical localization in rats as compared with that in human, selected points included 29/45 cephalocaudal acupoints in stomach-Meridian, and Li-11 (quchi) acupoint of Large-intestine-meridian in forelimb, Cv-6 (qihai), Cv-12 (zhongwan) acupoints of conception-vessel in abdomen, Bl-13 (feishu, $T_3$ spinal segment), Bl-17 (geshu, $T_7$), Bl-21 (weishu, $T_{12}$), Bl-27 (xiaochangshu, $S_1$) acupoints of Bladder-meridian in dorsum, Sp-6 (sanyingjiao) of spleen-meridian in hindlimb and the auriculo-concha, non-acupoint in tail (the location of acupoints is shown in Figure 2, Figure 3, Figure 4).

### *Evans blue dye injection and gastric inflammation*

This experiment was performed in ten SD rats including six for mineral oil of injection in intragastric mucous membrane and four for the control of saline injection. Fifteen minutes after the surgery was completed, Evans

blue dye (Sigma) dissolved in sterile water (5 mg per 100 mL) was injected through the arterial catheter. Ten minutes after the dye injection, 0.3-0.5 mL (volume depending on injected points of the stomach) of 10% mustard oil (Sigma, dissolved in mineral oil) was injected into the intragastric mucosa and visceral peritoneum covering the stomach in several areas, in order to induce a chemical inflammation of the gastric mucous membrane. Skin color changes were observed for 1-2 h, and the area of dye extravasation was sketched on the body charts for assessment. Dye extravasation was quantified by counting the number of blue dots in the skin over frontal and dorsal body areas. In control rats, a similar volume of physiological saline was injected into the intragastric mucous membrane. Evans blue dye injection was the same as mustard oil experimental animals.

### Experimental procedures

The rats were kept in supine position; gastric motor activity was first analyzed visually to detect cyclic waves of contractions. When gastric background activity is well recorded, the response of gastric motor to skin stimulation and acupuncture is tested separately. A standard protocol was employed for the determination of effects of acupuncture and somatic stimuli on the gastric motility response. A gastric background activity was recorded for 5-10 min followed by a test of their responses to manual acupuncture at various acupoint or natural stimulation on the skin was applied for 30 s. After the acupuncture or somatic stimulation the gastric motility response was recorded for another 5-10 min. After the series of experiments was finished, rats were sacrificed under an overdose deep anesthesia (urethane, > 2 g/kg ip).

### Statistical analysis

The data obtained before and after intervention in the same group was compared statistically by an independent *t* test. $P < 0.05$ was considered as a statistical significance. All data are expressed as mean ± SE.

## RESULTS

### Number and location of dye extravasation in skin on gastric inflammation

Injection of mustard oil into intragastric mucous membrane resulted in massive mucous inflammation evidenced on histological examination, which showed that the intragastric mucosa had become edematous, dilated blood vessels and ulceration of the endogastric lining. In all six rats that received mustard oil injections into intragastric mucosa (Figure 1), small blue dots appeared in the skin over the whole abdomen, but mainly in peri-midline upper- and middle- abdomen, middle-back, a few in thigh and groin. The number and distribution of blue dots varied considerably among rats. The dots started to appear about 20 min after injection of mustard oil, and the majority of dots were visible within 50 min. The dots were very small, usually ranging from 1-3 mm in diameter. However, several dots appeared in the state of stick-like in length of 3-6 mm (Figure 1). In contrast, two of four control rats that received saline injections into the intragastric mucous membrane showed no skin color changes at all. The remaining two control rats showed only 3-5 small blue dots over the middle-abdomen (Figure 1), perhaps this extravasation restricted to abdominal skin in some of the control rats might be associated with the abdominal surgical incision. It may speculate that cutaneous innervations of blue dots had same distribution as gastric segmental innervations.

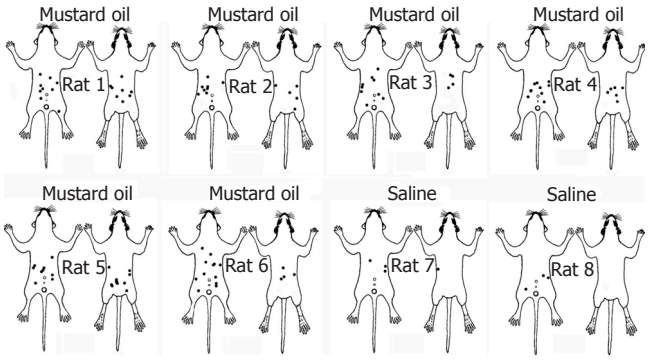


**Figure 1** Schematic drawing of number and location of blue dots in rats. Dye extravasation in skin after inflammation of intragastric mucous membrane and rare small blue dots over abdominal area of control rats.

### Gastric motor characteristics

Gastric motor characteristics were observed in 38 rats under normal anesthetic condition. When the intrapyloric balloon pressure was increased from 0 to about 80-200 mm$H_2O$ levels, the rhythmic wave of contractions in pyloric area were observed in 32 rats, at least in the periodic course during recording. Others showed no cyclic contraction during recording. Among the total, the contractile amplitude of rhythmic wave was about 5-50 mm$H_2O$, whereas strong contractions exceeded to 300 mm$H_2O$. When the pressure in an intragastric balloon was maintained at about 100 mm$H_2O$ by expanding the volume of the balloon with warm-water, rhythmic contractions occurred at a rate of 4-6 per minute. With regard to gastric motor characteristics, it was noteworthy both the changes of intragastric pressure and rhythmic contraction in present study. Generally, the intragastric pressure represents the index of gastric tone motility and rhythmic contraction represents then gastric peristalsis induced by circular muscle contractions, similar to slow-wave of gastric motor activity.

Effects of noxious mechanical or hot cutaneous stimulation of various sites on gastric motility were studied in 14 rats. As illustrated in Figure 2, pinching of the abdominal skin always produced strong inhibition (10/11 rats, 100.09 ± 2.73 *vs* 87.82 ± 3.68, $P < 0.05$) in the pyloric pressure; the maximum response of decrease was obtained about 10-s after the onset of stimulation. Pinching the middle dorsal skin in some, but not all cases, produced slight or moderate inhibition or only post-inhibition ($n$ = 11, 102.3 ± 3.36 *vs* 100 ± 3.33, $P < 0.05$). Interestingly, pinching in fore- or hind-limb induced mainly moderate increase of gastric motility ($n$ = 8, 104.56 ± 1.29 *vs* 113.78

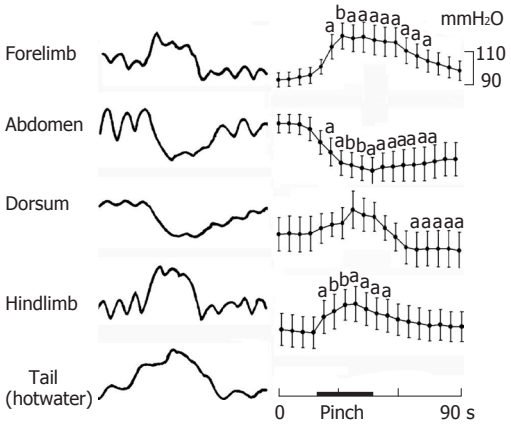


**Figure 2** Gastric motility in response to cutaneous natural stimuli. $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* control gastric motility of pre-stimuli.

± 5.37, $P < 0.05$; $n = 9$, 97.13 ± 4.63 *vs* 104.88 ± 5.03, $P < 0.05$; respectively), meanwhile, hot water bath (50℃) on the tail could produce moderate augmentation of gastric motility.

### *Gastric motility in response to stomach-meridian acupoint stimuli*

Figure 3 shows the gastric motility in response to majority craniocaudal stomach-meridian acupoint stimuli was systemically observed in four rats. Manual acu-stimulation in 9 acupoints of face (St-2, St-3, St-4, St-5 and St-7), neck (St-9) and cephalic-chest (St-12, St-13 and St-15), induced significantly moderate increase of gastric motility. This facilitation could be observed about 2-4 s after application and continued to augment during 30 s acu-stimulation. On seven acupoints of lower-chest (St-18) and whole-abdomen (St-19, St-21, St-23, St-25, St-26 and St-28), acu-stimulation produced moderate-strong suppressive responses on gastric motility, which started to suppress about 2 s delay of onset and lasted throughout the period of application of the acu-stimulation. The inhibitory magnitude on gastric motility was arranged from fadein to fadeout among these cephalocaudal acupoints. Acu-stimulation in hindlimb (St-30, St-31, St-32, St-34, St-35, St-36, St-37, St-39, St-40, St-41 and St-45) evoked moderate augmentation of gastric motility. However, in between acupoints of St-16 (in the 5$^{th}$ intercostal space) and St-30 (slightly above the inguinal groove), acupuncture could not bring about clear response of excitation/inhibition on gastric movement in most cases. It was noteworthy that, from the above observations, manual acu-stimulation applied in the Stomach-meridian acupoints from the lower-breast to the groin suppressed generally the gastric motor activity, whereas acu-stimulation in other acupoints of Stomach-meridian (i.e. face, neck, cephalic-chest and hindlimb) brought about exciting responses in different magnitudes on gastric motility. These results suggested the effective regularity of site-special acupoint on gastric motility. Next study focused at acupoint stimuli in different meridians and different parts of the body, and compared their effects on responses of gastric motility.

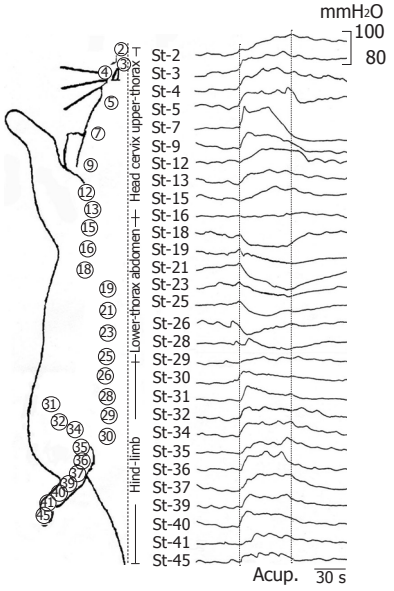


**Figure 3** The gastric motility in response to craniocaudal stomach-meridian acupoints stimuli.

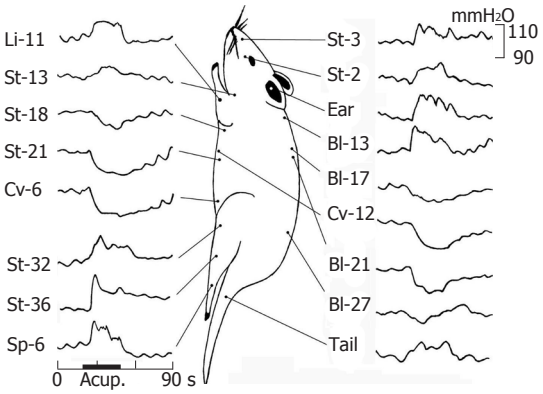


**Figure 4** Gastric motility in response to acu-stimulation.

### *Gastric motility in response to acupoint stimuli in different parts of body*

The acu-stimulation at 16 acupoints in the different parts of the body and one non-acupoint in tail on responses of gastric motility was systemically observed. A total of 27 rats were studied. Most of them (22/27) exhibited the rhythmic wave of contractions in the periodic course, and the pyloric pressure maintained at about 80-200 mmH$_2$O. Gastric motility in the most rats showed various responses to acu-stimulation applied to some representative acupoints in several meridians and different parts of the body. An individual example of various kinds of responses was illustrated in manual acu-stimulation applied to acupoints either St-2 (sibai), St-3 (juliao) of Stomach-meridian and the auriculo-concha in face, Li-11 (quchi) in forelimb, St-13 (qihu) in upper-breast, Bl-13 (feishu) in upper-dorsum, Bl-27 (xiaochangshu) in lower-dorsum, or St-32 (futu), St-36 (zusanli), Sp-6 (sanyinjiao) in hindlimb and non-acupoint in tail, which gave rise to slight-moderate facilitation of gastric motility with a rapid onset, followed by a tonic motor that lasted throughout the period of acu-stimulation. These responses were followed in several cases by after-effects of promotion; this was particularly clear when the auriculo-concha, Li-11, St-36 and Sp-6 acupoints

were acu-stimulated but was also obvious in the other cases (except from Bl-27). However it was interesting to emphasize that, the effects in both abdomen and middle-dorsum on modulation of gastric motility differed basically from those in the head, upper chest/dorsum and fore-/hind-limbs. The same stimulus applied to the acupoints of St-18 (rugen, lower breast) and St-21 (liangmen), Cv-6 (qihai) and Cv-12 (zhongwan) in the abdomen, and Bl-17 (geshu) and Bl-21 (weishu) in the middle dorsum, resulted in high suppression of gastric tonic motility with a rapid onset, followed by a obvious inhibition of rhythmic wave of contractions. These suppressions lasted throughout the period of acu-stimulation and followed in most cases after inhibition.

At a quantitative level, the facilitative/inhibitory responses on the gastric motility were evaluated before 20 s, during 30 s and after 40 s of acu-stimulation. The mean values are shown in Figure 5. Acu-stimulation in acupoints of head-neck, limbs upper chest-dorsum and lower-dorsum induced markedly augmentation of gastric motility (acupoints on head-neck such as St-2: $n = 16$, 105.19 ± 1.36 *vs* 112.25 ± 2.02 and St-3: $n = 14$, 101.5 ± 1.75 *vs* 109.36 ± 1.8; acupoints on limbs such as Sp-6: $n = 19$, 100.74 ± 1.54 *vs* 110.26 ± 3.88; St-32: $n = 17$, 103.59 ± 1.64 *vs* 108.24 ± 2.41; St-36: $n = 16$, 104.81 ± 1.72 *vs* 110.81 ± 2.74 and Li-11: $n = 17$, 106.47 ± 2.61 *vs* 114.77 ± 3.77, $P < 0.05$-0.001 respectively). Notable inhibitory responses of gastric motility induced by acu-stimulation applied in acupoints of whole abdomen and middle-dorsum were significantly different as compared with the control before acu-stimulation (abdomen acupoints such as Cv-12: $n = 11$, 109.36 ± 2.09 *vs* 101 ± 2.21; Cv-6: $n = 18$, 104.39 ± 1.42 *vs* 91.83 ± 3.22 and St-21: $n = 12$, 107 ± 2.97 *vs* 98.58 ± 2.81; acupoints on middle-dorsum such as Bl-17: $n = 19$, 100.63 ± 1.4 *vs* 92.21 ± 2.07 and Bl-21: $n = 19$, 103.84 ± 1.48 *vs* 97.58 ± 2.16, $P < 0.001$ respectively). Acu-atimulation in the tail of non-acupoint could also increase gastric motor. Such regular regulatory effects both of facilitation/inhibition on gastric motility did appear to be somatotopically organized in the acupoints of the whole body and suggested the effective regularity of site-special acupoints on gastric motility.

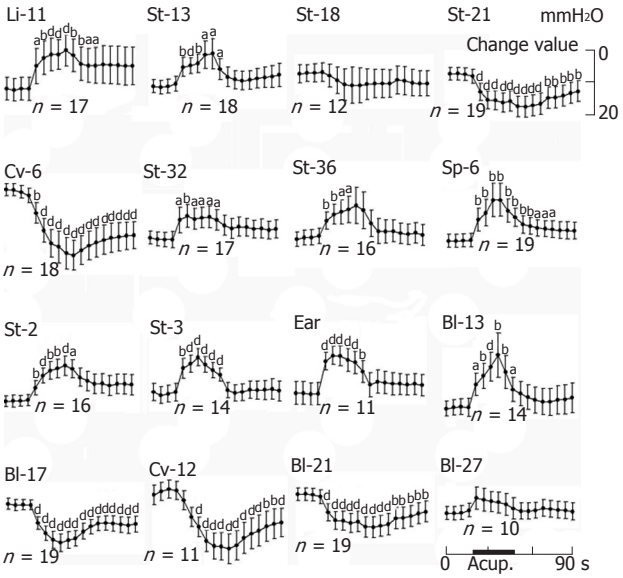


**Figure 5** The quantitative analysis of gastric motility in response to acu-stimulation. $^aP < 0.05$, $^bP < 0.01$, $^dP < 0.001$ *vs* control gastric motility of pre-stimuli.

## DISCUSSION

Recent studies have begun exploring the effects of acupuncture on gastrointestinal physiology and its potential for the treatment of gastrointestinal diseases[2,20-22]. However, despite its well-known effects on nausea and vomiting, relatively little attention has been paid to its potential for the management of disorders of gastrointestinal motility[13,14]. The purpose of this part of the study was to investigate the cutaneous distributions in association with gastric segmental innervations. Discomfort and pain were the sensory experiences most commonly evoked from visceral organs. Many forms of visceral pain were felt in the regions of the body other than the organ whose stimulation caused pain (referred pain, or viscero-somatic reflex).

Stomachal innervation has clear anatomical data, but recent study has further developed. Retrograde axonal transport of horseradish peroxidase (HRP) was applied to the ventral surface of the cat stomach. Hino *et al*[23] investigated the number, size and distribution of HRP-positive cells in spinal ganglia. The unexpected finding was the wide distribution of these cells from $T_3$ down to $L_3$. Spinal ganglion cells innervating the stomach of the rats were demonstrated using the somatopetal HRP transport technique. After injection of the tracer into the anterior wall of the stomach, labelled neurons were observed bilaterally within spinal ganglia $T_4$-$L_1$. They were most numerous in ganglia $T_8$-$T_{10}$[24]. The distribution of sensory neurons innervating the peritoneum was studied using axonal transport of fluoro-gold. The tracer was injected into parietal peritoneum and visceral peritoneum covering the stomach and small intestine. Many neurons in the nodose ganglia in addition to somata in the dorsal root ganglia from $T_4$ to $T_{13}$ were labeled when the tracer was placed on the peritoneum lining the stomach, small intestine or caecum[25].

In agreement with gastric (-intestinal) segmental innervations, chemical gastric inflammation with mustard oil in rats results in neurogenic plasma extravasation in the skin over whole abdomen and middle- and lower-back; neuroanatomically, these somatic territories belong to $T_4$-$L_1$ dermatomes[26]. It may speculate that cutaneous innervations of blue dots distribution were similar to the gastric segmental innervations.

Our results showed that, noxious mechanical (pinch) stimulation applied to limbs or tail as well as noxious heat (hot water bath at 50℃ on the tail) induced gastric excitation. It was remarkable that (electro-) acu-stimulation applied to the acupoints of six in face, two in neck, one in forelimb, five in upper chest-dorsum and one in hindlimb, which were distributed in the remote somite (hetero-segment) with gastric segmental innervation, produced a facilitative response of gastric motility followed by the activities increasing slightly in vagal and/or inhibiting in

sympathetic nerves innervated to stomach.

It is generally believed that acupuncture at different points (acupoints) shows different effects. Previous studies presented the site-specific inhibitory or stimulatory effects of acupuncture on gastric motility[10]. During 2-3 Hz of electroacupuncture on St-36 acupoint, there was a significant increase in the percentage of normal frequency. The percentage of normal frequency in the post-acupuncture period was also increased[16,17]. The pericardium Pc-6 (neiguan) at wrist and stomach St-36 are the common acupoints used for treating gastric symptoms such as nausea and vomiting[12,13]. On the basis of our studies and current results, it is conceivable therefore, that acupuncture at total acupoints, with exception in homo-somite with gastric segmental innervation, may encourage gastric motility and cure some disorders of gastroparesis[27], gastroadynamics or gastroatonia.

In contrast within the remote somite (hetero-segment) with gastric segmental innervations, the present study showed that the mechanical (pinch) stimulation applied at abdomen-dorsum induced gastric inhibition. Interestingly, acu-stimulation applied to the homotopic acupoints in the lower-chest, middle-dorsum and whole abdomen induced an inhibitory response of gastric motility followed by the activities of increasing in sympathetic and/or inhibiting slightly in vagal nerves innervated to stomach. These effects involved in intact preparation of sympathetic (splanchnic) nerves, but did not require intact preparation of bilateral vagal nerves and high spinal cord. Sato *et al*[10] showed that when it was applied to the abdomen and lower chest region, acupuncture-like stimulation elicited suppressive response on gastric motility. The authors proposed that acupuncture provoked a reflex that had cutaneous and muscle nerves as its afferent pathway, and the sympathetic gastric branches as its efferent pathway; and the acu-stimulation in abdomen inhibited gastric motility by increasing the activity of the efferent nerves of sympathetic gastric branches, abdomen acu-stimulation-induced suppression of gastric motility persisted in spinalized rats. The inhibitory duodenal response by electro-acupuncture stimulation in abdomen was a propriospinal reflex response involving splanchnic excitatory nerves, and cutting the vagal nerve branches innervating the duodenum did not affect the suppressed duodenal response[28].

Acupuncture on the abdomen has been used for treating abdominal pain[2-4], suggesting that acupuncture at this point may inhibit gastric motility and/or reduce gastrospasm or gastrohypertonics. It is conceivable that, the inhibition in gastric motility and the increase in sympathetic activity could appear only in electro-acupuncture stimulation applied to the abdomen and middle-dorsum acupoints, e.g., the homo-segmental somatic territory with gastric innervations. The results of this experiment suggest that, regardless of any meridian-acupoints, manual acupuncture at the acupoints of widespread territory except homo-segmental innervations with gastric innervations, induce a facilitative response on gastric motility; it is effective for the treatment of disturbances of gastrointestinal hypomotility such as gastroparesis, gastroptosis, gastroadynamic, enteroparesis, constipation or gastroatonia. However, acu-stimulation at the acupoints of local territory of homo-segmental innervations with gastric innervations, induces inhibitory response on gastric motility and is effective for the treatment of disturbances of gastrointestinal overmotility such as gastralgia, gastrospasm, diarrhea or enterospasm. In conclusion, acu-stimulation applied to the distant acupoints, such as in face, neck, forelimbs, upper chest-dorsum and hindlimbs, produced a facilitative response on gastric motility; whereas acu-stimulation applied to the local acupoints, such as in lower-chest, middle-dorsum and whole abdomen, induced an inhibitory response on gastric motility.

## ACKNOWLEDGMENTS

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*BASIC RESEARCH*

# Construction of a fusion protein expression vector MK-EGFP and its subcellular localization in different carcinoma cell lines

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## Abstract

**AIM:** To construct an expression plasmid encoding human wild-type midkine (MK) and enhanced green fluorescence protein (EGFP) fusion protein (MK-EGFP), and to analyze the subcellular localization of MK in different carcinoma cell lines.

**METHODS:** Two kinds of MK coding sequences with or without signal peptide were cloned into plasmid pEGFP-N2, and the recombinant plasmids constructed were introduced into HepG2, MCF7 and DU145 cells, respectively, by transfection. With the help of laser scanning confocal microscopy, the expression and subcellular localization of MK-GFP fusion protein could be detected.

**RESULTS:** Compared with the GFP control, in which fluorescence was detected diffusely over the entire cell body except in the nucleolus, both kinds of fusion protein MK-GFP were localized exclusively to the nucleus and accumulated in the nucleolus in the three kinds of cancer cell lines.

**CONCLUSION:** This study reveals the specific nucleolar translocation independent of signal peptide, which may be involved in the mechanism that MK works. It provides valuable evidence for further study on the functions of MK in nucleus and its possible mechanisms, in which ribosomal RNA transcription and ribosome assembly are involved.



**Key words:** Midkine; Subcellular localization; Laser scanning confocal microscopy

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## INTRODUCTION

Midkine (MK) is a 13 kDa protein originally found to be a secretory heparin-binding growth factor that is involved in cell growth, migration and survival. However, a number of subsequent studies showed that it is expressed at high levels in a variety of human carcinomas[1-5], indicating that it may play important roles in carcinogenesis. Recent studies demonstrated that MK can promote the growth of Wilm's tumor cells and fibroblasts[6,7], the transformation of NIH3T3 cells[8], anti-apoptotic activity of Wilm's tumor cells treated with cisplatin[9], and induction of a strong angiogenic response in rabbit corneal assay[10]. All of these suggest the multifunction of MK in carcinogenesis, cell growth, differentiation and apoptosis, and the study of its functional mechanism might lead to a new approach for cancer therapy including gastrointestinal tract cancers, since a number of recent studies have demonstrated that MK is not only highly expressed in gastrointestinal cancers, but also modulates biological phenotypes for gastric cancer growth and metastasis[2,11-13]. Moreover, it has been shown that MK could serve as a marker for the diagnosis and treatment of gastrointestinal cancers[14,15].

In addition, several subcellular localization studies have demonstrated that MK localizes in nucleus[16-18], such as in the nucleolus of PHA-activated peripheral blood lymphocytes[19], indicating that MK may serve as a transcriptional factor, or is involved in nuclear function. Nuclear transcriptional factors have been known to play roles in cell growth, differentiation, apoptosis and carcinogenesis[20-22]; however, whether MK plays roles in these functions is yet to be elucidated. In the present study, we found that MK was localized to the nucleolus of HepG2, MCF7 and DU145 cells by using MK-green fluorescence protein (GFP) fusion proteins as tracking molecules. The results showed that both MKs with or without signal peptide were exclusively localized to the nucleus and accumulated in the nucleolus of all the three carcinoma cells, while the fluorescence of GFP control was detected all over the cell except the nucleolus. Our findings may provide valuable evidence for further studies

on the functions of MK in nucleus and its mechanisms, in which ribosomal RNA transcription and ribosome assembly might be involved.

## MATERIALS AND METHODS

### Cell lines and culture

The following three cell lines were obtained from American Type Culture Collection (ATCC, M anassas, VA): hepatoma cell line (HepG2), prostate carcinoma cell line (DU145) and breast cancer cell line (MCF7). They were cultured in Dulbecco's modified Eagle's medium (DMEM, Hyclone Laboratories, Logan, UT) supplemented with 10% fetal bovine serum (FBS, Hyclone Laboratories, Logan, UT) at 37℃, with 5% humidified $CO_2$ and passaged every 3 d by trypsinization.

### Cloning of MK cDNA by RT-PCR

Total RNA was extracted from the 12-wk abortive fetal liver (provided by our own hospital) with RNeasy mini kit (Qiagen Corp), and full-length human MK gene with or without signal peptide was obtained by reverse transcription polymerase chain reaction (RT-PCR). The upstream primer of MK gene without the signal peptide is 5'-AAAGAAAGATAAGGTGAAGAAGGGCGG-3', which has the *Eco*RI site and the downstream primer is 5'-GGGATCCGGTCCTTTCCCTTCCCTTTCTTG-3', which has the *Bam*HI site. The upstream primer of MK gene with signal peptide is 5'-GGAATTCATGCAGCA CCGAGGCTTCCT-3', which has the *Eco*RI site and the downstream primer is the same with the former. After PCR amplification, the resulting fragments were digested with *Eco*RI and *Bam*HI and identified by electrophoresis and sequenced by ABI 3700 (Sangon Bioengineering Company, Shanghai). Ethical approval for the use of fetal liver tissue was obtained.

### Construction of recombinant plasmids and transfection

Two MK gene fragments with or without signal peptide were separately inserted into pEGFP-N2 plasmids (enhanced green fluorescent protein N-terminal protein fusion vector, Clontech Laboratories, Palo Alto, CA), and the recombinant plasmids were used to transform *E.coli* DH 5α and the product was amplified in LB medium, Qiaprep® Spin Plasmid MINIPREP KIT (Qiagen, Hilden, Germany). After that, two recombinant plasmids pEGFP-MKS (EGFP fused with MK with signal peptide) and pEGFP-MKN (EGFP fused with MK without signal peptide) were obtained. Transient transfections were performed with Effectene Transfection Reagent (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Cells were plated at a density of $1 \times 10^5$ cells per well on a 35 mm dish 24 h before transfection and were transfected with 0.4 μg of pEGFP-MKS, pEGFP-MKN and pEGFP-N2, respectively.

### Western blotting analysis

The cells in microplates were collected into 1.5 mL Eppendorf tubes on ice, centrifuged at 2000 r/min for 5 min at 4℃, resuspended in 100 μL cell lysis buffer, and incubated on ice for 1 h. The lysates were centrifuged at 12000 r/min for 10 min at 4℃; the supernatants were collected and stored at -20℃ for electrophoresis. Proteins in conditioned media were separated by electrophoresis on 13% SDS-PAGE gels and transferred electrophoretically onto nitrocellulose membranes. After being blocked for 2 h with 5% skim milk, blots were incubated with 1:10 diluted rabbit antihuman MK (Biovendor Laboratory Medicine, Inc.) and 1:5000 diluted β-actin (Santa Cruze, CA, USA) at room temperature for 2 h. After being washed three times with TBST, the membrane was separately incubated with donkey anti-rabbit MK (Santa Cruze, USA) at a dilution of 1:5000 and anti-mouse GAPDH (Santa Cruze, CA, USA) at a dilution of 1:5000. Finally, the protein bands were visualized with an ECL kit (Pierce, USA). Quantitative analysis of the blots was performed with an imaging densitometer. Beta-actin was used as a control.

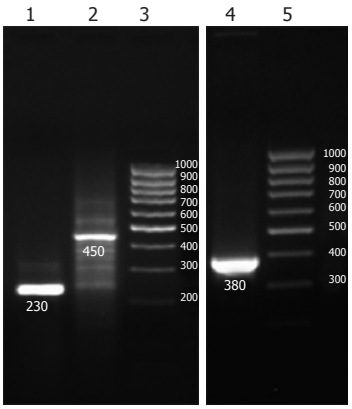


**Figure 1** Electrophoresis analysis of RT-PCR products of MKS and MKN genes. L1: β-actin (positive control); L2: fragment of MKS; L4: fragment of MKN; L3 and 5: 100 bp DNA ladder.

### Subcellular localization analysis

Ten hours after transfection, the cells were observed dynamically with laser scanning confocal microscopy (Leica, Heidelberg, Germany) for live imaging and the live images were obtained using a 40 × 1 NA oil immersion objective.

### Immunostaining assay

To visualize the subcellular localization of MK, HepG2 cells were washed three times with PBS, fixed with 4% paraformaldehyde (PFA) in PBS for 30 min at 4℃ and permeabilized with 0.1% Triton X-100. After blocked with 3% bovine serum albumin (BSA) in PBS at room temperature for 1 h, the cells were stained with 1:100 diluted goat anti-MK antibody (Santa Cruz, CA, USA) at 4℃ overnight. The treated cells were incubated with 1:100 diluted FITC-conjugated mouse anti-goat antibody (Vector Laboratories, Ltd, Peterborough, England) at 20℃ for 45 min. Finally, they were extensively washed with PBS and examined with a fluorescence microscope.

## RESULTS

### Identification of recombinant plasmids

Two expected fragments of 450 bp (MKS) and 380 bp (MKN) were clearly shown in 1.5% agarose gel electrophoresis by RT-PCR and restriction endonucleases (*Eco*RI and *Bam*HI) digestion identification, suggesting that the desired

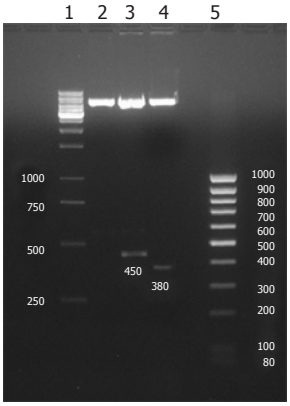


**Figure 2** Identification of constructed plasmids by digestion with restriction endonucleases (*Eco*RI and *Bam*HI). L1: 1000 bp marker; L2: pEGFP-N2; L3: pEGFP-MKS; L4: pEGFP-MKN; L5: 100 bp marker.

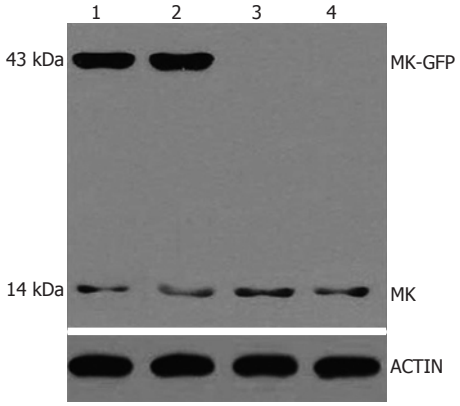


**Figure 3** Western blotting analysis of MK-GFP expressed in HepG2 cells transfected with various plasmids. Cells were transfected with pEGFP-MKS (L1), pEGFP-MKN (L2), pEGFP-N2 (L3) and with no plasmids (L4).

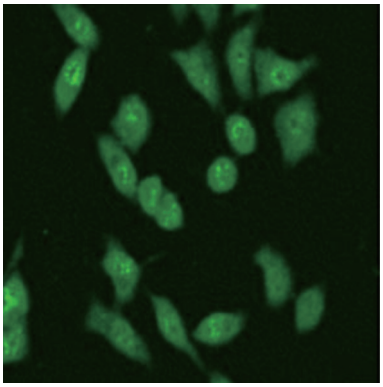

**Figure 4** Presence of MK in the nucleoli of HepG2 cells. The cells were stained with goat anti-MK antibody (1:1000). Cells were fixed with PFA and the secondary antibody FITC-conjugated mouse anti-goat antibody was added. The staining (green for MK) is presented (× 400).

products were obtained (Figures 1 and 2). Subsequent sequence analysis also proved that the obtained fragments were exactly the same as the sequence of MK gene from GenBank.

### *Western blotting and immunostaining assay*

To examine whether the MK-GFP fusion proteins were expressed in the cells transfected with pEGFP-MKN and pEGFP-MKS, 24 h after transfection, Western blotting and immunostaining assay were performed. The results revealed that MK-GFP fusion proteins were highly expressed in all the cells transfected with pEGFP-MKS and pEGFP-MKN in comparison with the low levels of native MK proteins expressed by tumor cells themselves (Figure 3, Lanes 1 and 2), whereas no signals were seen in the cells transfected with pEGFP-N2 (Figure 3, Lane 3) and with no plasmid cell controls (Figure 3, Lane 4). By immunostaining, MK was clearly detectable in the nucleoli in HepG2 cells (Figure 4), thus providing evidence for subcellular localization of MK by laser scanning confocal microscopy.

### *Dynamic subcellular localization analysis by confocal microscopy*

Dynamic subcellular localization process of fusion protein MK-GFP was observed by laser scanning confocal microscopy at different time points using the fusion protein MKN-GFP in HepG2 cells. The results showed that 11 h after transfection, the fusion protein MKN-GFP began to appear in the nucleus where it accumulated in the nucleolus (Figure 5). At 16 h 40 min time point, it was degraded in the nucleolus and finally disappeared.

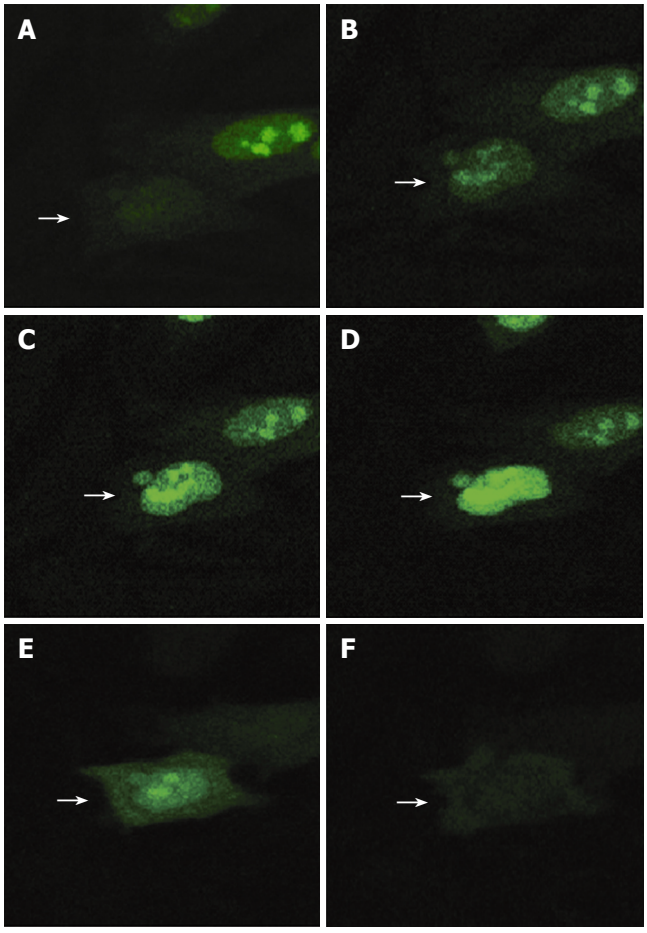


**Figure 5** Dynamic imaging of the subcellular localization of MK-GFP fusion protein in HepG2 cells captured by laser scanning confocal microscopy (marked by an arrow). **A-F**: 11 h 0 min, 11h 30 min, 12 h, 12 h 40 min, 15 h 40 min, 16 h 40 min after transfection, respectively.

### *Subcellular localization of MK in different carcinoma cell lines and the effect of signal peptide on MK*

The effect of signal peptide on MK localization was carefully observed in HepG2, DU145 and MCF7 cell lines, respectively. The results showed that 24 h after transfection, both fusion proteins MKS-GFP and MKN-GFP in the two cell lines, HepG2 and DU145, were localized exclusively to the nucleus and accumulated in the nucleolus, while GFP control was detected diffusely over the entire cell body except in the nucleolus, indicating that

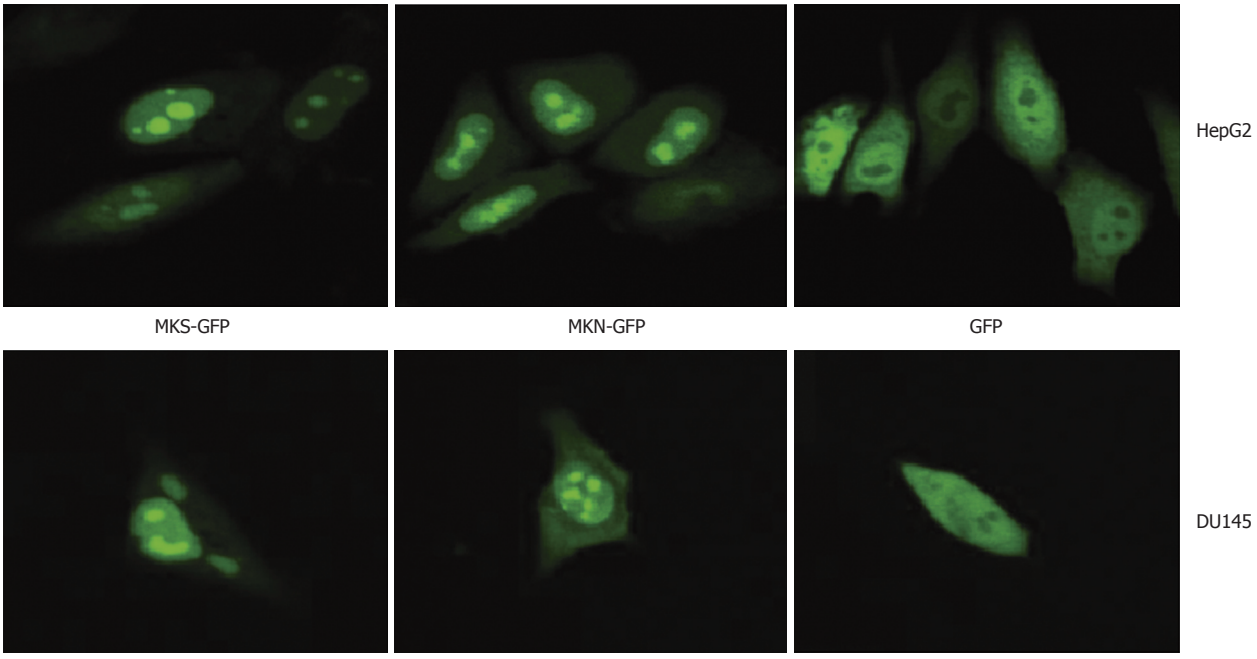


**Figure 6** Subcellular localization analysis of MKS-GFP and MKN-GFP fusion proteins in carcinoma cell lines. Twenty-four hours after transfection HepG2 cells (upper pannel)and DU145 cells (bottom pannel) were separately transfected with EGFP-MKS and EGFP-MKN. MKS-GFP and MKN-GFP fusion proteins were localized to nucleus and accumulated in nucleolus, but rarely localized to cytoplasm. The high light "spots" in the center of nuclei are nucleoli. HepG2 cells (upper panel) and DU145 cells (bottom panel) were also transfected with pEGFP-N2 for control. It seemed that GFP protein could diffuse freely in the whole cell except the nucleoli. The whole cell is light and the low light "spots" are nucleoli.

the signal peptide in pEGFP-MKS did not participate in the nuclear and nucleolar localization (Figure 6). Similar results were obtained in MCF7 cell line (data not shown).

## DISCUSSION

GFP protein is stable *in vivo* and has been fused to the C or N terminus of many cellular and extracellular proteins without a loss of activity, thereby permitting the tagging of proteins for gene regulation analysis, protein localization, or specific organelle labeling[23]. Proteins below 40 kDa, such as GFP, without any specific location signal can diffuse freely through the nuclear pore complex, and thus GFP can be used as a perfect positive control to detect the localization information of MK. In our study, based on the findings that GFP had no effect on the subcellular localization of the fusion protein, we confirmed that it was MK protein that drove the fusion protein into the nucleus, and to be exclusively localized in the nucleolus.

Localization of proteins in subcellular structures is important for the study of their functions. Nuclear located proteins usually act as transcription factors involved in the regulation of cell proliferation, differentiation and apoptosis. The localization of MK to the nucleolus of three carcinoma cell lines suggests that MK may act as a nuclear factor associated with carcinogenesis. In addition, considering the functions of nucleolus, MK may be involved in the transcription of rRNAs and its subsequent manipulation in cellular proliferation and other biological activities.

Nuclear translocation is mainly mediated by signal mechanisms[24], in which nuclear localization signal (NLS) helps extra-nuclear proteins bind with certain carriers to be transported through the nuclear pore complex and enter the nucleolus[25,26]. However, we have revealed similar distributions of both MKN-GFP and MKS-GFP fusion proteins in the HepG2 and DU145 cell lines, indicating that there is no such kind of a "signal" in the peptides of MK. Therefore, further research is needed to detect the possible "NLS" that leads MK to enter the nucleolus and to elucidate the precise translocation mechanism.

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## COMMENTS

### Background

MK (Midkine, MK) is a 13 kDa protein originally found to be a secretory heparin-binding growth factor that is involved in cell growth, migration and survival. Subsequent studies have suggested that it may play important roles in carcinogenesis. MK has been shown to be localized in the nucleus or cytosol.

### Research frontiers

Owing to the multifunction of MK in carcinogenesis, cell growth, differentiation and apoptosis, the study of its functional mechanism might lead to a new approach for cancer therapy. Further studies on the functions of MK in carcinoma cells and its possible mechanisms are important for understanding the mechanism by which MK works.

### Innovations and breakthroughs

In this study, we analyzed the subcellular localization of MK in different carcinoma cell lines by using MK-GFP fusion proteins as tracking molecules. The results showed that both MK with or without signal peptide were exclusively localized to the nucleus and accumulated in the nucleolus of all the three carcinoma cell lines, while the fluorescence of GFP control was detected all over the cell except the nucleolus.

### Applications

The results provide valuable evidence for further study on the functions of MK in nucleus and its mechanisms, in which ribosomal RNA transcription and ribosome assembly are involved.

### Terminology

NLS: nuclear localization signal which helps extra-nuclear proteins bind with certain carriers to be transported through the nuclear pore complex and enter the nucleus.

### Peer review

In this manuscript by Dai et al., the authors report the subcellular localization of MK in different carcinoma cell lines. They employed an approach based on the visualization of a fluorescent fusion protein of EGFP with two different variants of MK, with or without signal peptide. The authors observed nuclear localization and nucleolar accumulation of the fusion proteins, regardless of the presence or absence of signal peptide. Their findings may be important for the study of the mechanism(s) of nuclear import of MK, and shed new light on the function of this protein.

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*CLINICAL RESEARCH*

# Double-balloon endoscopy in the diagnosis and management of GI tract diseases: Methodology, indications, safety, and clinical impact

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## Abstract

**AIM:** To prospectively evaluate the indications, methodology, safety, and clinical impact of double-balloon endoscopy.

**METHODS:** A total of 60 patients with suspected or documented small- or large-bowel diseases were investigated by double balloon endoscopy. A total of 103 procedures were performed (42 from the oral route, 60 from the anal route, and 1 from the stoma route). The main outcome measurements were the time of insertion and the entire examination, complications, diagnostic yields, and the ability to successfully perform treatment.

**RESULTS:** Observation of the entire small intestine was possible in 10 (40%) of 25 patients with total enteroscopy. The median insertion time was 122 min (range, 74-199 min). Observation of the entire colon was possible in 13 (93%) of 14 patients after failure of total colonoscopy using a conventional colonoscope. Small-intestine abnormalities were found in 20 (43%) of 46 patients with indications of suspected or documented small bowel diseases, obscure GI tract bleeding, or a history of ileus. Endoscopic procedures including tattooing ($n$ = 33), bite biopsy ($n$ = 17), radiographic examination ($n$ = 7), EUS ($n$ = 5), hemostasis ($n$ = 1), polypectomy ($n$ = 5), balloon dilatation ($n$ = 1), endoscopic mucosal resection ($n$ = 1) and lithotripsy ($n$ = 1) were all successfully performed. No relevant technical problems or severe complications were encountered.

**CONCLUSION:** Double balloon endoscopy is a feasible technique that allows adequate small and large bowel examination and potentially various endoscopic procedures of small-intestinal lesions. It is safe, useful, and also provides a high clinical impact.



**Key words:** Double-balloon endoscopy; GI tract disease; Endoscopic therapy

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## INTRODUCTION

Due to the development of endoscopic instruments and techniques, endoscopy has played an important role in the diagnosis and treatment of disorders in the esophagus, stomach, duodenum, and colon. However, diseases occurring in more hidden areas of the gastrointestinal tract have long remained a gastroenterological problem. Unfortunately, endoscopic small bowel examination has commonly been limited to the proximal jejunum[1,2]. Therefore, more distal lesions have not usually been identified endoscopically. Recently, a new wireless capsule endoscope was introduced. Capsule endoscopy can provide an endoscopic image from the entire GI-tract without requiring a surgical laparotomy. It is undoubtedly an important improvement in diagnostic endoscopy of the small intestine and also offers advances the diagnostic workup of small bowel diseases[2,3]. Nevertheless, this method is limited because it cannot provide for air insufflations, tissue rinsing, biopsies, or therapeutic interventions. Double-balloon endoscopy (DBE), developed by Yamamoto and colleagues in 2001[4], is an exciting new endoscopic technique that allows for complete visualization and therapeutic interventions in the entire small intestine. Preliminary experiences with DBE have illustrated the capability to perform total small-bowel enteroscopy with a good safety profile and patient tolerance for this procedure[4-11]. The aim of this study was to prospectively determine the indications, safety, diagnostic and therapeutic impact of DBE in patients with known or suspected GI-tract disorders.

## MATERIALS AND METHODS

### Patients

Between July 2004 and November 2005, 103 endoscopies, including 42 antegrade and 61 retrograde procedures, were performed on 60 patients in our hospital using the DBE system. The patients were being tested for gastrointestinal bleeding, abdominal pain, diarrhea, inflammatory bowel diseases, suspected small-bowel tumors or polyps, and unsuccessful total colonoscopy using a conventional colonoscope (Table 1). Most patients had undergone at least one esophagogastroduodenoscopy and colonoscopy. DBE was performed when small bowel disease was suspected after negative upper and lower endoscopy and radiographic evaluation of the GI tract, or was previously documented by various imaging investigation [small bowel enema study ($n$ = 20), transabdominal US ($n$ = 10), CT ($n$ = 22), angiography ($n$ = 4), and radionuclide scanning ($n$ = 7)]. In some patients, more than 1 enteroscopy was performed for endoscopic treatment of small-intestinal lesions or patients were reexamined for obscure GI bleeding by DBE previously. The median age of the 60 patients (35 men, 25 women) was 64 years (range, 19 to 83 years). Written informed consent was obtained from all patients. The indications included the following: suspected or documented small intestinal lesions (24 patients), obscure GI bleeding (20 patients), unsuccessful total colonoscopy using a conventional colonoscope (14 patients), and other indications (2 patients). This study was reviewed and approved by our institutional review board.

### Methods

The DBE system (Fujinon-Toshiba ES system Co, Tokyo, Japan) is a high resolution video endoscope with a flexible overtube. The videoendoscope has a working length of 200 cm and a detachable balloon at its tip. It is used with a soft overtube measuring 145 cm in length with another balloon at the distal end. The endoscope and overtube balloons are made from latex that is 0.1 mm thick, very soft, and can be inflated or deflated by a specially designed air pump controller with one-touch controls while monitoring air pressure. The balloons are used at 45 mmHg, which is the lowest possible pressure needed to hold the intestine for endoscope insertion, and the balloon is designed not to cause pain or discomfort to the subjects due to balloon dilation (PB-10, Fujinon-Toshiba ES system Co, Tokyo, Japan)[4]. There are two types of DBE, i.e., one for general use (EN-450P5) and one for treatment (EN-450T5) (Figure 1). The major difference between the EN-450P5 and EN-450T5 is the diameter. EN-450P5 is a thinner endoscope with an external diameter of 8.5 mm and forceps channel diameter of 2.2 mm. It is used with an overtube that has an external diameter of 12.2 mm and an internal diameter of 10 mm. The EN-450T5 has an external diameter of 9.4 mm and forceps channel diameter of 2.8 mm and it is used with an overtube that has an external diameter of 13.2 mm and an internal diameter of 11 mm.

Antegrade DBE was performed transorally after overnight fasting. Retrograde DBE was performed transanally after the patients were prepared with the same oral electrolyte lavage solution as that used for regular colonoscopy. The patients were prepared by continuous intravenous infusion and then were examined by DBE under conscious sedation with intravenous flunitrazepam coupled with 35 mg of petidine chloride. During DBE, blood pressure and oxygen saturation were monitored and, when necessary, intravenous sedatives were added. DBE was carried out under fluoroscopy by experienced endoscopists, and the endoscope was advanced as far as possible in the manner described below. With the endoscope in the intestine

**Table 1 Indications for and the clinical Impact of DBE**

| Indications | DBE diagnosis | *n* (%) | Clinical impact |
|---|---|---|---|
| Suspected or documented small bowel diseases | | 24 (40) | |
| | Crohn's disease | 5 (8) | Medical and Endoscopic treatment |
| | Jejunal cancer | 2 (3) | Surgical treatment |
| | Malignant lymphoma | 1 (1.7) | Medical treatment |
| | Enterolithiasis | 1 (1.7) | Endoscopic treatment |
| | Peutz-Jeghers syndrome | 1 (1.7) | Endoscopic treatment |
| | Intestinal Behçet disease | 1 (1.7) | Medical treatment |
| | Cronkhite-Canada syndrome | 1 (1.7) | Medical treatment |
| | Jejunal lipoma | 1 (1.7) | No treatment |
| | NSAIDs ulcers | 1 (1.7) | Medical treatment |
| | Stomal ulcer | 1 (1.7) | Medical treatment |
| | Negative findings | 9 (15) | Symptomatic approaches |
| Obscure GI tract bleeding | | 20 (33) | |
| | Ileal angiodysplasia | 1 (1.7) | Endoscopic treatment |
| | Jejunal cancer | 1 (1.7) | Surgical treatment |
| | NSAIDs ulcer | 1 (1.7) | Medical treatment |
| | Solitary Peutz-Jeghers type polyp | 1 (1.7) | Surgical treatment |
| | Idiopathic small intestinal ulcer | 1 (1.7) | Medical treatment |
| | Negative findings | 15 (25) | Follow-up |
| Incomplete conventional colonoscopy | | 14 (23) | |
| | Colonic polyp | 5 (8) | Endoscopic treatment |
| | Colonic diverticula | 3 (5) | Synmptomatic approaches |
| | Colonic cancer | 1 (1.7) | Surgical treatment |
| | Colonic submucosal tumor | 1 (1.7) | Follow-up |
| | Colonic tuberculosis | 1 (1.7) | Medical treatment |
| | Negative findings | 3 (5) | Follow-up |
| History of ileus | | 2 (3) | |
| | Negative findings | 2 (3) | Follow-up |

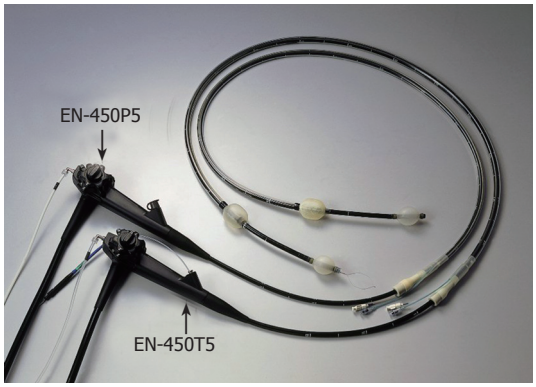


**Figure 1** Two types of double-balloon videoenteroscopes (EN-450P5 and EN-450T5).

**Table 2 Clinical data of patients who underwent DBE**

| Indications | Approach | *n* | Median insertion time (range, min) | Successful insertion |
|---|---|---|---|---|
| Panenteroscopy | Antegrade + Retrograde | 25 | 122 (74-199) | 40% (10/25) |
| Partial enteroscopy for suspected or documented lesion | Antegrade | 9 | 40 (11-99) | 67% (6/9) |
| | Retrograde | 12 | 55 (10-87) | 75% (9/12) |
| | Total | 21 | 46 (10-99) | 71% (15/21) |
| Total colonoscopy | Retrograde | 14 | 47 (15-78) | 93% (13/14) |

and the balloon of the overtube inflated and anchoring the position, the endoscope can thus be advanced. When the endoscope is advanced to its most distal point, the endoscope balloon is inflated to hold the insertion point. The overtube can be advanced after deflating the overtube balloon until the proximal end of the overtube reaches a set mark on the endoscope (150 mm); this corresponds to the distal end striking the rubber ring that holds the balloon onto the endoscope (which acts as a stopper for overtube advancement). The overtube balloon is inflated to maximum pressure and then both the enteroscope and the overtube are slowly withdrawn to reduce the GI-tract loops on the overtube and straighten the lumen for advancement. The endoscope balloon is then deflated, and insertion into the GI tract continues. This process is repeated until advancing the endoscope any further becomes difficult. The insertion route was chosen according to the estimated location of the suspected lesions, e.g., antegrade DBE was chosen when jejunal lesions were suspected and retrograde DBE was chosen when ileal lesions were suspected. A combination of both approaches was used if the indication required inspection of the whole length of the small intestine. However, examination of the entire small intestine is uncertain because there is no landmark in the small intestine. Therefore, an india-ink tattoo is left as a landmark for a subsequent retrograde DBE[4]. Fluoroscopy was used when advancement came to an unexpected halt. Gastrografin (Nihon Schering, Osaka, Japan) was injected via the working channel to allow for a radiographic assessment of the nature of the lesion. Instrumentation with a wide range of instruments is possible; e.g., ultrasound catheter probe, snares, biopsy, clip or injection needle.

## RESULTS

### *Technical aspects*

In our study, endoscopic observation of the entire small intestine was successful in 10 (40%) of the 25 patients with total enteroscopy by a combination of both approaches (anterograde and retrograde) (Table 2). The median insertion time was 122 (range, 74-199) min. Insertion of the endoscope and endoscopic observation of the target lesion was successfully achieved using DBE in 15 (71%) of the 21 patients with partial enteroscopy for suspected or documented lesion. The main reasons for failing to complete total enteroscopy or to reach the region of the target lesion was marked intestinal adhesion caused by previous laparotomy. The median insertion time was 46 (range, 10-99) min. Observation of the entire colon was possible in 13 (93%) of 14 patients in whom total colonoscopy failed using a conventional colonoscope. The median insertion time was 47 (range, 15-78) min.

**Table 3 Managements during or after DBE in 60 patients**

| | *n* | % |
|---|---|---|
| Endoscopic | 9 | 15 |
| Surgical | 5 | 8 |
| Medical | 12 | 20 |
| Symptomatic approaches or follow-up | 34 | 56 |

**Table 4 Endoscopic procedures using DBE**

| Procedure | *n* | % |
|---|---|---|
| Tattooing | 33 | 55 |
| Biopsy | 17 | 28 |
| Radiographic examination | 7 | 12 |
| EUS | 5 | 8 |
| Polypectomy | 5 | 8 |
| Hemostasis | 1 | 2 |
| Balloon dilation | 1 | 2 |
| Lithotripsy | 1 | 2 |
| Endoscopic mucosal resection | 1 | 2 |

### *Indications and clinical impacts of DBE*

The indications and clinical impact of DBE are presented in Table 1. Small-intestine abnormalities were found in 20 patients (43%); i.e., 15 (63%) of 24 patients with suspected or documented small bowel diseases, 5 (25%) of 20 patients with obscure GI bleeding and neither (0%) of two patients with a history of ileus. Colonic abnormalities were observed in 11 (79%) of 14 patients who underwent incomplete conventional colonoscopy. DBE resulted in a therapeutic intervention (endoscopic, medical or surgical therapy, excluding symptomatic approaches) in 43% of the patients (26/60) (Table 3). In 9 patients (15%), an endoscopic intervention was carried out during the DBE procedure (polypectomy, $n = 5$; endoscopic injection of hypertonic saline-solution-epinephrine, $n = 1$; endoscopic balloon dilation, $n = 1$; endoscopic lithotoripsy, $n = 1$; endoscopic mucosal resection, $n = 1$). In one patient with Peutz-Jeghers syndrome, it was possible to resect seven small intestinal polyps (without complications in three DBE sessions) by endoscopic polypectomy with clipping, with the sizes of the polyps ranging from 0.7 to 2 cm (Figure 2). In one patient with obstructive symptoms due to a huge enterolith (7 cm), it was possible to crush the enterolith using a large polypectomy snare (Captivator Ⅱ, Boston Scientific Japan, Tokyo, Japan) and remove it (Figure 3). Five patients (8%) underwent surgery for a resection of jejunal cancer (Figure 4), with one patient incurring a resection of a large solitary Peutz-Jeghers type polyp of the jejunum and another incurring a resection of advanced ascending colon cancer.

### *Endoscopic procedures using DBE*

DBE intervention and therapeutics are summarized in Table 4. Thirty-three underwent tattooing, 17 underwent

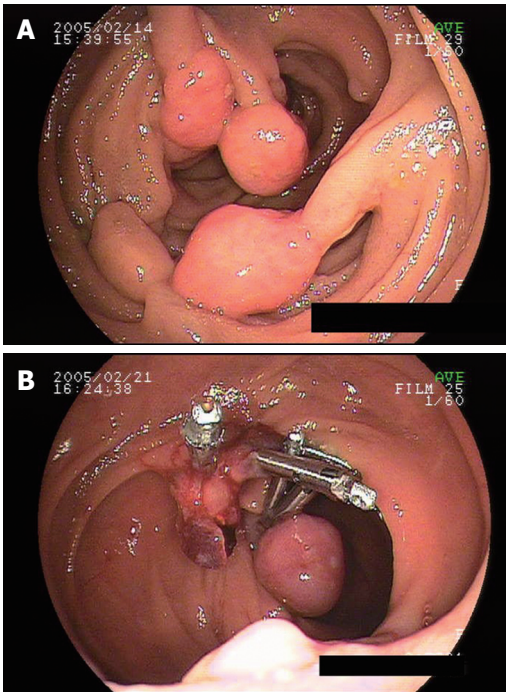


**Figure 2** The successful endoscopic removal of polyps from the mid small bowel in a patient with Peutz-Jeghers syndrome. **A**: Endoscopic view of multiple pedunculated small intestinal polyps; **B**: Endoscopic view of the region after endoscopic polypectomy using clipping.

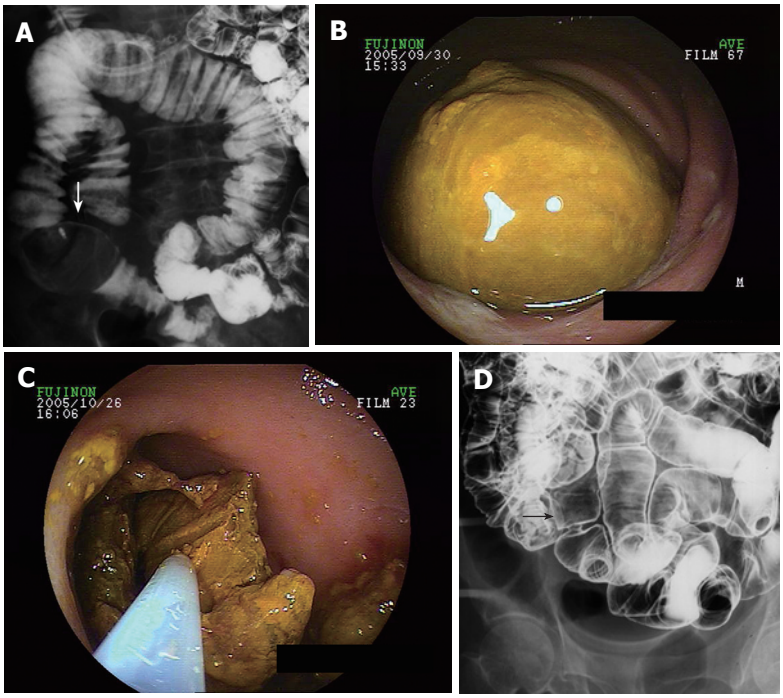


**Figure 3** Successful endoscopic lithotripsy for a huge enterolithiasis. **A**: Radiographic view of the ileum showing a huge enterolith (arrow); **B**: Endoscopic view of a huge enterolith; **C**: Endoscopic view of the lesion during lithotripsy; **D**: Radiographic view of the region after endoscopic lithotripsy showing no enterolith (arrow).

bite biopsy, 7 underwent a selective radiographic examination (Figure 4B), 5 underwent EUS using an ultrasound catheter probe (Figure 4C), 5 underwent endoscopic polypectomy, 1 underwent endoscopic hemostasis (Figure 5), 1 underwent endoscopic balloon dilation, 1 underwent endoscopic lithotripsy using a polypectomy snare, and 1 underwent endoscopic mucosal resection. All endoscopic procedures were successfully performed.

### *Complications*

No severe complications, such as perforation, occurred. DBE was well tolerated by all patients.

## DISCUSSION

Although EGD and total colonoscopy have become unequivocal procedures for the diagnosis and the treatment of GI tract diseases, enteroscopy has not yet been widely accepted by gastroenterologists. This may be partly explained by failures in total enteroscopy and the inconvenience of the procedure in push enteroscopy[1,2,12]. While video capsule endoscopy now permits the direct and painless visualization of small bowel mucosa, it does not provide a histological diagnosis and treatment of small-intestinal pathology at present[4,5,12]. A new insertion method of enteroscopy, i.e., the DBE method, has been reported to enable the endoscopic scrutiny of the entire small bowel with intervention capabilities[4-11].

Total enteroscopy using the antegrade route alone is not usually achieved, but it is possible in rare cases[4,9]. This was not achieve in this study. The strategy of combining antegrade and retrograde approaches with DBE allows total enteroscopy more frequently. To achieve this, the deepest point reached during the antegrade procedure is marked by injecting india ink so that the same point can be reached again using the retrograde approach. When total enteroscopy is intended, previous studies[4-6,8-11] have demonstrated that this mark can be reached in from 0% to 86% of all cases. In our study, this point could only be reached in 40% of the cases. The median insertion time required for panenteroscopy was 122 minutes, which was almost the same as the time reported by Yamamoto *et al* (123 min)[4]. This is certainly longer than with push enteroscopy, in which the examination times are usually approximately 30 min[10,12]. As a result, DBE is certainly a time-consuming procedure that also requires a high level of staffing, with two assistants needed in addition to a well-trained endoscopist (one assistant to help perform the DBE and one to provide patient care). However, we think that, if possible, total enteroscopy is as desirable as total colonoscopy because small bowel diseases sometimes have multiple lesions. To minimize the total effort and costs of this procedure, further technical and mechanical refinements are required.

At present, the main indications for DBE are the investigation of gastrointestinal bleeding and inflammatory bowel disease[4-12]. Other indications for DBE include an evaluation of suspected small-bowel diarrhea, abdominal pain, abnormal radiographic studies, small-bowel intussusception, the removal of small-bowel polyps, treatment for angiodysplasias, the retrieval of tissue samples, accessing Roux-en-Y anastomoses, and most

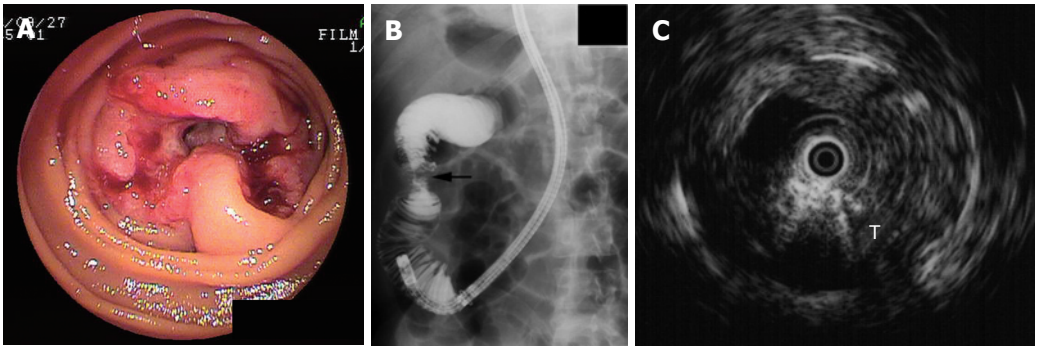


**Figure 4** Primary advanced jejunal cancer. **A**: Endoscopic image; **B**: Selective radiographic image using DBE revealing a stenotic lesion (arrow) suggestive of jejunal cancer; **C**: EUS image using ultrasound catheter probe showing a hypoechoic tumor (T), which extended into the serosal layer (T3).

recently, the evaluation of abnormal capsule endoscopy findings[4-6,8-11]. The indications for the patients examined in our study included suspected or documented small bowel diseases, obscure GI tract bleeding, incomplete conventional colonoscopy, and history of ileus. At present, conventional colonoscopy is the reference standard for evaluating the colon, especially for the screening, diagnosis, and treatment of colorectal tumors. However, the entire colon cannot be visualized during endoscopic colonoscopy in 5%-15% of patients[13,14]. Even an experienced endoscopist may be unable to intubate the colon in its entirety as far as the cecum for a variety of reasons, including a redundant or tortuous colon, marked diverticulosis, angulation or fixation of colonic loops, or spasm[14]. In this study, observation of the entire colon was possible in 93% of the patients for whom total colonoscopy failed using a conventional colonoscope, even though the median insertion time was 47 min. Using the DBE technique, especially *via* the anal route, it is possible to reach deep portions of the terminal ileum. As a result, it is considered to be an effective alternative method for patients who previously underwent incomplete colonoscopy using a standard colonoscope. DBE is much thinner, longer and less stiff than a conventional colonoscope. Therefore, colonoscopy in a "normal"patient appears to be more difficult with the DBE than with the standard colonoscope. We think that the best indication of DBE for total colonoscopy is in difficult cases when using a standard colonoscope.

In the report of Yamamoto *et al* (123 patients)[4], multiple perforations occurred after DBE in a patient with lymphoma who was actively undergoing chemotherapy. A second patient, with Crohn's disease, developed abdominal pain and fever after DBE, but no perforation was found after further investigation. May *et al* (137 patients)[10] demonstrated DBE to be a safe procedure with none of the patients experiencing bleeding or perforation. In the Heine series (275 patients)[9], severe complications were recognized in three patients, all cases involved pancreatitis. In this study, we encountered no complications. Complications have been reported relatively rarely. The reported incidence of severe complications associated with DBE has ranged from 0% to 2.5%[4-12,15].

DBE was clinically useful for making an endoscopic and histologic diagnosis as well as for providing appropriate therapy[4-12,16-21]. DBE was found to be a useful and safe method for obtaining tissue specimens, EUS images using an ultrasound catheter probe, selective

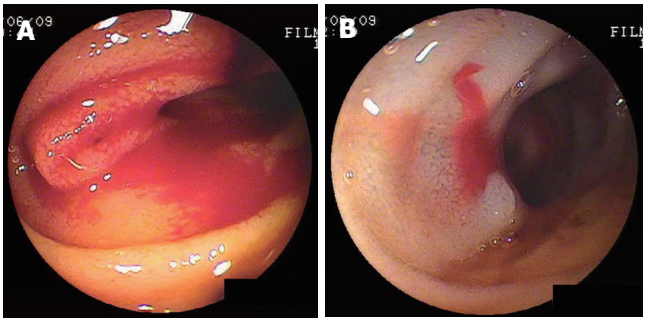


**Figure 5** Endoscopic hemostasis using hypertonic-saline solution epinephrine injection. **A**: Endoscopic image of bleeding angiodysplasia; **B**: Endoscopic image of the region after hemostasis.

radiologic images for making a diagnosis, for perfoming hemostasis, crushing enterolithiasis, performing balloon dilation, and carrying out polypectomies. Furthermore, all patients who underwent these endoscopic therapeutic procedures had an excellent outcome, thereby allowing them to avoid surgical intervention. More importantly, beyond its diagnostic value, the therapeutic value of DBE and its impact on clinical decision-making were favorable in 43% of the patients studied. This technique was also confirmed to have a high diagnostic yield in comparison to previous methods of small-bowel imaging and evaluation[4-12,20,21]. The therapeutic impact of the procedure was evident since the findings associated with this new modality resulted in decisions to start new treatments, change existing ones, and carry out surgical intervention or perform therapeutic endoscopy.

Because DBE is a new diagnostic and therapeutic procedure, it is currently not known when it should be performed during the evaluation of the small bowel. If information from prior tests is available, it is helpful in choosing the type of enteroscope (i.e. thinner type scope, EN-450P5 or therapeutic type scope, EN-450T5) and the type of approach (i.e. from oral route or anal route). Compared with other diagnostic radiologic tests, wireless capsule endoscopy has a significantly higher yield in patients[3,22]. While capsule endoscopy appears to be a reasonable initial diagnostic imaging test, miss rates of up to 36% have been reported with the capsule because of the limited 140° field of view[23]. It is limited in its inability to provide diagnostic sampling and therapeutic intervention for small-bowel lesions. Therefore, if the lesion was suspected or found by capsule endoscopy, the subsequent DBE is vital for confirming the lesion,

including biopsy, and if possible performing endoscopic therapy. Tests prior to DBE are time consuming and increase costs. At present, there are no investigation algorithms for suspected small bowel diseases. From the results of previous studies[4-12, 16-21] and ours, DBE is safe and the most accurate diagnostic modality and is able to deliver endoscopic biopsy and therapy in addition. We believe that DBE should be performed as soon as possible if small bowel disease is suspected after negative upper and lower endoscopy. However, assessing the significance of DBE in small-bowel diseases requires prospective studies comparing DBE with other currently available imaging modalities[24].

In our preliminary study, this new method was found to assist in the diagnosis and treatment of small intestine diseases and any part of the GI tract where conventional endoscopic access is otherwise difficult. It is likely that the indications for this procedure will increase in the future and that novel uses of DBE will be developed for diagnosing, monitoring, and treating entire GI tract diseases.

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*CLINICAL RESEARCH*

# Sampling variability of computer-aided fractal-corrected measures of liver fibrosis in needle biopsy specimens

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## Abstract

**AIM:** To assess the sampling variability of computer-aided, fractal-corrected measures of fibrosis in liver biopsies.

**METHODS:** Samples were derived from six to eight different parts of livers removed from 12 patients with clinically and histologically proven cirrhosis undergoing orthotopic liver transplantation. Sirius red-stained sections with a thickness of 2 μm were digitized using a computer-aided image analysis system that automatically measures the surface of fibrosis, as well as its outline perimeter, fractal surface and outline dimensions, wrinkledness, and Hurst coefficient.

**RESULTS:** We found a high degree of inter-sample variability in the measurements of the surface [coefficient of variation (CV) = 43% ± 13%] and wrinkledness (CV = 28% ± 9%) of fibrosis, but the inter-sample variability of Hurst's exponent was low (CV = 14% ± 2%).

**CONCLUSION:** This study suggests that Hurst's exponent might be used in clinical practice as the best histological estimate of fibrosis in the whole organ, and evidences the fact that biopsy sections, which are fundamental for the qualitative diagnosis of chronic hepatitis, play a key role in the quantitative estimate of architectural changes in liver tissue.



**Key words:** Cirrhosis; Hepatitis C virus; Inter-sample variability; Extra-cellular matrix; Image analysis

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## INTRODUCTION

The discovery of specific antiviral and antifibrotic treatments and the current debate concerning the possibility of reversing liver fibrosis[1-5] emphasize the need for rigorous quantitative methods capable of detecting small differences in the liver extra-cellular matrix (ECM). Such methods should provide, on the basis of biopsy section analysis, a representative index of the status of the whole organ.

Although the use of a panel of biochemical markers or other non-invasive approaches has been proposed[5-13], liver biopsy remains the "reference standard" for confirming a clinical diagnosis of chronic hepatitis, assessing disease severity, and monitoring the efficacy of therapeutic interventions[14-17].

All of the semi-quantitative systems for defining the histological stage of chronic liver disease[18-21] have been extensively criticized because their scores generate ordinals to label subjective categories of severity that are inevitably influenced by the skill and experience of the observer[22-24]. In addition, these ordinals are not continuous variables because the absolute distances between the labeled categories are unknown, restricting their application to the most widely used methods of mathematical and statistical analyses[25,26].

Available quantitative morphometric methods[27-29] have all the primary defects in their use of the Euclidean geometry, which is inexact to describe irregular objects, and the international system (IS) meter which is unsuitable for measuring highly irregular objects.

To investigate the dynamics underlying chronic hepatitis we have recently developed a computer-aided

method based on the principles of fractal geometry that automatically measures some of the geometric characteristics of the irregularly shaped collagen fragments making up liver fibrosis[30-33]. However, in applying this quantitative method, we reflected upon the patchy (irregularly shaped or considered fractal) deposition of ECM components (Figure 1). Thus, we decided to revisit previous investigations that focused on evaluating whether a biopsy section reflects the fibrotic status of the whole liver[34-41].

We have studied biopsy sections of liver samples derived from patients undergoing orthotopic liver transplantation (OLT) with clinically and histologically proven cirrhosis. We used these samples to assess the degree of sampling variability of the quantitative fractal geometric parameters of liver fibrosis.

We found a high degree of inter-sample variability in the measurements of the surface and wrinkledness of fibrosis, but a low variability of Hurst's exponent, which gives a measure of the heterogeneous distribution of a set of irregularly-shaped objects. With the support of the Euclidean and fractal dimensions, it is capable of evaluating the loss of natural order occurring in the histological picture as a result of the disruption of the natural quantitative relationship between the metric spaces covered by fibrosis and the parenchyma[42]. This gives the alterations in the significance of a physical variable and makes Hurst's exponent the descriptor of the configurational disorder of hepatic tissue.

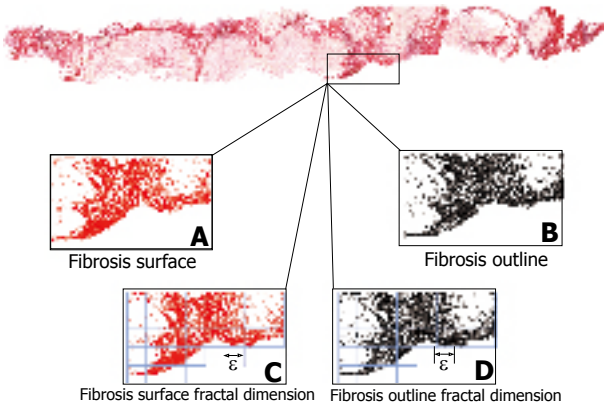


**Figure 1** Fractal morphometry of liver fibrosis in two-dimensional biopsy sections. The software automatically selects the fibrosis surface (**A**) on the basis of RGB colour segmentation, and its outline perimeter (**B**) whose calculated length was subsequently used to estimate the wrinkledness index. The fractal surface (**C**) and outline (**D**) dimensions of fibrosis were estimated using the box-counting algorithm. Covering an irregular surface requires the intersection of contiguous and non-overlapping two-dimensional boxes with a side length of $\varepsilon$: We counted the number of boxes that contain at least one point of the object $N(\varepsilon)$ for different box sizes $\varepsilon$ and then determined the fractal dimension ($D_B$) by using Equation (1).

## MATERIALS AND METHODS

### Biopsy Specimens

The study was conducted in accordance with the guidelines of the Ethics Committee of the Ospedale Maggiore IRCCS (Milan, Italy), where patients were treated.

The study was carried out on livers removed from 12 patients with clinically and histologically proven cirrhosis during OLT in order to define the variability of measured parameters in multiple specimens sampled in different sites of each liver's mass. From each liver, 6 to 8 biopsy specimens (> 10 mm in length) were collected and analyzed. All patients were positive for hepatitis C virus (HCV) infection.

### Histochemistry

Two consecutive 2 μm-thick sections were cut from each formalin-fixed, paraffin-embedded specimen. One was subsequently stained with hematoxylin-eosin solution, and the other was stained with a freshly prepared Sirius red collagen staining solution[43].

### Semi-quantitative analysis

The sections were independently staged by two expert hepatopathologists using Knodell's histology activity index (HAI)[18], and the Sheuer[19], Ishak[20] and METAVIR[21] semi-quantitative scoring systems.

### Image analysis and quantitative evaluations

For image analysis the Sirius red-stained sections were digitized at 20 × objective magnification by using a Leica DMLA microscope (Leica, Italy) equipped with an *x-y* translator table, a digital camera (Leica DC200, Leica, Italy), and an Intel Dual-Pentium Ⅲ, 660 MHz computer with incorporated ad hoc image analysis software. This software automatically selects fibrosis on the basis of RGB color segmentation[32-33]. The same image intensity level was used throughout the study. The surface area and perimeter of the fibrosis, together with its fractal surface and outline dimensions, wrinkledness, and Hurst exponent, were automatically calculated for each digitized histological section as previously described[33]. The entire procedure (patent pending) was very speedy as it digitized and furnished 11 parameters concerning the physical state of fibrosis in 10 s/mm$^2$ of tissue biopsy surface. In brief, we geometrically defined the following:

(a) Fibrosis as a set of irregularly shaped objects (collagen fragments or islets) that could be distinguished from the remaining tissue by their chemical affinity to Sirius red dye (Figure 1A).

(b) Area of fibrosis as the sum of the areas of the collagen islets (Figure 1B), expressed as a percentage of the true liver biopsy section surface area excluding any unfilled natural spaces (vascular and biliary cavities, and sinusoidal spaces) and tissue-free spaces resulting from specimen manipulation. As collagen islets are irregularly shaped, the measures of their surface areas and outline perimeters were automatically corrected by using a fractal-reference meter[32]. The fractal-rectified fibrosis surface area ($A_F$) is given by the formula:

$$A_F \cong A_M + \lambda_A (D - D\gamma) (A_B - A_M) \quad (1)$$

where $A_M$ is the morphometric area of fibrosis, $D$ the fractal surface dimension, $D\gamma$ the Euclidean dimension, $A_B$ the liver biopsy section surface area, $(A_B - A_M)$ the area of the remaining tissue, and $[\lambda_A (\Delta - \Delta\gamma)]$ the probability

factor. $\lambda_A$ was found to be $\cong 0.01$[32,33]. Equation (1) also makes it possible to consider the smallest "probably present" collagen islets (which were invisible at the magnification used in the study)[32,33] in the measurement process.

(c) Fractal-rectified length of the perimeter of fibrosis ($P_F$) as the sum of the lengths of the perimeters of each collagen islet (Figure 1B) given by the formula:

$$P_F \cong P_M [1 + \lambda_P (D - D\gamma)] \quad (2)$$

where $P_M$ is the morphometric perimeter of fibrosis, $\lambda_P$ is the dilation constant for the perimeter of the collagen islets depending only on the scale of observation[32,33], and was found to be $\cong 4.5$[32,33]. The obtained $P_F$ values were subsequently used to determine the wrinkledness of the fibrosis.

(d) Fractal surface and outline dimensions of fibrosis as measures of its space-filling properties (Figure 1C), which were automatically estimated by means of the box-counting method using the formula (Figure 2):

$$D_B = \lim_{\varepsilon \to 0} \frac{Log N(\varepsilon)}{Log(1/\varepsilon)} \quad (3)$$

where $D_B$ is the box-counting fractal dimension, $\varepsilon$ the side length of the box, and $N(\varepsilon)$ the smallest number of boxes of side $\varepsilon$ required to cover the complete surface or the outline of the object (Figure 1D). As the zero limit cannot be applied to biological objects, the dimensions were calculated as D = d, where d is the slope of the graph of log $[N(\varepsilon)]$ against log $1/\varepsilon$. The log-log graphs were plotted, the linear segments were identified using least squares regression, and their gradients were calculated using an iterative resistant line method[30-33,44-46].

(e) Wrinkledness (W) of fibrosis as a form-factor, given by the formula (Figure 3):

$$W = \frac{P_F}{2\sqrt{\pi A_F}} - R \quad (4)$$

where R is the roundness coefficient[32,33].

(f) Hurst exponent of fibrosis as a measure of the heterogeneous distribution of a set of irregularly-shaped objects (in this case, collagen islets), obtained using the relationship:

$$H = D\gamma + 1 - D \quad (5)$$

where $D$ is the fractal dimension of the outline of all of the collagen islets making up the fibrosis[33,47-49].

### Statistical analyses

All data are expressed as mean ± SD and were analyzed using Statistica software (StatSoft Inc., Tulsa, OK, USA).

The sampling variability of the fibrosis surface, wrinkledness, and Hurst exponent was evaluated in each liver by using the coefficient of variation (CV) given by the formula:

$$CV = \left(\frac{SD}{mean}\right) \times 100\% \quad (6)$$

The obtained data were analyzed using the Student $t$-test. $P$ values of less than 0.05 were considered statistically significant.

## RESULTS

### Semi-quantitative evaluations

All histological sections were staged as cirrhosis and classified in the maximum category of each of the 4 systems used in the study (HAI, and the Scheuer, Ishak and METAVIR scoring systems).

### Fractal morphometric evaluations

Table 1 shows the fractal morphometric measures of each investigated liver. The minimum and maximum values of $A_F$ obtained by measuring the 88 biopsy sections were 4.95% and 55.67% of the specimen area, with a mean value of 20.46% ± 10.55%.

The same 88 biopsy sections gave $P_F$ values ranging from 907.6 μm and 16341.1 μm, with a mean value of 6073.6 ± 3299.6 μm.

The values of the fractal surface dimension of fibrosis ranged from 1.47 to 1.84 (mean 1.66 ± 0.07), and those of the fractal outline dimension ranged from 1.41 to 1.77, with a mean of 1.58 ± 0.07.

The mean W value was 1041.6 ± 321.25 (range: 460-1834.8). The H values ranged from 0.23 to 0.59, with a mean of 0.41 ± 0.07.

### Sampling variability

Table 2 shows the inter-sample variability determined in the 6 to 8 biopsy samples that were taken from each of the 12 livers. High CV values were obtained for both the surface (CV = 43% ± 12%, range, 19.79-57.28) and the wrinkledness of fibrosis (CV = 28% ± 9%, range, 14.13-40.22), whereas the variability of Hurst's exponent was low (CV = 14% ± 2%, range, 10.71-17.74). Statistical comparisons of the mean surface, wrinkledness, and Hurst's exponent CVs showed that Hurst exponent was characterized by a smaller range and that Hurst exponent mean CV was statistically different from that of the fibrosis surface ($P < 0.00005$) and wrinkledness ($P < 0.00005$) (Figure 4).

## DISCUSSION

During the course of chronic hepatitis, the net result of the balance between ECM synthesis (fibrogenesis) and degradation (fibrolysis) is the irregular shape and patchy distribution of collagen fragments. Currently, the lack of robust and representative markers of fibrosis is the single greatest factor limiting both the validation of progression or regression of fibrosis, and the testing of antifibrotic therapies in clinical trials. This observation prompted us to investigate whether fractal-rectified measures of fibrosis recognized in two-dimensional liver biopsy sections provide concrete scalars that can represent the state of the whole organ. This aim was reinforced by recent proposals to use methods of analyzing blood stream molecules as a means of scoring liver fibrosis. However, none of these methods has inspired sufficient confidence to displace our reliance on hepatic histology[33,50-57].

To obtain rigorous measures of irregularly shaped collagen islets, a computer-aided method was developed that uses a fractal-corrected meter to provide close-to-reality

**Table 1 Fractal parameters of liver fibrosis measured in biopsies taken at different sites (mean ± SD)**

| Liver | Sections (*n*) | $A_F$ (%) | $P_F$ (μm) | D | | W | H |
|---|---|---|---|---|---|---|---|
| | | | | $D_A$ | $D_P$ | | |
| 1 | 6 | 19.17 ± 10.98 (9.1-38.4) | 6964.74 ± 3128.95 (4646.2-12328.6) | 1.64 ± 0.06 (1.6-1.8) | 1.56 ± 0.06 (1.5-1.7) | 1178.82 ± 198.79 (897.9-1453.3) | 0.35 ± 0.06 (0.3-0.5) |
| 2 | 8 | 22.00 ± 7.99 (8.5-35.1) | 3570.14 ± 1170.41 (1365.9-5240.7) | 1.66 ± 0 06 (1.5-1.7) | 1.57 ± 0.06 (1.4-1.7) | 748.94 ± 129.68 (1365.9-5240.7) | 0.34 ± 0.06 (0.4-0.6) |
| 3 | 8 | 15.62 ± 8.73 (5.5-33.8) | 4673.66 ± 4405.36 (907.6-15037.6) | 1.62 ± 0.07 (1.5-1.8) | 1.55 ± 0.07 (1.4-1.7) | 881.40 ± 354.46 (460-1641) | 0.37 ± 0.07 (0.3-0.6) |
| 4 | 8 | 32.83 ± 6.35 (25-44.2) | 7727.38 ± 4215.44 (2949.9-16341.1) | 1.74 ± 0.04 (1.6-1.8) | 1.64 ± 0.04 (1.6-1.7) | 1103.06 ± 307.68 (701.7-1649.3) | 0.26 ± 0.04 (0.3-0.4) |
| 5 | 6 | 19.53 ± 11.06 (4.95-32.9) | 6833.67 ± 3057.82 (3057.8-11213.4) | 1.64 ± 0.07 (1.5-1.7) | 1.56 ± 0.06 (1.5-1.6) | 1172.91 ± 342.08 (666.1-1690.3) | 0.35 ± 0.07 (0.4-0.5) |
| 6 | 8 | 31.42 ± 15.22 (13.1-55.67) | 7035.59 ± 3871.56 (2290.7-14153.6) | 1.74 ± 0.07 (1.6-1.8) | 1.66 ± 0.06 (1.6-1.8) | 1155.17 ± 367.12 (793.7-1713.5) | 0.25 ± 0.04 (0.2-0.4) |
| 7 | 6 | 12.81 ± 5.26 (6.5-20.8) | 6576.58 ± 4024.44 (2626.9-12294.2) | 1.61 ± 0.05 (1.5-1.7) | 1.55 ± 0.05 (1.5-1.6) | 1121.75 ± 429.47 (717.8-1796) | 0.38 ± 0.07 (0.4-0.5) |
| 8 | 6 | 13.52 ± 6.09 (5.5-21.7) | 5224.22 ± 3583.102 (1599.6-11107.4) | 1.57 ± 0.07 (1.5-1.7) | 1.49 ± 0.07 (1.4-1.6) | 908.53 ± 363.51 (547.1-1363.4) | 0.42 ± 0.07 (0.4-0.6) |
| 9 | 8 | 12.34 ± 6.87 (6.7-23.9) | 5087.80 ± 1619.29 (3081.7-6801.2) | 1.62 ± 0.05 (1.6-1.7) | 1.55 ± 0.05 (1.5-1.6) | 1076.95 ± 15.20 (902.5-1328.3) | 0.37 ± 0.05 (0.4-0.5) |
| 10 | 8 | 18.87 ± 5.54 (9.8-25) | 8133.72 ± 3459.80 (2699.6-12552.2) | 1.68 ± 0.05 (1.6-1.8) | 1.61 ± 0.06 (1.5-1.7) | 1276.35 ± 421.98 (635.9-1834.8) | 0.31 ± 0.05 (0.3-0.5) |
| 11 | 8 | 18.74 ± 4.93 (9.7-25.2) | 5760.81 ± 1698.43 (2602.6-7805.2) | 1.63 ± 0.05 (1.6-1.7) | 1.56 ± 0.05 (1.5-1.6) | 958.96 ± 173.98 (632.4-1147.6) | 0.34 ± 0.05 (0.4-0.5) |
| 12 | 8 | 25.26 ± 11.35 (9.7-44.4) | 5621.00 ± 2603.91 (1637.6-9839.8) | 1.71 ± 0.06 (1.6-1.8) | 1.62 ± 0.05 (1.5-1.7) | 970.14 ± 255.81 (609.3-1455.8) | 0.28 ± 0.06 (0.3-0.5) |

N: Number of biopsy sections; $A_F$: Fractal rectified area; $P_F$: Fractal rectified perimeter; $D_A$: Ssurface fractal dimension; $D_P$: Ooutline fractal dimension; W: Wrinkledness; H: Hurst's exponent.

**Table 2 Coefficent of variability of fibrosis surface, wrinkledness and Hurst's exponent calculated in biopsy specimens taken at different liver sites**

| Patient | Sections (*n*) | CV (%) | | |
|---|---|---|---|---|
| | | $A_F$ | W | H |
| 1 | 6 | 57.28 | 16.86 | 13.90 |
| 2 | 8 | 36.31 | 17.31 | 15.27 |
| 3 | 8 | 55.9 | 40.22 | 16.86 |
| 4 | 8 | 19.79 | 27.89 | 10.71 |
| 5 | 6 | 56.64 | 29.17 | 14.87 |
| 6 | 8 | 48.44 | 31.78 | 17.74 |
| 7 | 6 | 41.05 | 38.29 | 11.67 |
| 8 | 6 | 45.05 | 40.01 | 15.41 |
| 9 | 8 | 55.67 | 14.13 | 11.45 |
| 10 | 8 | 29.38 | 33.06 | 16.13 |
| 11 | 8 | 26.31 | 18.14 | 12.68 |
| 12 | 8 | 44.94 | 26.37 | 15.13 |

N: number of biopsy sections; $A_F$: fractal rectified area; W: wrinkledness; H: Hurst's exponent; CV: coefficient of variability.

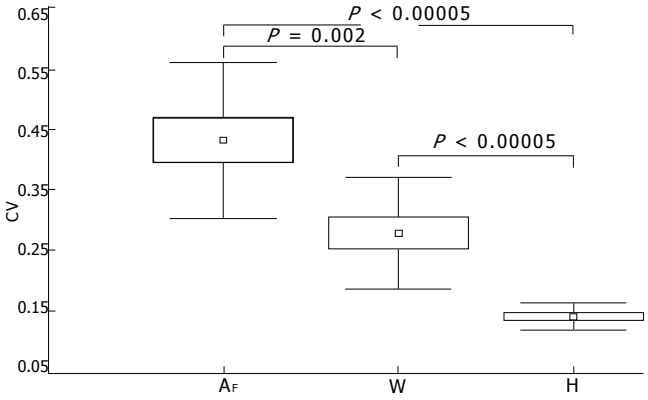


**Figure 4** Comparative analyses of the mean coefficients of variability of the surface, wrinkledness and Hurst's exponent of liver fibrosis.

measurements[30-33]. Such measurements cannot be obtained using the Euclidean meter, which is able to quantify only smooth objects (e.g. geometrical figures) that are practically unknown in Nature[30-33,44-46].

To determine the extent to which a histological section represents the whole liver we compared the scalar number generated by our quantitative method representing the magnitude of the area, wrinkledness and Hurst's exponent of the fibrosis recognized in two-dimensional sections taken from different parts of 12 explanted livers. The present study was restricted to explanted cirrhotic livers as they could make it possible to sample multiple specimens from the same liver, but impossible to obtain from the liver of living patients.

In a previous study, we found a high degree of intra-sample variability in the surface area of fibrosis that revealed the three-dimensional spatial irregularity of fibrosis detectable in the sample that reveals the non-representativity of the sample respect in the whole liver tissue[33]. In line with this finding, our analysis of the surface area (CV = 43% ± 12%) and wrinkledness (CV = 28% ± 9%) of the fibrosis detected in two-dimensional sections (Table 2) taken from multiple biopsies excised in different sites of each liver mass, revealed a high degree of variability and thus a low possibility to predict the state of a sample and that of the whole organ (i.e. liver).

Chronic hepatitis is an irregular dynamical process

complex both in time and in space, and its end stage known as cirrhosis is characterized by a wide distortion of the normal parenchyma configuration and secondary vascular events that might contribute to the heterogeneous distribution of ECM components[58].

The lowest variability (CV = 14% ± 2%) was detected for Hurst exponent in our study. We have introduced this parameter to measure the loss of natural liver tissue harmony[33]. This concept results from the ratio of two quantitative canons normally found in disease-free livers: 2% of ECM belonging to the bearing system of the liver tissue, and 98% of parenchymal tissue.

Hurst's exponent which gives a measure of the heterogeneous distribution of a set of irregularly-shaped objects, is capable of evaluating the loss of natural order that occurs in the histological picture as a result of the disruption of the natural quantitative relationship between the metric spaces covered by fibrosis and the parenchyma[42]. This is because Hurst exponent derives from the interaction between the Euclidean and fractal dimensions, becoming a measure unit. Both Euclidean and fractal dimensions separately express only the position of an object in the Euclidean space.

The present results suggest that this quantitative parameter might be more useful in clinical practice as a means of estimating fibrosis and its progression or reversibility after therapy, than the surface and wrinkledness of fibrosis.

Our results also underscore the key role of biopsy analyses both in diagnosing the histology of chronic hepatitis and in quantifying changes in the geometry of ECM islets. The clinical relevance of any of the parameters issued from morphometry is however not known yet. This incompleteness may be due to the low rigor in the actual detection of the data based on the Euclidean geometry and the use of linear IS meter to measure very irregular objects[30-33].

In summary, our method offers the following theoretical and experimental contributions. Hurst's exponent can be used to provide a good estimate of tissue alterations. Objective and rigorous metric measures of the fibrosis of a section of biopsied tissue do not fully represent the status of the organ as a whole. Hurst's exponent of liver tissue in a histological section is a good descriptor of the architectural condition of the whole liver.

Hurst's exponent therefore gives scientific value to the liver tissue status indicated by the term of architectural change, originally used by Ishak *et al*[20] in their definition of the staging of chronic viral hepatitis.

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*RAPID COMMUNICATION*

# Blockade of high mobility group box-1 protein attenuates experimental severe acute pancreatitis

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## Abstract

**AIM:** To examine the effects of anti-high mobility group box 1 (HMGB1) neutralizing antibody in experimental severe acute pancreatitis (SAP).

**METHODS:** SAP was induced by creating closed duodenal loop in C3H/HeN mice. SAP was induced immediately after intraperitoneal injection of anti-HMGB1 neutralizing antibody (200 μg). Severity of pancreatitis, organ injury (liver, kidney and lung), and bacterial translocation to pancreas was examined 12 h after induction of SAP.

**RESULTS:** Anti-HMGB1 neutralizing antibody significantly improved the elevation of the serum amylase level and the histological alterations of pancreas and lung in SAP. Anti-HMGB1 antibody also significantly ameliorated the elevations of serum alanine aminotransferase and creatinine in SAP. However, anti-HMGB1 antibody worsened the bacterial translocation to pancreas.

**CONCLUSION:** Blockade of HMGB1 attenuated the development of SAP and associated organ dysfunction, suggesting that HMGB1 may act as a key mediator for inflammatory response and organ injury in SAP.



**Key words:** Severe acute pancreatitis; High mobility group box-1; Neutralizing antibody; Inflammatory response; Organ dysfunction; Bacterial translocation

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## INTRODUCTION

In severe acute pancreatitis (SAP), multiple organ dysfunction syndrome (MODS) in the early phase[1,2] and complications of infection (infected pancreatic necrosis and sepsis) in the late phase are contributors to high mortality in SAP[3,4]. MODS is a consequence of the systemic inflammatory response syndrome, and it is conceivable that release of humoral mediators from the excessive activated macrophages/monocytes and neutrophils may lead to the remote organ injury. Complications of infection are thought to be a result of bacterial translocation from the gastrointestinal tract, and breakdown of intestinal integrity is considered to be implicated in the mechanism[5-7].

High mobility group box 1 (HMGB1) protein, originally discovered 30 years ago as a nuclear DNA binding protein[8-10], was recently identified as a late-acting mediator of endotoxin lethality[11]. Injection of HMGB1 itself was lethal, and serum levels of HMGB1 increased from 8 to 32 h after the administration of endotoxin, when the tumor necrosis factor (TNF) peak had already occurred[11]. Antibodies to HMGB1 attenuated the mortality associated with endotoxemia, even when the antibodies were administered 2 h after the onset of endotoxemia[11]. HMGB1 was also found to have the capacity to induce cytokines and activate inflammatory cells when it was applied extracellularly[11-13]. This implicates HMGB1 as a proinflammatory mediator. Recent investigations reported that serum HMGB1 levels increased in patients with sepsis/endotoxemia[11,14,15], hemorrhagic shock[16], acute lung injury[17,18], rheumatoid arthritis[19] and disseminated intravascular coagulation[20]. It has been demonstrated that HMGB1 is secreted actively by living inflammatory cells such as stimulated macrophages/monocytes, and is released passively from necrotic or damaged cells[21-23]. Therefore, HMGB1 may be related to inflammation and necrosis in SAP, and may be an important mediator for multiple organ failure.

In a recent study, we have first demonstrated that

serum HMGB1 levels were significantly elevated in patients with SAP on admission, and were correlated with the severity[24,25] of the disease. The HMGB1 levels were higher in patients with organ dysfunction and infection during the clinical course. The HMGB1 levels in non-survivors were higher than those in survivors. These results suggest that HMGB1 may play a pivotal role in the pathogenesis of SAP, and that HMGB1 may act as a key mediator for inflammation and organ failure in this disease. In the present study, to clarify the role of HMGB1 in the pathophysiology of SAP, effects of anti-HMGB1 neutralizing antibody were investigated in SAP in mice.

## MATERIALS AND METHODS

### Animals

Female C3H/HeN mice (weighing 20-22 g, 9 weeks old) were purchased from CLEA Japan (Tokyo, Japan). The protocol for this animal experiment was approved by the Institutional Animal Committee of Kobe University Graduate School of Medical Sciences.

### Anti-HMGB1 neutralizing antibody

Anti-HMGB1 neutralizing antibody (chicken anti-HMGB1 polyclonal antibody) was obtained from Shino-Test Corporation (Sagamihara, Japan). This antibody recognizes mouse HMGB1. The specificity and neutralizing activity of this antibody was confirmed by western blot analysis.

### Model for acute necrotizing pancreatitis (closed duodenal loop-induced pancreatitis)

Under general anesthesia with a subcutaneous injection of carbamic acid ethyl ester (urethane) at a dosage of 1.5 g/kg, a midline laparotomy was performed, and a closed loop (2 cm in length) was created by ligating the duodenum at 1 cm proximal and distal sides to the biliopancreatic duct outlet. Only laparotomy was performed in sham-operated mice.

### Experimental design

Saline (0.2 mL) or anti-HMGB1 neutralizing antibody (200 μg, 0.2 mL) was injected intraperitoneally, and immediately closed duodenal loop-induced pancreatitis was made. Mice were divided into three groups as follows. Group A: Sham, laparotomy with saline injection. Group B: SAP, severe acute pancreatitis with saline injection. Group C: HMGB1 Ab + SAP, severe acute pancreatitis with anti-HMGB1 antibody injection. Mice were sacrificed 12 h after induction of SAP. Pancreas and lung tissue was removed, fixed in 10% formalin, and stained with hematoxylin and eosin for light microscopic analysis. Blind analysis was carried out for all histological studies. Blood sample was drawn from heart. Serum amylase, aspartate aminotransferase (AST), alanine aminotransferase (ALT), lactate dehydrogenase (LDH), blood urea nitrogen (BUN), and creatinine (Cr) levels were measured using standard clinical automated analyzer.

### Bacterial culture of pancreas

The skin was cleaned with 10% povidone iodine. Pancreas was obtained 0, 4, 8, and 12 h after induction of SAP under sterile conditions, and processed for culture of aerobic and anaerobic organisms using a standardized method. Specimens were inoculated onto agar plates including BTB agar, sheep blood agar, chocolate agar (Nippon Becton Dickinson Co. Ltd., Tokyo, Japan), brucella HK agar (Kyokuto Pharmaceutical Co. Ltd., Tokyo, Japan), and GAM (Gifu Anaerobic Medium) agar (Nissui Pharmaceutical Co. Ltd., Tokyo, Japan). BTB agar was incubated in the aerobic chamber at 37℃, sheep blood agar and chocolate agar were incubated in the $O_2/CO_2$ incubator, and brucella HK agar was incubated in the anaerobic chamber for 48 h, respectively. GAM agar was incubated in the ambient chamber at 37℃ for 72 h. When the colony forming was detected, it was considered to be positive for bacterial translocation.

**Table 1 Blood biochemical parameters**

| Parameter | Sham ($n$ = 6) | SAP ($n$ = 20) | HMGB1 Ab + SAP ($n$ = 12) |
|---|---|---|---|
| Amylase (IU/L) | 2220 ± 707 | 52155 ± 14449[a] | 8994 ± 1623[c] |
| AST (IU/L) | 1499 ± 335 | 5056 ± 545[a] | 4193 ± 561 |
| ALT (IU/L) | 395 ± 288 | 1215 ± 118[a] | 848 ± 145[c] |
| LDH (IU/L) | 6743 ± 1206 | 16403 ± 1072[a] | 15220 ± 4687 |
| BUN (mg/dL) | 32 ± 3 | 93 ± 6[a] | 94 ± 7 |
| Cr (mg/dL) | 0.10 ± 0.00 | 0.42 ± 0.10[a] | 0.16 ± 0.03[c] |

Blood samples were obtained 12 h after induction of SAP. AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; LDH: Lactate dehydrogenase; BUN: Blood urea nitrogen; Cr: Creatinine. [a]$P < 0.05$ *vs* Sham group; [c]$P < 0.05$ *vs* SAP group.

### Statistical analysis

The results are expressed as mean ± SE. The Mann-Whitney $U$ test and Chi-square test were used to evaluate differences between two groups. A $P$ value < 0.05 was considered statistically significant.

## RESULTS

### Serum amylase level

Twelve hours after induction of SAP, serum amylase levels were significantly elevated in SAP group, and anti-HMGB1 neutralizing antibody significantly reduced its elevation (Table 1).

### Morphology of pancreas and lung

Twelve hours after induction of SAP, HE staining of the pancreas showed edema, hemorrhage, leukocyte infiltration, and necrosis. Anti-HMGB1 neutralizing antibody improved the histological alterations of pancreas (Figure 1A). Twelve hours after induction of SAP, HE staining of the lung showed edema, inflammatory infiltration, hemorrhage and thickening of the alveolar membrane. In contrast, anti-HMGB1 neutralizing antibody ameliorated the histological changes in the lungs (Figure 1B).

### Hepatic and renal dysfunction

Twelve hours after induction of SAP, serum AST, ALT,

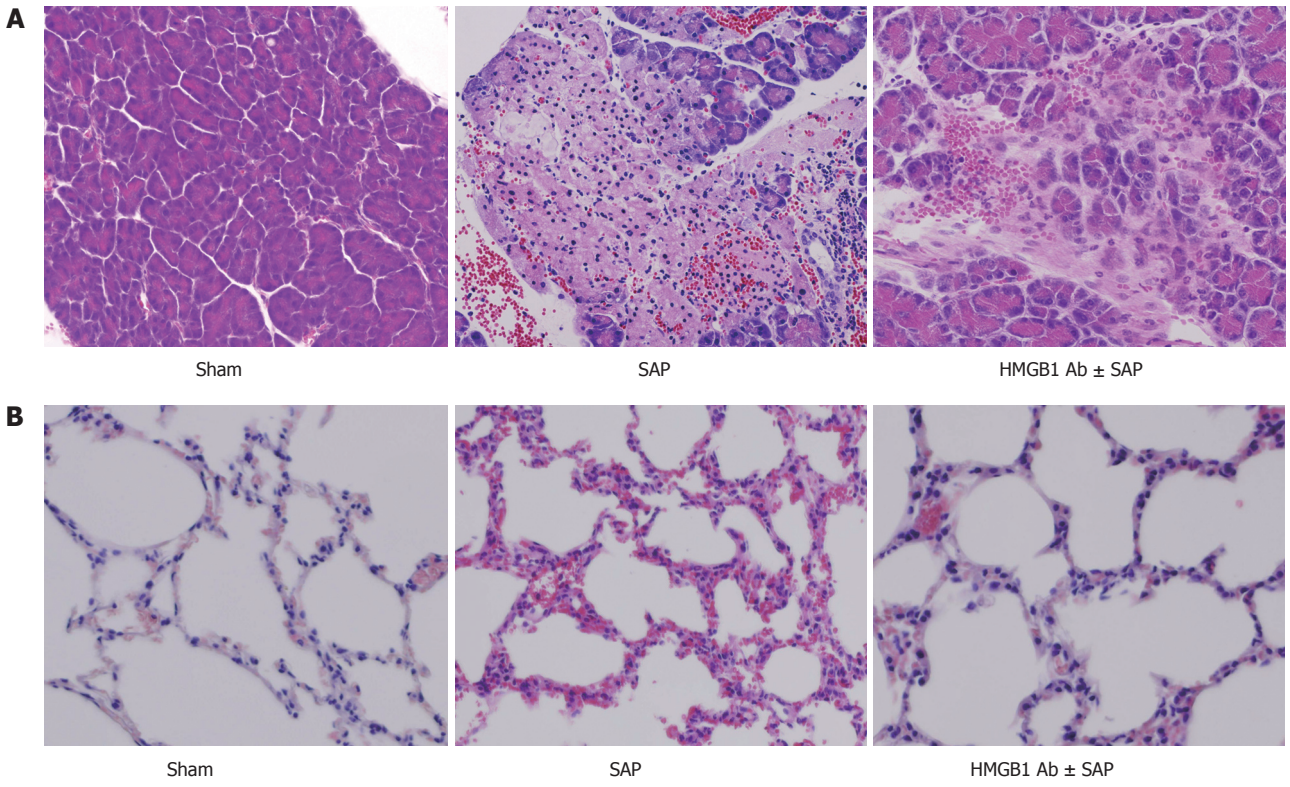


**Figure 1** Effect of anti-HMGB1 antibody on the morphology of pancreas and lung in mouse severe acute pancreatitis (SAP). Tissue samples were obtained 12 h after induction of closed duodenal loop pancreatitis. **A**: HE staining of pancreas (× 200); **B**: HE staining of lung (× 200).

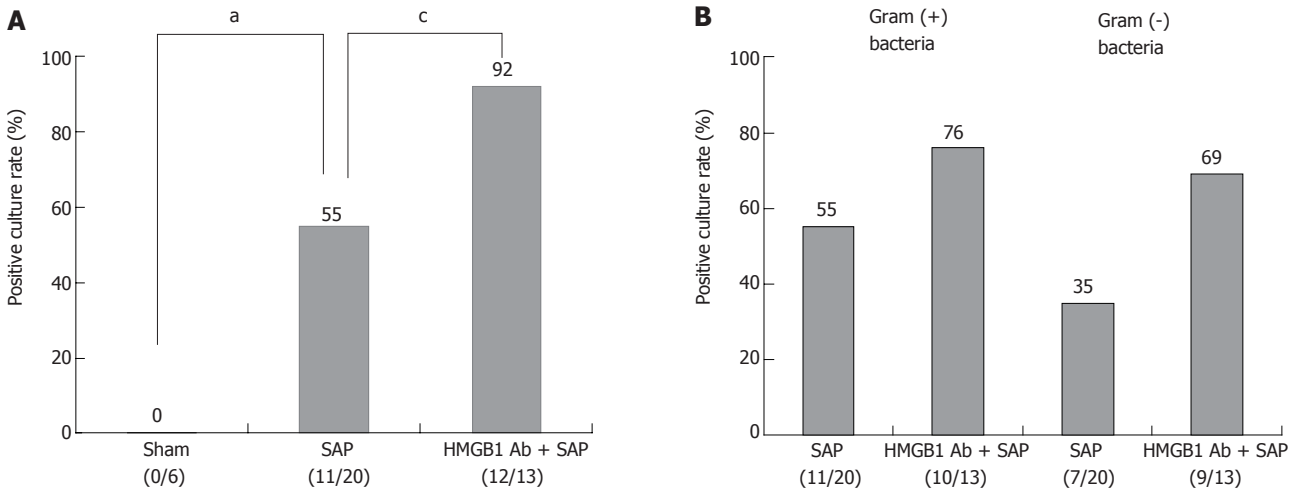


**Figure 2** Effect of anti-HMGB1 antibody on the bacterial translocation to pancreas in mouse severe acute pancreatitis (SAP). Bacterial culture of pancreas was examined 12 h after induction of SAP. **A**: Positive rate of bacterial culture of pancreas (overall). $^{a}P < 0.05$ *vs* Sham group; $^{c}P < 0.05$ *vs* SAP group; **B**: Positive rate of bacterial culture of pancreas (Gram-positive and Gram-negative bacteria).

LDH, BUN, and Cr levels were significantly elevated in SAP group, and anti-HMGB1 neutralizing antibody significantly improved the elevated ALT and Cr (Table 1).

### *Bacterial translocation to pancreas*

Bacterial translocation to pancreas was not observed in Sham group (Figure 2A), but could be detected 12 h after the induction of SAP. In earlier periods (0, 4, and 8 h), it was not detected. In SAP group, 55% of mice (11/20) exhibited positive bacterial culture. Anti-HMGB1 antibody significantly increased the positive culture rate to 92% (12/13) (Figure 2A). Positive rate of gram-positive and gram-negative bacterial culture in SAP group was 55% (11/20) and 35% (7/20), respectively. Anti-HMGB1 antibody increased them to 76% (10/13) and 69% (9/13), respectively, but no significant difference was observed (Figure 2B).

## DISCUSSION

Extracellular HMGB1 was recently identified as a novel proinflammatory cytokine. In a previous study, we dem-

onstrated that serum HMGB1 levels were significantly elevated in patients with SAP, and were correlated with disease severity[24]. In this study, we have for the first time demonstrated that blockade of HMGB1 attenuated the development of SAP and associated organ dysfunction, suggesting that HMGB1 may act as a key mediator for inflammatory response and organ injury in SAP. We think that raised HMGB1 may represent a cause of aggravation of SAP (progression to SAP) and associated organ dysfunction as well as a consequence of SAP. On the other hand, HMGB1 can promote alterations in gut barrier function by increasing the permeability in enterocytic monolayers and increasing bacterial translocation in mice[26]. Similar contributions of HMGB1 to SAP were supposed, but blockade of HMGB1 adversely worsened the bacterial translocation against our expectation.

There have been several reports concerning effects of anti-HMGB1 neutralizing antibody in other pathological conditions. It has been demonstrated that anti-HMGB1 antibody protected against organ injury and improved survival in murine sepsis[14] and rat sepsis[27]. Tsung *et al*[28] clarified that inhibition of HMGB1 with neutralizing antibody significantly decreased liver damage after ischemia/reperfusion, whereas administration of recombinant HMGB1 worsened it. It has been reported that anti-HMGB1 antibody improved lipopolysaccharide (LPS)-induced acute lung injury in mice[18], and ventilator-induced lung injury in rabbits[29]. These observations together with our results in this study indicate that HMGB1 is one of the deteriorating factors in the development of organ injury.

Concerning the elevation of serum HMGB1 levels in SAP, two possible mechanisms can be assumed[24]. First, HMGB1 may be produced and released by macrophages/monocytes in response to inflammatory mediators. In SAP, it is conceivable that release of humoral mediators from the excessive activated macrophages/monocytes may lead to the remote organ injury. Thus, release of HMGB1 from activated macrophages/monocytes may participate in tissue injury and organ failure in SAP. Second, HMGB1 may be produced and released by injured pancreas or other damaged organs. Recent investigations demonstrated that HMGB1 mRNA expression was significantly increased in liver and lung after rat thermal injury[30], that HMGB1 concentration was increased in lung epithelial lining fluid of patients with acute lung injury[18], and that HMGB1 expression was up-regulated in the liver after murine liver ischemia-reperfusion[28]. Therefore, it is likely that HMGB1 is produced and released by damaged organs in SAP. Change of HMGB1 expression in various tissues should be investigated in SAP.

It was recently clarified that extracellular HMGB1 acts as a cytokine by signaling *via* the receptor for advanced glycated end-products (RAGE)[31,32] and/or *via* members of the toll-like receptor (TLR) family (TLR2 and 4)[33]. Activation of RAGE and TLR leads to the induction of inflammatory responses *via* NF-κB. Tsung *et al*[28] demonstrated that anti-HMGB1 antibody failed to provide protection in TLR4-defective mice, but successfully reduced liver damage after ischemia/reperfusion in wild-type mice, suggesting that TLR4 is involved in the process as one of the receptors. As TLR4 recognizes LPS of gram-negative bacilli[34,35], interactions of HMGB1 with TLR4 may provide an explanation for the ability of HMGB1 to generate inflammatory responses that are similar to those initiated by LPS. Moreover, TLR is involved in not only inflammatory response but also host defense mechanism, and it is postulated that TLR may function defensively against infection. In our result, anti-HMGB1 antibody worsened the bacterial translocation (especially gram-negative bacteria) in SAP, suggesting that HMGB1 may function at least partially *via* TLR (especially TLR4).

Results obtained here raise the possibility that blockade of HMGB1 in the early phase is useful as a new therapeutic option against the inflammatory response and MODS in patients with SAP. Further investigations should be performed to elucidate the role of HMGB1 and HMGB1 signaling in the mechanism of inflammatory response, organ injury, and infection in SAP.

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*RAPID COMMUNICATION*

# Defensive medicine practices among gastroenterologists in Japan

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## Abstract

**AIM:** To clarify the prevalence of defensive medicine and the specific defensive medicine practices among gastroenterologists in Japan.

**METHODS:** A survey of gastroenterologists in Hiroshima, Japan, was conducted by mail in March 2006. The number of gastroenterologists reporting defensive medicine behaviors or changes in their scope of practice and the reported defensive medicine practices, i.e., assurance and avoidance behaviors, were examined.

**RESULTS:** A total of 131 (77%) out of 171 gastroenterologists completed the survey. Three (2%) respondents were sued, and most respondents (96%) had liability insurance. Nearly all respondents (98%) reported practicing defensive medicine. Avoidance behaviors, such as avoiding certain procedures or interventions and avoiding caring for high-risk patients, were very common (96%). Seventy-five percent of respondents reported often avoiding certain procedures or interventions. However, seasoned gastroenterologists (those in practice for more than 20 years) adopted avoidance behaviors significantly less often than those in practice for less than 10 years. Assurance behaviors, i.e., supplying additional services of marginal or no medical value, were also widespread (91%). Sixty-eight percent of respondents reported that they sometimes or often referred patients to other specialists unnecessarily.

**CONCLUSION:** Defensive medicine may be highly prevalent among gastroenterologists throughout Japan, with potentially serious implications regarding costs, access, and both technical and interpersonal quality of care.



**Key words:** Defensive medicine; Gastroenterologist; Japan; Survey; Clinical practice

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## INTRODUCTION

The number of negligence claims against physicians is increasing continuously, not only in Western countries but also in Japan[1-9], where a 10-fold increase in malpractice litigations, from 102 to 1019 cases per year, was observed between 1960 and 2003[7]. Although the number of medical lawsuits in Japan is relatively small in comparison to that in the United States of America (USA), the situation in Japan is gradually becoming more like that in the USA[10,11].

Defensive medicine is a deviation from sound medical practice that is induced primarily by the threat of liability claims[12,13]. Defensive medicine consists of two general behaviors. One is assurance behavior (sometimes called "positive" defensive medicine), which involves supplying additional services of marginal or no medical value with the aim of reducing adverse outcomes, deterring patients from filing malpractice claims, or persuading the legal system that the standard of care is met. The other is avoidance behavior (sometimes called "negative" defensive medicine), which refers to physicians' efforts to distance themselves from sources of legal risk. It was recently reported that nearly all (93%) physicians in the USA practice defensive medicine[12]. Defensive medicine has been reported in other countries[14,15], but the prevalence and specific behaviors of defensive medicine in Japan, particularly among gastroenterologists, remain unclear. Therefore, we studied the prevalence of defensive medicine and specific defensive behaviors in a proportion of gastroenterologists in Japan.

## MATERIALS AND METHODS

### Survey questionnaire and administration

A random sample of 171 Japanese gastroenterologists was drawn from the Hiroshima Medical Association Physician File. A questionnaire with items pertaining to clinical decisions, liability insurance, and malpractice claims, was developed. Respondents were asked to rate on a four-point scale (never, rarely, sometimes, often) how often concerns about malpractice liability claims caused them to engage in each of 4 forms of assurance behavior: (1) order more tests than medically indicated, (2) prescribe more medications than medically indicated, (3) refer patients to specialists unnecessarily, and (4) suggest invasive procedures against their professional judgment. Respondents used the same scale to rate the frequency with which they practiced two forms of avoidance behavior: (1) avoid conducting certain procedures/interventions, and (2) avoid caring for high-risk patients[12]. In addition, respondents were asked in consecutive questions whether they had reduced or eliminated various high-risk aspects of their medical practice in the last 3 years.

The questionnaire was mailed in March 2006 to the 171 randomly selected gastroenterologists in Hiroshima, Japan. One hundred and thirty-one (77%) of these gastroenterologists completed the survey.

### Statistical analysis

Survey responses were analyzed in relation to the number of years respondents had been in practice or to the type of practice. Differences between groups were analyzed by chi-square test or Fisher's exact probability test. $P < 0.05$ was considered statistically significant.

## RESULTS

### Respondent characteristics

The characteristics of respondents are shown in Table 1. Respondents were experienced gastroenterologists (82% with > 9 years in practice). They practiced in hospitals (81%), solo settings (14%), and other contexts (5%). Two percent of respondents had been sued in the past. Most respondents (96%) had obtained liability insurance, either directly from a commercial carrier (93%) or through a hospital (7%). Most respondents perceived their insurance premiums to be financially burdensome, with 33% classifying the burden as major.

**Table 1 Characteristics of gastroenterologists surveyed (*n* = 131)**

| Characteristic | Gastroenterologists *n* (%) |
|---|---|
| Sex | |
| Male | 110 (84) |
| Female | 21 (16) |
| Years in practice | |
| 1-10 | 33 (25) |
| 11-19 | 67 (51) |
| 20-29 | 26 (20) |
| 30 | 5 (4) |
| Practice type | |
| Solo | 18 (14) |
| Hospital clinic | 107 (81) |
| Other | 6 (5) |
| Perceived burden of liability insurance premiums | |
| Not a burden | 14 (11) |
| Minor | 17 (13) |
| Medium | 57 (43) |
| Major | 3 (33) |
| Claims experience | |
| Sued | |
| ≤ 3 yr ago | 1 (1) |
| > 3 yr ago | 2 (1) |
| Never sued | 128 (98) |

**Table 2 Frequency of assurance and avoidance behaviors among gastroenterologists surveyed**

| | Often *n* (%) | Never/rarely *n* (%) |
|---|---|---|
| Assurance behavior | | |
| Order more tests than medically indicated | 7 (5) | 83 (64) |
| Prescribe more medications (e.g., antibiotics) than medically indicated | 0 (0) | 110 (84) |
| Refer patients to other specialists unnecessarily | 36 (27) | 41 (32) |
| Suggest invasive procedures (e.g., biopsy) to confirm diagnosis | 21 (16) | 61 (47) |
| Avoidance behavior | | |
| Avoid certain procedures or interventions | 24 (18) | 32 (24) |
| Avoid caring for high-risk patients | 27 (21) | 32 (24) |

### General findings

Most respondents (98%) reported that they sometimes or often engaged in at least one of the six forms of defensive medicine outlined in the survey.

### Assurance behavior

One-hundred and nineteen (91%) of the 131 respondents reported that they sometimes or often engaged in at least one of the four forms of assurance behavior. Sixty-eight percent of respondents reported that they sometimes or often referred patients to other specialists unnecessarily (Table 2). Fifty-four percent of respondents reported that they sometimes or often suggested invasive procedures that were unwarranted. Thirty-six percent of respondents reported that they sometimes or often ordered more diagnostic tests than medically indicated. Sixteen percent of respondents reported sometimes or often prescribing more medications than were medically indicated.

### Avoidance behavior

One-hundred and twenty-six (96%) of the 131 respondents reported that they sometimes or often engaged in at least one of the two forms of avoidance behavior. Seventy-five percent of respondents reported that they often avoided certain procedures or interventions (Table 2). Fifty-three percent of respondents reported that they sometimes or often avoided caring for high-risk patients.

### Assurance and avoidance behaviors in relation to years in practice

The relation between assurance and avoidance behaviors and years in practice is shown in Table 3. Seasoned

**Table 3 Relation between assurance and avoidance behaviors and years in practice**

| | Number of yearsin practice | Often/sometimes *n* (%) |
|---|---|---|
| Assurance behavior | | |
| Order more tests than medically indicated | 1-9 | 14/33 (42) |
| | 10-19 | 26/67 (39) |
| | 20- | 8/31 (26) |
| Prescribe more medications (e.g., antibiotics) than medically indicated | 1-9 | 7/33 (21)[a] |
| | 10-19 | 13/67 (19)[c] |
| | 20- | 1/31 (3) |
| Refer patients to other specialists unnecessarily | 1-9 | 24/33 (73) |
| | 10-19 | 47/67 (70) |
| | 20- | 19/31 (61) |
| Suggest invasive procedures (e.g., biopsy) to confirm diagnosis | 1-9 | 21/33 (64) |
| | 10-19 | 36/67 (54) |
| | 20- | 13/31 (42) |
| Avoidance behavior | | |
| Avoid certain procedures or interventions | 1-9 | 28/33 (85) |
| | 10-19 | 52/67 (78) |
| | 20- | 19/31 (61)[e] |
| Avoid caring for high-risk patients | 1-9 | 28/33 (85) |
| | 10-19 | 52/67 (78) |
| | 20- | 19/31 (61)[e] |

[a]$P = 0.033$, [c]$P = 0.027$ *vs* 20 yr in practice; [e]$P = 0.032$ *vs* 1-9 yr in practice.

**Table 4 Relation between assurance and avoidance behaviors and practice types**

| | Practicetype | Often/sometimes *n* (%) |
|---|---|---|
| Assurance behavior | | |
| Order more tests than medically indicated | Solo | 2/18 (11)[a] |
| | Hospital clinic | 43/107 (40) |
| | Other | 3/6 (50) |
| Prescribe more medications (e.g., antibiotics) than medically indicated | Solo | 1/18 (6) |
| | Hospital clinic | 19/107 (18) |
| | Other | 1/6 (17) |
| Refer patients to other specialists unnecessarily | Solo | 18/18 (100)[b] |
| | Hospital clinic | 69/107 (64) |
| | Other | 3/6 (50) |
| Suggest invasive procedures (e.g., biopsy) to confirm diagnosis | Solo | 10/18 (56) |
| | Hospital clinic | 57/107 (53) |
| | Other | 3/6 (50) |
| Avoidance behavior | | |
| Avoid certain procedures or interventions | Solo | 12/18 (67) |
| | Hospital clinic | 82/107 (77) |
| | Other | 5/6 (83) |
| Avoid caring for high-risk patients | Solo | 14/18 (78) |
| | Hospital clinic | 80/107 (75) |
| | Other | 5/6 (83) |

[a]$P = 0.013$, [b]$P = 0.0006$ *vs* hospital clinic.

gastroenterologists (20 years in practice or more) prescribed medications that were not medically indicated significantly more often than other gastroenterologists ($P = 0.033$ by Fisher's exact probability test *vs* gastroenterologists in practice for 1-9 years and $P = 0.027$ *vs* gastroenterologists in practice for 10-19 years). Seasoned gastroenterologists were also significantly less likely than those who had been in practice for fewer than 10 years to avoid certain procedures or interventions [19/31 (61%) *vs* 28/33 (85%), $P = 0.032$ by Fisher's exact probability test] and to avoid caring for high-risk patients [19/31 (61%) *vs* 28/33 (85%), $P = 0.032$ by Fisher's exact probability test].

### Assurance and avoidance behaviors in relation to practice type

Adoption of assurance and avoidance behaviors in relation to practice types is shown in Table 4. Gastroenterologists in solo practices ordered significantly less often more tests than medically indicated than gastroenterologists in hospital clinics [2/18 (11%) *vs* 43/107 (40%), $P = 0.013$ by Fisher's exact probability test]. However, they referred patients to other specialists unnecessarily significantly more often than those in hospital clinics [18/18 (100%) *vs* 69/107 (64%), $P = 0.0006$ by Fisher's exact probability test].

### Most recent defensive act

Ninety percent (118 of the 131) of gastroenterologists who reported practicing defensive medicine detailed their most recent defensive act. Specific practices reported by these respondents are summarized in Table 5. The defensive act most frequently reported was recording interactions with a patient in considerable detail (35%). This was followed by unnecessary referral (33%), avoiding certain procedures or interventions, and ordering an unnecessary test (11%).

**Table 5 Specific defensive medicine practices among gastroenterologists ($n = 118$)**

| Most recent act of defensive medicine | Gastroenterologists *n* (%) |
|---|---|
| Recorded interaction with patient in considerable detail | 41 (35) |
| Referred patient to another physician | 39 (33) |
| Avoided certain procedure or intervention | 25 (21) |
| Ordered more tests than medically indicated | 13 (11) |

## DISCUSSION

We found that defensive medicine practices were as widespread among the gastroenterologists we surveyed in Japan as they are among physicians in the USA. In the USA, 88% of physicians have a lawsuit filed against them, reflecting the high rate of litigation in that country[12]. Studdert *et al*[12] reported that 93% of physicians in the USA practice defensive medicine, and that assurance behaviors, such as ordering tests, performing diagnostic procedures, and referring patients for consultation, are very common (92%). In Japan, the number of negligence claims against

gastroenterologists is still very limited. Only 2% of our respondents have been sued, a finding similar to that reported by the Hiroshima Prefectural Medical Association from a survey of all its members[16]. Despite the relatively low frequency of lawsuits, nearly all (98%) gastroenterologists we surveyed reported practicing defensive medicine, and both assurance and avoidance behaviors were very common (91% and 96%, respectively). This may be due to the continuous increase in the number of litigations. With sensational mass media reporting on medical malpractice in Japan, gastroenterologists in Japan have begun to focus on risk management activities, leading them to practice defensive medicine[7,17]. These findings support the idea that individual physicians' propensity to practice defensive medicine is not associated with objective measures of physicians' exposure to and experience with liability claims[18-20]. It has been suggested that the signal to practice defensive medicine may have been broadcasted so widely that individual experience is overshadowed by collective anxiety[18].

Although the prevalence of defensive medicine in Japan is similar to that in the USA, individual components of this trend differ between the two countries. In a USA study of physicians in 6 specialties (not including gastroenterology)[12], almost one-third of respondents reported that they often describe more medications than medically indicated, whereas only 16% of our survey respondents, all gastroenterologists, reported doing so. In addition, 32% of physicians in the USA often suggest invasive procedures to confirm diagnoses, whereas only 16% of gastroenterologists we surveyed did so. Our findings suggest that assurance behavior is less prevalent among gastroenterologists in Japan than among physicians in the USA. In contrast, our findings suggest that the prevalence of avoidance behavior among gastroenterologists in Japan is similar to that among physicians in the USA. This may be due to differences in the structure of the medical economic system between the two countries[21]. In Japan, medical examination fees are considerably lower than those in the USA. Therefore, physicians in Japan may be more apt to suggest invasive procedures to confirm diagnoses.

Avoidance behaviors we surveyed were significantly less common among seasoned gastroenterologists than among those in practice less than 10 years. It is not surprising that less experienced physicians tend to avoid difficult procedures and interventions and caring for high-risk patients. It is reasonable that high-risk patients should be treated by fully experienced doctors.

Respondents who worked in hospital clinics ordered more tests than medically indicated significantly more often than respondents in solo practices. In Japan, patients desiring a thorough examination tend to visit a hospital clinic. To meet patients' expectations, doctors in hospital clinics may order more tests than medically indicated. However, doctors in hospital clinics reported referring patients to other specialists unnecessarily less frequently than doctors in solo practices. Doctors in hospital clinics may refer less frequently because of easy access to specialized examinations or to other specialists, especially in general hospitals.

Our study was limited in several ways. First, measurement and identification of defensive medicine by the physicians themselves are difficult because distinctions between inappropriate and appropriate care are not clear in many clinical situations[22]. Moreover, it can be difficult to disentangle liability-related motivational factors from other factors that influence clinical decision-making, such as physicians' general desire to meet patients' expectations, preserve trust, and avoid conflict[23,24]. To the extent that the responding gastroenterologists attributed their decisions to liability concerns when in fact they were driven primarily by other considerations, our results are exaggerated. Second, the gastroenterologists' self-reports of defensive medicine may have been biased toward giving a socially desirable response or achieving political goals. This may have led respondents to overestimate the frequency of their adoption of forms of defensive medicine.

An increased incidence of defensive medical practices is part of the social cost of a health-care system. The most frequent form of defensive medicine we found is suggestion of invasive procedures to confirm diagnoses, which seems not only wasteful but also likely to increase the risks of medical complications. Efforts to reduce the practice of defensive medicine in Japan should focus both on educating patients and gastroenterologists regarding appropriate care in the gastroenterological context that most commonly prompts defensive medicine, and on developing and disseminating clinical guidelines that target common defensive practices.

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## COMMENTS

### *Background*

The prevalence of defensive medicine and the specific defensive medicine practices among gastroenterologists in Japan are unclear.

### *Research frontiers*

Two percentage respondents have been sued, and most respondents (96%) had liability insurance. Nearly all respondents (98%) reported practicing defensive medicine, and avoidance behaviors, such as avoiding certain procedures or interventions and avoiding caring for high-risk patients, were very common (96%). Defensive medicine may be highly prevalent among gastroenterologists throughout Japan, with potentially serious implications regarding costs, access, and both technical and interpersonal quality of care.

### *Applications*

Efforts to reduce the practice of defensive medicine in Japan should focus both on educating patients and gastroenterologists regarding appropriate care in the gastroenterological context that most commonly prompts defensive medicine, and on developing and disseminating clinical guidelines that target common defensive practices.

### *Terminology*

Defensive medicine, a deviation from sound medical practice, is induced primarily by the threat of liability claims.

### *Peer review*

This study is interesting and provides useful information on the characteristics of defensive medicine among gastroeneterologists in Japan.

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# Meta-analysis of short-term outcomes after laparoscopy-assisted distal gastrectomy

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## Abstract

**AIM:** To elucidate the current status of laparoscopy-assisted distal gastrectomy (LADG) with regard to its short-term outcomes by comparing it with conventional open distal gastrectomy (CODG).

**METHODS:** Original articles published from January 1991 to August 2006 were searched in the MEDLINE, EMBASE, and Cochrane Controlled Trials Register. Clinical appraisal and data extraction were conducted independently by 2 reviewers. A meta-analysis was performed using a random effects model.

**RESULTS:** Outcomes of 1611 procedures from 4 randomized controlled trials and 12 retrospective studies were analyzed. Compared to CODG, LADG was a longer procedure (weighted mean difference [WMD] 54.3; 95% confidence interval [CI] 38.8 to 69.8; $P < 0.001$), but was associated with a lower associated morbidity (odds ratio [OR] 0.54; 95% CI 0.37 to 0.77; $P < 0.001$); this was most significant for postoperative ileus (OR 0.27; 95% CI 0.09 to 0.84; $P = 0.02$). There was no significant difference between the two groups in anastomotic, pulmonary, and wound complications and mortality. Duration from surgery to first passage of flatus was faster (WMD -0.68; 95% CI -0.85 to -0.50; $P < 0.001$) and the frequency of additional analgesic requirement (WMD -1.36; 95% CI -2.44 to -0.28; $P = 0.01$), and duration of hospital stay (WMD -5.51; 95% CI -7.61 to -3.42; $P < 0.001$) were significantly lower after LADG. However, a significantly higher number of lymph nodes were dissected by CODG (WMD -4.35; 95% CI -5.73 to -2.98; $P < 0.001$).

**CONCLUSION:** LADG for early gastric cancer is associated with a lower morbidity, less pain, faster bowel function recovery, and shorter hospital stay.



## INTRODUCTION

Many surgeons are interested in laparoscopic surgery for gastric cancer because it has been proved that laparoscopic surgery has several advantages over conventional open surgery[1-3]. Since 1991, laparoscopy-assisted distal gastrectomy (LADG) has been adopted by Kitano[4] for the treatment of early gastric cancer, and it has been performed worldwide, especially in Japan and Korea. In 1997, Goh *et al*[5] published the early results of 118 LADGs; they sent a questionnaire to 16 surgeons across 12 countries and found that 10 of these surgeons claimed LADG to be superior to conventional open distal gastrectomy (CODG) because of a faster recovery, reduced pain, and better cosmesis[5]. Many studies comparing LADG with CODG with respect to their short-term outcomes have been performed. However, the feasibility and advantages of LADG have not been thoroughly evaluated thus far. Therefore, we performed a meta-analysis by comparing LADG with CODG with regard to their short-term outcomes to elucidate the current status of LADG. Unfortunately, only 4 prospective randomized controlled trials (RCTs) have been published[6-9]. Lack of RCTs may be due to the difficulty encountered in conducting a large RCT in Japan, where LADGs are most frequently performed because the Japanese are disinclined toward enrolling in RCTs and show a strong preference for a specific type of treatment[9]. Therefore, performing a reasonable meta-analysis of only RCTs may not be currently justified. Nevertheless, summarizing all the published data is important because it may help surgeons in choosing a better approach for the management of individual patients with gastric cancer. This meta-analysis is not only limited solely to RCTs but also includes retrospective trials that have compared LADG with CODG. We have also analyzed the RCTs separately in addition to analyzing all the included studies.

## MATERIALS AND METHODS

### *Literature search*

From January 1991 to August 2006, a thorough search of the MEDLINE, EMBASE, and Cochrane Controlled Trials Register databases was performed. The following keywords were used: "laparoscopic," "laparoscopy-assisted/laparoscopic-assisted," and "gastrectomy." The search was limited to studies published in English; all titles and abstracts were scanned and appropriate citations were reviewed.

### *Inclusion and exclusion criteria*

Inclusion criteria were as follows: (1) study type-RCTs and non-randomized prospective and retrospective studies; (2) studies that analyzed both LADG and CODG for the treatment of gastric cancer; (3) studies with any sample size; and (4) when we found several studies reporting the same patients, we included only the most recent study; however, if an older study in this category was an RCT, then it was included in our meta-analysis.

### *Methods of review*

Clinical appraisal and data extraction were conducted independently by 2 authors (S.H. and Y.A.). Discrepancies between the authors were resolved by consensus. The primary outcome measures were operative findings, postoperative complications and operation-related mortality, and postoperative clinical course. The following operative findings were analyzed: operating time, blood loss, and total number of dissected lymph nodes. The following postoperative complications were analyzed: overall complications, anastomotic leakage and stenosis, postoperative ileus, pulmonary complications, and wound infection. Overall complications were evaluated based on the total number of postoperative events. Anastomotic leakage included duodenal stump leakage; postoperative ileus included both mechanical and paralytic ileus; and pulmonary complications included pneumonia, pleural effusion, and atelectasis. Postoperative clinical course was analyzed in terms of bowel function recovery, frequency of additional analgesic requirement, number of days with body temperatures more than 37℃, duration of postoperative hospital stay, and WBC counts and C-reactive protein (CRP) levels on postoperative d 1, 3, and 7. Bowel function recovery was assessed by calculating the time interval between surgery and the first passage of flatus. Data were obtained from individual trials using the most reliable data available. Raw data were considered the most reliable, followed by derivation from the graph. It was assumed that all definitions in the included trials were synonymous, unless specified otherwise.

### *Statistical analysis*

Weighted mean differences with 95% confidence intervals were used for analyzing continuous variables that were presented in the same scale (e.g., operating time, blood loss, and postoperative hospital stay). When the trials had reported medians and ranges instead of means and standard deviations, we assumed that the difference in medians is equal to that in means, and the estimated standard deviation was considered equivalent to a quarter of the reported range. If neither a range nor any other measure of dispersion was available, then the standard deviation was estimated by halving the mean or the median. For dichotomous variables, odds ratios with 95% confidence intervals were calculated using a random effects model. If a particular outcome measure was reported in more than 2 RCTs, we conducted data analyses of these RCTs as well as of the overall studies. All statistical calculations were performed using the computer software Review Manager (RevMan) version 4.2.8 provided by Cochrane Collaboration. A value of $P < 0.05$ was considered significant. Heterogeneity was evaluated by using the $\chi^2$ test; $P < 0.1$ was considered significant for heterogeneity.

## RESULTS

Through our database searches, we found 4 RCTs[6-9] and 12 non-randomized retrospective studies[10-21] that compared LADG with CODG. We performed a meta-analysis of all the 16 studies using the data obtained from 1611 patients (837 and 774 patients who underwent LADG and CODG, respectively). The characteristics of the studies included in our meta-analysis are listed in Table 1.

### *Operative findings*

All studies[6-21] had reported the operating time, and 15 studies[6-17,19-21] had reported blood loss. Most studies claimed that CODG was superior to LADG in terms of operating time; however, some[8,10,12,13,16] did not report such an advantage. Pooled data obtained from the weighted mean difference revealed that an additional 54 min was required to perform LADG ($P < 0.001$), and that the blood loss was decreased ($P < 0.001$) when compared with CODG. The number of dissected lymph nodes had been reported in 14 studies[6-9,11,12,14-21], including 4 RCTs[6-9]. There were different levels of lymphadenectomy (Table 1). Analyses of the pooled data of only the RCTs as well as of all the studies revealed that a significantly higher number of lymph nodes were dissected during CODG ($P < 0.001$) (Figure 1). A significant heterogeneity was observed among all the studies; however, heterogeneity was not detected among the 4 RCTs. A summary of the pooled results with regard to the operative findings is presented in Table 2.

### *Morbidity and mortality*

Thirteen studies[6-14,16,18,19,21], which included 1054 patients, provided data regarding the overall postoperative complications. Overall complications after LADG (58/535) were significantly less than that after CODG (97/519; $P < 0.001$; Figure 2). Seven studies[8,9,11,12,16,18,21], which included 750 patients, provided data regarding anastomotic leakage. The incidence of anastomotic leakage was not different between LADG (2/385; 0.5%) and CODG (10/365; 2.7%) ($P = 0.10$). Similarly, the incidence of anastomotic stenosis was also not different between LADG (6/172; 3.5%) and CODG (5/163; 3.1%) ($P = 0.86$) in 5 studies[7,9,11,12,16]. Six studies[7,10,12,13,16,18] reported that postoperative ileus was significantly less frequent after LADG (2/267; 0.75%) than that after CODG (13/264; 4.9%) ($P = 0.02$) (Figure 3). Wound infection was observed in 9 studies[7,8,11,12,14,16,18,19,21], which included 869 patients. There was no difference

**Table 1 Trials included in the meta-analysis**

| Ref. | Yr | Country | *n* | | Level of lymph node dissection | Participants in LADG |
|---|---|---|---|---|---|---|
| | | | LADG | CODG | | |
| Prospective randomized controlled trials | | | | | | |
| 6 | 2002 | Japan | 14 | 14 | D1 + α | Patients with EGC in whom EMR was not indicated |
| 7 | 2005 | Korea | 24 | 23 | D2 | Patients with preoperatively diagnosed mucosal or submucosal cancer |
| 8 | 2005 | Italy | 30 | 29 | D1, D2 | Patients who presented with metastatic tumor or with tumor extension beyond the distal stomach were excluded. |
| 9 | 2005 | Japan | 14 | 14 | D1 + α | Patients with EGC assumed to infiltrate the mucosa or submucosa |
| Retrospective studies | | | | | | |
| 10 | 2000 | Japan | 49 | 53 | D1 + α | Patients with EGC assumed to infiltrate the mucosa or submucosa |
| 11 | 2000 | Japan | 21 | 31 | D1 + α | Patients with EGC assumed to infiltrate the mucosa |
| 12 | 2001 | Japan | 24 | 35 | D1 + α | Patients with preoperatively diagnosed mucosal or submucosal cancer; those with deep submucosal cancer were excluded. |
| 13 | 2003 | Japan | 10 | 17 | D1 + α | Patients with EGC assumed to infiltrate the mucosa or submucosa |
| 14 | 2005 | Japan | 37 | 31 | D2 | Patients with EGC assumed to infiltrate the mucosa or submucosa, and those diagnosed with advanced cancer without lymph node metastasis |
| 15 | 2005 | Japan | 235 | 200 | D2 | Patients with preoperatively diagnosed gastric cancer assumed to be confined to the muscular layer without lymph node metastasis |
| 16 | 2005 | Japan | 89 | 60 | D1 + β | Patients with preoperatively diagnosed EGC assumed to infiltrate the mucosa or submucosa without lymph node metastasis |
| 17 | 2005 | Japan | 20 | 22 | D1 + α, D1 + β | Patients with preoperatively diagnosed EGC assumed to infiltrate the mucosa or submucosa without lymph node metastasis |
| 18 | 2005 | Korea | 71 | 76 | D1 + α, D1 + β, D2 | Patients with mucosal cancer indicated for EMR were excluded. |
| 19 | 2005 | Korea | 16 | 16 | D2 | Patients with EGC assumed to infiltrate the mucosa or submucosa |
| 20 | 2006 | Japan | 47 | 33 | D1 + β | Patients with EGC assumed to infiltrate the mucosa |
| 21 | 2006 | Korea | 136 | 120 | D1 + α, D1 + β, D2 | Patients with preoperatively diagnosed EGC |

LADG: Laparoscopy-assisted distal gastrectomy; CODG: Conventional open distal gastrectomy; EGC: Early gastric cancer; D1: Perigastric lymph nodes; D1 + α: Perigastric lymph nodes and lymph nodes along the left gastric artery; D1 + β: Perigastric lymph nodes and lymph nodes along the left gastric artery and the celiac axis; D2: Perigastric lymph nodes and lymph nodes along the left gastric, common hepatic, proper hepatic, celiac, and splenic arteries; EMR: Endoscopic mucosal resection.

**Table 2 Operative findings**

| Outcome | Type of studies included in the meta-analysis | Trials (*n*) | Patients (*n*) | Pooled results WMD (95% CI) | Interpretation | Test for heterogeneity |
|---|---|---|---|---|---|---|
| Operating time | RCTs | 4 | 162 | 83.1 (40.5, 125.6)<br>$Z = 3.83$, $P < 0.001$ | L > C | $\chi^2 = 91.9$, df = 3<br>$P < 0.001$, $I^2 = 96.7\%$ |
| | Overall | 16 | 1611 | 54.3 (38.8, 69.8)<br>$Z = 6.88$, $P < 0.001$ | L > C | $\chi^2 = 620.9$, df = 15<br>$P < 0.001$, $I^2 = 97.6\%$ |
| Blood loss | RCTs | 4 | 162 | -104.3 (-189.0, -19.5)<br>$Z = 2.41$, $P = 0.02$ | L < C | $\chi^2 = 13.6$, df = 3<br>$P = 0.004$, $I^2 = 77.9\%$ |
| | Overall | 15 | 1464 | -145.6 (-181.4, -109.9)<br>$Z = 7.99$, $P < 0.001$ | L < C | $\chi^2 = 280.4$, df = 14<br>$P < 0.001$, $I^2 = 95.0\%$ |
| No. of Lymph nodes dissected | RCTs | 4 | 162 | -4.34 (-6.66, -2.02)<br>$Z = 3.66$, $P < 0.001$ | L < C | $\chi^2 = 1.68$, df = 3<br>$P = 0.64$, $I^2 = 0\%$ |
| | Overall | 14 | 1482 | -4.35 (-5.73, -2.98)<br>$Z = 6.20$, $P < 0.001$ | L < C | $\chi^2 = 34.8$, df = 13<br>$P = 0.001$, $I^2 = 62.7\%$ |

L: Laparoscopy-assisted distal gastrectomy; C: Conventional open distal gastrectomy; RCTs: Randomized controlled trials; WMD: Weighted mean difference; CI: Confidence intervals; df: Degree of freedom.

in wound infection rate between LADG (9/448; 2.0%) and CODG (13/421; 3.1%). Only 2 of the 16 studies[8,18] reported on mortality; however, there was no difference between LADG and CODG with regard to mortality. A summary of the pooled results for morbidity and mortality is presented in Table 3.

### Postoperative clinical course

Bowel function recovery was evaluated by counting the number of days from surgery to the first passage of flatus in 12 studies[6,7,9-12,14,15,17-19,21], which included 1296 patients. Our analysis showed that bowel function recovery was achieved 0.7 d earlier by the LADG patients than by the CODG patients ($P < 0.001$) (Figure 4). Postoperative pain was assessed by determining the frequency of additional analgesic requirement for postoperative pain in only 6[6,7,9,10,17,18] of the 16 studies. The requirement of additional analgesics after LADG was 3.3 times less frequent

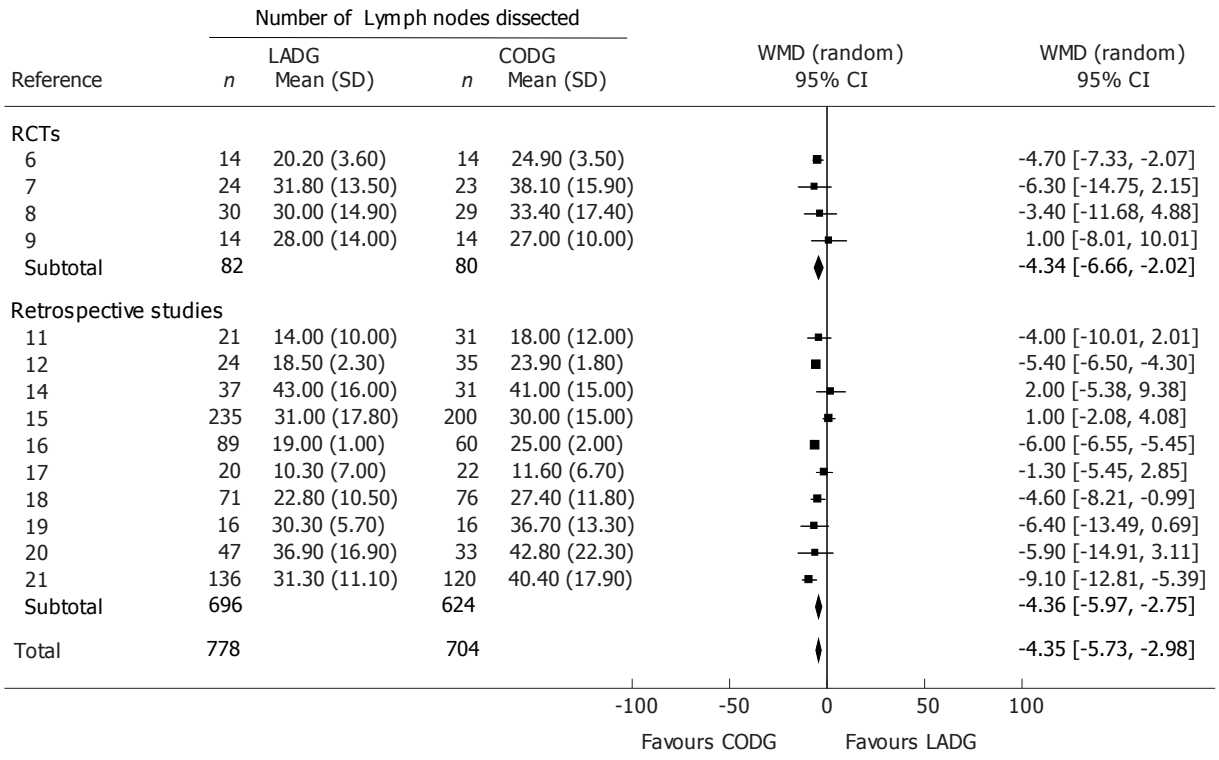


**Figure 1** Analysis of the number of lymph nodes dissected. Weighted mean differences (WMDs) are shown with 95% CI.

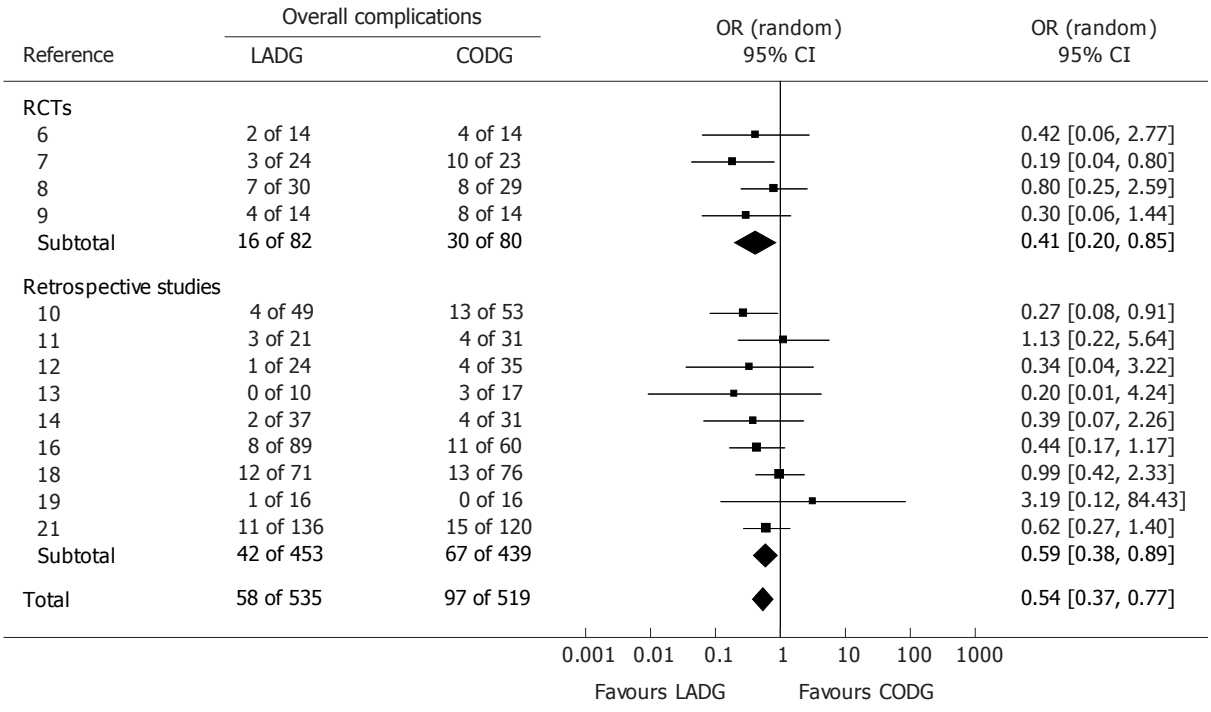


**Figure 2** Analysis of overall complications. Weighted mean differences (WMDs) are shown with 95% CI.

than that after CODG. Data regarding the duration of postoperative hospital stay was provided in 15 studies[6-19,21], which included 1531 patients. From our analysis, this duration was 5.5 d shorter for LADG patients than for CODG patients ($P < 0.001$). Variability of the duration of postoperative hospital stay was extremely high with a mean duration ranging from 7.8 to 29.2 d in LADG and from 11.2 to 40.7 d in CODG. However, most individual studies, except 4 studies[6,7,13,19], reported a significantly shorter hospital stay after LADG than after CODG. The number of days with body temperatures more than 37℃ was reported in 5 studies[9-11,14,17]. Body temperatures normalized 1.3 d earlier in LADG patients than in CODG patients. WBC counts on postoperative d 1, 3, and 7 were

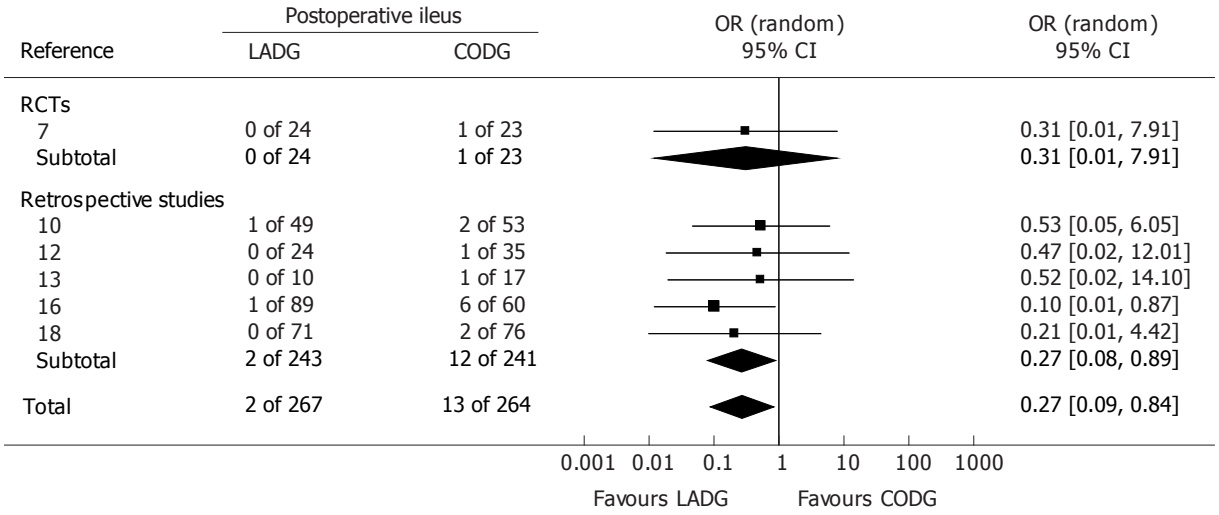


**Figure 3** Analysis of postoperative ileus. Odds ratios (ORs) are shown with 95% CI.

**Table 3 Morbidity and mortality**

| Outcome | Type of studies included in the meta-analysis | Trials (*n*) | LADG | | CODG | | Pooled results OR (95% CI) | Interpretation | Test for heterogeneity |
|---|---|---|---|---|---|---|---|---|---|
| | | | Events | Patients (*n*) | Events | Patients (*n*) | | | |
| Overall complications | RCTs | 4 | 16 | 82 | 30 | 80 | 0.41 (0.20, 0.85) $Z = 2.41$, $P = 0.02$ | L < C | $\chi^2 = 2.52$, df = 3 $P = 0.47$, $I^2 = 0\%$ |
| | Overall | 13 | 58 | 535 | 97 | 519 | 0.54 (0.37, 0.77) $Z = 3.37$, $P < 0.001$ | L < C | $\chi^2 = 9.11$, df = 12 $P = 0.69$, $I^2 = 0\%$ |
| Anastomotic leakage | RCTs | 2 | 0 | 44 | 3 | 43 | 0.23 (0.02, 2.18) $Z = 1.28$, $P = 0.20$ | L = C | $\chi^2 = 0.07$, df = 1 $P = 0.80$, $I^2 = 0\%$ |
| | Overall | 7 | 2 | 385 | 10 | 365 | 0.38 (0.12, 1.18) $Z = 1.67$, $P = 0.10$ | L = C | $\chi^2 = 0.96$, df = 6 $P = 0.99$, $I^2 = 0\%$ |
| Anastomotic stenosis | RCTs | 2 | 0 | 38 | 2 | 37 | 0.31 (0.03, 3.11) $Z = 1.00$, $P = 0.32$ | L = C | $\chi^2 = 0.00$, df = 1 $P = 1.00$, $I^2 = 0\%$ |
| | Overall | 5 | 6 | 172 | 5 | 163 | 1.11 (0.35, 3.54) $Z = 0.18$, $P = 0.86$ | L = C | $\chi^2 = 2.01$, df = 4 $P = 0.73$, $I^2 = 0\%$ |
| Postoperative ileus | Overall | 6 | 2 | 267 | 13 | 264 | 0.27 (0.09, 0.84) $Z = 2.26$, $P = 0.02$ | L < C | $\chi^2 = 1.40$, df = 5 $P = 0.92$, $I^2 = 0\%$ |
| Pulmonary complications | RCTs | 4 | 10 | 82 | 19 | 80 | 0.47 (0.20, 1.12) $Z = 1.70$, $P = 0.09$ | L = C | $\chi^2 = 1.95$, df = 3 $P = 0.58$, $I^2 = 0\%$ |
| | Overall | 8 | 12 | 260 | 22 | 271 | 0.54 (0.25, 1.15) $Z = 1.59$, $P = 0.11$ | L = C | $\chi^2 = 3.66$, df = 7 $P = 0.82$, $I^2 = 0\%$ |
| Wound infection | RCTs | 2 | 3 | 54 | 3 | 52 | 0.96 (0.18, 5.01) $Z = 0.05$, $P = 0.96$ | L = C | $\chi^2 = 0.05$, df = 1 $P = 1.00$, $I^2 = 0\%$ |
| | Overall | 9 | 9 | 448 | 13 | 421 | 0.69 (0.30, 1.57) $Z = 0.89$, $P = 0.37$ | L = C | $\chi^2 = 3.00$, df = 8 $P = 0.93$, $I^2 = 0\%$ |
| Mortality | Overall | 2 | 2 | 101 | 3 | 105 | 0.67 (0.11, 4.24) $Z = 0.43$, $P = 0.67$ | L = C | $\chi^2 = 0.19$, df = 1 $P = 0.66$, $I^2 = 0\%$ |

L: Laparoscopy-assisted distal gastrectomy; C: Conventional open distal gastrectomy; OR: Odds ratio; CI: Confidence intervals; RCTs: Randomized controlled trials; df: Degree of freedom.

reported in 7[9-11,13,14,17,18], 5[9,10,13,17,18], and 4[9,10,17,18] studies, respectively; serum CRP levels on postoperative d 1, 3, and 7 were reported in 5[9,10,13,14,17], 4[9,10,13,17], and 3[9,10,17] studies, respectively. The increase in both WBC counts and CRP levels on postoperative d 1 and 3 was significantly less in LADG patients than in CODG patients; however, there was no significant difference with regard to WBC counts and CRP levels on postoperative d 7 between these two groups. A summary of the pooled results with regard to the postoperative clinical course is presented in Table 4.

## DISCUSSION

In this meta-analysis, we compared the feasibility and advantages of LADG with those of CODG to elucidate the current status of LADG. Although LADG was found to be a significantly longer procedure, its associated morbidity rate was lower than that of CODG. This observation was most significant with regard to postoperative ileus. There was no significant difference between the two groups with regard to anastomotic, pulmonary, and wound complications and mortality.

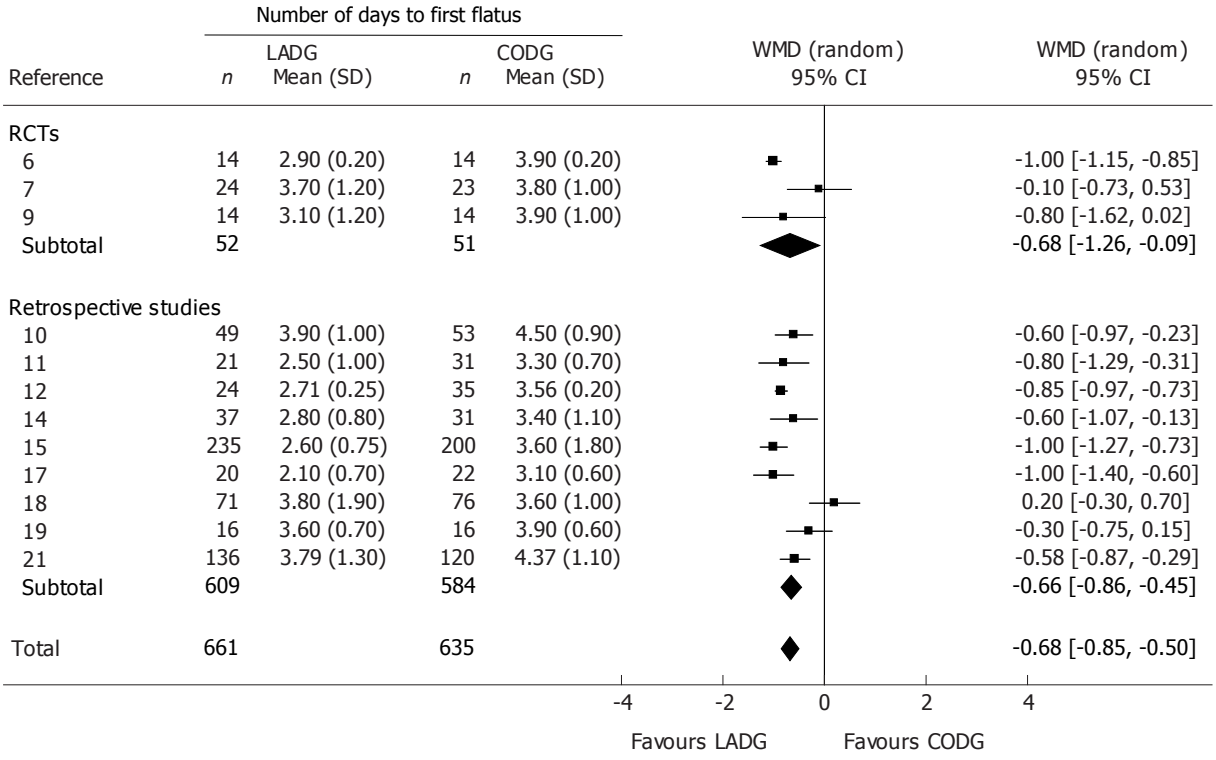


**Figure 4** Analysis of the number of days from surgery to the first passage of flatus. Weighted mean differences (WMDs) are shown with 95% CI.

**Table 4 Clinical course after operation**

| Outcome | Type of studies included in the meta-analysis | Trials (*n*) | Patients (*n*) | Pooled results WMD (95% CI) | Interpretation | Test for heterogeneity |
|---|---|---|---|---|---|---|
| Bowel function recovery | RCTs | 3 | 103 | -0.68 (-1.26, -0.09)<br>$Z = 2.27$, $P = 0.02$ | L < C | $\chi^2 = 7.55$, df = 2<br>$P = 0.02$, $I^2 = 73.5\%$ |
| | Overall | 12 | 1296 | -0.68 (-0.85, -0.50)<br>$Z = 7.63$, $P < 0.001$ | L < C | $\chi^2 = 39.6$, df = 11<br>$P < 0.001$, $I^2 = 72.2\%$ |
| Frequency of analgesic requirement | RCTs | 3 | 103 | -1.69 (-2.18, -1.21)<br>$Z = 6.82$, $P < 0.001$ | L < C | $\chi^2 = 0.12$, df = 2<br>$P = 0.94$, $I^2 = 0\%$ |
| | Overall | 6 | 394 | -1.36 (-2.44, -0.28)<br>$Z = 2.48$, $P = 0.01$ | L < C | $\chi^2 = 40.6$, df = 5<br>$P < 0.001$, $I^2 = 87.7\%$ |
| No. of days with temperatures more than 37℃ | Overall | 5 | 292 | -1.25 (-1.69, -0.82)<br>$Z = 5.64$, $P < 0.001$ | L < C | $\chi^2 = 2.05$, df = 4<br>$P = 0.73$, $I^2 = 0\%$ |
| Duration of hospital stay | RCTs | 4 | 162 | -3.32 (-7.69, 1.05)<br>$Z = 1.49$, $P = 0.14$ | L = C | $\chi^2 = 33.5$, df = 3<br>$P < 0.001$, $I^2 = 91.1\%$ |
| | Overall | 15 | 1531 | -5.51 (-7.61, -3.42)<br>$Z = 5.16$, $P < 0.001$ | L < C | $\chi^2 = 280.7$, df = 14<br>$P < 0.001$, $I^2 = 95.0\%$ |
| WBC (POD 1) | Overall | 7 | 466 | -1409.5 (-1934.6, -884.4)<br>$Z = 5.26$, $P < 0.001$ | L < C | $\chi^2 = 5.63$ , df = 6<br>$P = 0.47$, $I^2 = 0\%$ |
| WBC (POD 3) | Overall | 5 | 346 | -1028.1 (-1578.7, -477.4)<br>$Z = 3.66$, $P < 0.001$ | L < C | $\chi^2 = 4.18$, df = 4<br>$P = 0.38$, $I^2 = 4.2\%$ |
| WBC (POD 7) | Overall | 4 | 319 | -280.1 (-751.7, 191.5)<br>$Z = 1.16$, $P = 0.24$ | L = C | $\chi^2 = 1.73$, df = 3<br>$P = 0.63$, $I^2 = 0\%$ |
| CRP (POD 1) | Overall | 5 | 267 | -1.33 (-2.20, -0.46)<br>$Z = 3.01$, $P = 0.003$ | L < C | $\chi^2 = 7.24$, df = 4<br>$P = 0.12$, $I^2 = 44.8\%$ |
| CRP (POD 3) | Overall | 4 | 199 | -3.71 (-6.61, -0.80)<br>$Z = 2.50$, $P = 0.01$ | L < C | $\chi^2 = 24.4$, df = 3<br>$P < 0.001$, $I^2 = 87.7\%$ |
| CRP (POD 7) | Overall | 3 | 172 | -1.33 (-2.90, 0.25)<br>$Z = 1.65$, $P = 0.10$ | L = C | $\chi^2 = 8.36$, df = 2<br>$P = 0.02$, $I^2 = 76.1\%$ |

L: Laparoscopy-assisted distal gastrectomy; C: Conventional open distal gastrectomy; RCTs: Randomized controlled trials; CRP: C-reactive protein; POD: Postoperative day; WMD: Weighted mean difference; CI: Confidence intervals; df: Degree of freedom.

Gastrointestinal recovery was faster after LADG. Furthermore, the frequency of additional analgesic requirement, number of d with temperatures more than 37℃, duration of postoperative hospital stay, and acute inflammatory reaction in terms of WBC counts and CRP levels were significantly lower after LADG. However, the

number of lymph nodes dissected in CODG patients was significantly higher than that in LADG patients.

LADG with systematic lymphadenectomy is considered technically more complicated than other laparoscopic procedures such as laparoscopic cholecystectomy and colon resection because in LADG, large vessels have to be identified and extensive lymph node dissection has to be performed. Although the learning curve of LADG has reached a plateau, LADG remains a time-consuming procedure[22]. However, with improvements in instruments and techniques, the operating time for LADG would decrease[10,21]. Furthermore, Kim *et al*[23] claimed that the operating time for LADG is related to the knowledge of and familiarity with the laparoscopic system and instruments and the skill of the operating team. The prevalence of standard techniques and the development of an education and training system would be important in the future.

The Japanese Gastric Cancer Association guideline recommends the following optimal lymph node dissection levels for early gastric cancer: perigastric lymph nodes (D1) and lymph nodes along the left gastric artery for mucosal cancer and for submucosal cancer < 1.5 cm in diameter; D1 and lymph nodes along the left gastric artery and the celiac axis for preoperatively diagnosed submucosal cancer without lymph node metastasis and for early cancer < 2.0 cm in diameter with only perigastric lymph node metastasis; and D2 (D1 and lymph nodes along the left gastric, common hepatic, proper hepatic, celiac, and splenic arteries) for early cancer > 2.0 cm in diameter with lymph node metastasis[24]. On the other hand, many surgeons in the USA and other Western countries rarely perform extensive prophylactic lymphadenectomy because 2 European randomized trials[25,26] showed that there was no survival advantage of D2 over D1 lymphadenectomy, and that operative mortality and morbidity were higher after D2 than after D1 lymphadenectomy. Although there exists some controversy about whether D2 lymphadenectomy is superior to D1 lymphadenectomy in conventional open surgery for gastric cancer, some studies[7,8,14,15,19,27] reported that the mortality and morbidity rates after LADG with D2 lymphadenectomy were acceptable. However, with regard to the number of dissected lymph nodes, which was considered to reflect the quality of lymphadenectomy, our meta-analysis of all the published studies showed that CODG was superior to LADG; meta-analysis of only the RCTs also showed a similar result. Miura *et al*[27] demonstrated that a significantly higher number of lymph nodes were harvested by CODG, and that the difference was significant for the perigastric lymph nodes along the major curvature and the second tier nodes along the celiac and splenic arteries. One of the explainable reasons for these observations is that several studies included in our meta-analysis might have been conducted during the learning phase of LADG. However, Fujiwara *et al*[22] reported that there was no difference between their preliminary study and the study following the learning curve with regard to the number of dissected lymph nodes in LADG. To obtain a definitive answer, a well-designed RCT following the learning curve of LADG would be required. Our meta-analysis may indicate that during the learning phase, LADG should be performed for early gastric cancer with low potential of lymph node metastasis.

The principal advantage of laparoscopic surgery is the reduction in the stress induced by minimal manipulation of the small bowel and the use of a small incision, which accounts for early bowel function recovery and prevention for postoperative ileus. It is noteworthy that all the aforementioned advantages were obtained despite the longer operating time for LADG. Faster bowel function recovery could lead to early resumption of oral intake, and subsequently early hospital discharge. Furthermore, it could result in less postoperative nausea, vomiting, and abdominal discomfort. Mitigating surgical stress reduces the generalized inflammatory reaction; consequently, it might lead to a reduction in the overall complication rate.

The application of LADG for gastric cancer is still controversial because of the lack of clinical evidence regarding its long-term outcomes. Indeed, there is only 1 prospective randomized controlled trial of the long-term outcomes of LADG[8]. Huscher *et al*[8] noted that there was no significant difference in the 5-year overall survival and disease-free survival between LADG and ODG. Several retrospective studies reported comparable results[15,16,28]. However, the lack of statistical significance in the long-term outcomes may be attributable to the small sample size.

Our study demonstrated that all the comparative studies published in the English literature preferred LADG to CODG for the treatment of gastric cancer with regard to short-term outcomes. However, it is uncertain whether there is any need to perform a meta-analysis. The poor methodology of the available trials may be anticipated and their heterogeneity may be statistically proven later although no heterogeneity was observed with regard to the analysis of postoperative complications. The explainable reasons for the heterogeneity were the different levels of laparoscopic expertise; the issue related to the learning curve; different levels of lymphadenectomy; nonblinded assessment of outcomes; lack of randomization, except 4 RCTs; and the assumption regarding the mean and standard deviation. Based on these reasons, we employed the random effects model of DerSimonian and Laird[29] even when statistically significant heterogeneity was not detected. However, in the future, a well-designed RCT with a large sample size would be required to aptly compare the controversial outcome measures, particularly the operating time and quality of lymphadenectomy.

In conclusion, compared to CODG, LADG for early gastric cancer is associated with a lower morbidity, less pain, faster bowel function recovery, and a shorter hospital stay. To establish LADG as the standard treatment for gastric cancer, further studies should be conducted with regard to the following aspects: (1) the prevalence of standard techniques and the development of an education and training system and, (2) well-designed RCTs following the learning curve of LADG to increase the statistical power and elucidate oncological clearance, including the quality of lymphadenectomy and long-term outcomes.

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## COMMENTS

### *Background*

Nowadays, laparoscopy-assisted distal gastrectomy (LADG) for the treatment of early gastric cancer is a well-established procedure. However, the feasibility and advantages of LADG have not been thoroughly evaluated thus far. Therefore, we attempted a meta-analysis to elucidate the current status of LADG with regard to its short-term outcomes by comparing it with conventional open distal gastrectomy (CODG).

### *Research frontiers*

The application of LADG for gastric cancer is still controversial because of the lack of clinical evidence regarding its long-term outcomes.

### *Innovations and breakthroughs*

This paper summarizes all the published data comparing LADG with CODG.

### *Peer review*

This paper will provide useful information for the readers of the *World Journal of Gastroenterology* regarding the daily management of patients with gastric cancer.

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*RAPID COMMUNICATION*

# Differences in characteristics of colorectal neoplasm between young and elderly Thais

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## Abstract

**AIM:** To analyze the differences of clinical characteristics of colorectal neoplasm including polyps between the elderly and young Thai patients.

**METHODS:** Colonoscopy database from December 2000 to October 2004 was retrospectively analyzed. There were 1822 eligible patients who underwent colonoscopy (with a mean age of 56.6 years). Patients were classified into two groups: the older age group (aged ⩾ 60 years; *n* = 989) and the younger age group (aged < 60 years; *n* = 833). Data were recorded on age, colonoscopic indications, tumor location, colonoscopic findings and their related histological findings.

**RESULTS:** Colorectal malignancy related lesions were more often found in the older age group (21%) than in the younger age group (12%). Left-sided lesions were detected more commonly than right-sided in both age groups in approximately two-thirds of all cases. Hematochezia showed greater association with left-sided lesions in the elderly. No relationship was found between age and neoplasm staging and severity.

**CONCLUSION:** The chance of detecting colorectal neoplasm by colonoscopy was higher in the elderly than in the young Thais. However, both groups had the lesions predominantly located in the left side.



**Key words**: Colon neoplasm; Colonoscopy; Thais; Elderly

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## INTRODUCTION

Colorectal cancer is one of the most common cancers worldwide. It is also the most frequent form of cancers among elderly population[1]. In contrast, colorectal cancer is considered to be a rare disease in people younger than 40 years of age[2]. The incidence rate of colorectal cancer is highest in the US, Canada, Japan, and Western Europe, being approximately 40 and 25-30 cases per 100 000 population in male and female, respectively. It is relatively uncommon in many developing countries, particularly those in Africa and Asia, where the average rate among men is 5-10 cases per 100 000 population and even lower in women[3]. In Thailand, data showed that the incidence rates for colorectal cancer among men and women were 7.2 and 5.2 per 100 000 population, respectively[4].

Recently, the Tumor Registry of Thailand reported that colorectal cancer has become the third most common cancer in the country, and the majority of patients were at advanced stages[4]. Our recent study also revealed that more than half of colorectal cancer cases already had lymph nodes invasion, and their prognoses were poor[5]. These reports also showed that most of the patients were older than 60 years of age. However, no data have been reported before regarding the clinical characteristics of this tumor which include adenomatous polyps, cancer staging at diagnosis, site location, pathological cell types, and their relationship to patient symptoms. Furthermore, no previous studies have focused on the older population, that intends to examine the association between age and characteristics of colorectal neoplasm in Thailand.

Therefore, this study was aimed to analyze the relationship between age and clinical characteristics of colorectal neoplasm including polyps in both older and younger Thai patients.

## MATERIALS AND METHODS

### Subjects

This is a retrospective study based on the colonoscopic database information from all colonoscopic examinations performed at King Chulalongkorn Memorial Hospital, Bangkok, Thailand from December 2000 to October 2004.

These data were computerized recordings by attending colonoscopists ($n$ = 8) or by supervised trainees ($n$ = 12). This database consisted of standard information collected for clinical purposes at the Gastroenterology Unit, King Chulalongkorn Memorial Hospital. Patients, who underwent colonoscopy except those who were previously diagnosed with colorectal neoplasms, were included in this study. Exclusion criteria included: cases of colorectal neoplasm followed up, post-colectomy patients and patients who had contraindication for colonoscopy, e.g., bleeding tendency and colonic perforation. Eighty patients were excluded because of the above-mentioned reasons.

There were 1822 patients eligible for the study who underwent colonoscopy during this period (788 men, 1034 women, mean age of 56.6 years). Patients were classified into two age groups: older age group (aged ⩾ 60 years; $n$ = 989) and younger age group (aged < 60 years; $n$ = 833). Colonoscopies were generally performed if the following clinical indications were presented at interview: anemia (hemoglobin below 14 g/dL in male and 13 g/dL in female), abnormal radiography (by computerized tomography, ultrasonography, or barium enema), bowel habit change, family history of colorectal cancer or inherited colorectal cancer syndromes (e.g., familial adenomatous polyposis, hereditary non-polyposis colorectal cancer), positive stool occult blood test, abdominal pain, abnormal abdominal mass, and hematochezia. Additionally, in some cases, colonoscopy was used as a primary modality for investigation in the asymptomatic patients with personal awareness for colon cancer screening.

### Methods

The majority of patients had only one colonoscopic clinical indication but thirty-three patients had more than one clinical indication. In this situation, the most serious indication was put as the first indication on the list. Among the thirty-three patients, only the first indication was used for analysis. In patients who need to repeat colonoscopy in this study period (62 patients), only the information from the first examination was included in this study. The average waiting time for patients who referred to colonoscopy at our hospital was less than 4 wk.

Colonoscopes in the present study were all standard long colonoscopes (CF-140 and CF-160, Olympus, Tokyo, Japan). Some patients required pediatric colonoscopies (PCF-140, Olympus, Tokyo, Japan). No magnified colonoscope was available during this study. Chromoendoscopy was not routinely done in our hospital.

Bowel preparation was selected by attending physicians either using a standard 90 mL of sodium phosphate or at least 3 L of polyethylene glycol. Patients were asked to refrain from fruit and vegetable 2 d prior to the study date. Patients with suboptimal bowel preparation were required to take more of the above agents until the preparation was optimal for colonoscopy, otherwise he or she had to be rescheduled.

Conscious sedation was used for our colonoscopy with a combination of intravenous midazolam and meperidine. The dosage of these agents were titrated by colonoscopists. No other mode of sedation or general anesthesia was used in the present study.

**Table 1 General characteristics of patients**

| Characteristic | Men | Women | Total |
|---|---|---|---|
| $n$ | 788 | 1034 | 1822 |
| Mean age (yr) | 56.4 | 56.7 | 56.6 |
| Age range (yr) | 14-91 | 14-100 | 14-100 |

Data were recorded on age, gender, colonoscopic indications, colonoscopic findings and their related histological findings. Colorectal neoplastic lesions were categorized into colorectal polyp and colorectal cancer. Colorectal polyp is a colonic neoplasm which does not contain tumor cells. They were also classified into 2 types based on their histological findings and potential to become a cancer; 'hyperplastic polyps' contain only hyperplastic cells, with no potential to develop into a cancer. Adenomatous polyps contain tubulous, villous, or tubulovillous cells and have precancerous change. In this study, we termed "colorectal malignancy related lesions" for determining both colorectal adenoma and colorectal cancer.

Both colorectal cancer and polyps were designated as right-sided lesions if they were proximal to the splenic flexure (including cecum, ascending colon, transverse colon) and left-sided lesions if they were distal to splenic flexure (including descending colon, sigmoid colon, and rectum). If polyps or cancers were found in both sides on the same patient, these were classified as pancolonic distribution. Patient with synchronous polyp and cancer was registered as a cancer case. Colorectal cancer was staged based on Modified Dukes classification[6]. They were classified into 4 types based on their histological findings: well differentiated, moderately differentiated, poorly differentiated adenocarcinoma, and other forms of cell types. Incomplete data from computer database were reviewed manually from medical records.

### Statistical analysis

Descriptive statistics are expressed as number (%). Statistical analysis was performed by Chi-square or Fisher exact test. $P < 0.05$ was considered to be statistically significant. Data were analyzed with the Statistic Package for Social Sciences (SPSS 11.5), (Chicago, IL, USA).

## RESULTS

The colonoscopic records of 1822 patients were examined. There were 788 men (mean age 56 years) and 1034 women (mean age 57 years) (Table 1). The overall completion rate for colonoscopy was 96.4% ($n$ = 1756). Sixty-six patients had incomplete colonoscopy and subsequently underwent double contrast barium enema. Only two patients were found to have small polyps in the ascending colon. However, repeated (complete) colonoscopies were unable to detect such polyps.

There was a statistical difference in colonoscopic findings between age groups. Colorectal malignancy related lesions were more often found in the older age group (21%)

**Table 2 Age groups and colonoscopic findings**

| Colonoscopic finding | Younger age group (< 60 yr) (%) | Older age group (≥ 60 yr) (%) | Total | |
|---|---|---|---|---|
| | | | *n* | % |
| Normal | 42.9 | 33.6 | 704 | 38.6 |
| Colorectal polyps | 12.8 | 25.2 | 337 | 18.5 |
| -colorectal adenoma | 7.0 | 14.0 | 185 | 10.5 |
| -nonadenoma | 5.8 | 11.2 | 152 | 8.0 |
| Colorectal cancer | 5.1 | 7.1 | 109 | 6.0 |
| IBD | 2.4 | 1.0 | 33 | 1.8 |
| Other diagnoses | 36.8 | 33.0 | 639 | 35.1 |
| Total (%) | 100 | 100 | | 100 |
| N | 989 | 833 | 1822 | |

**Table 3 Age groups and site distribution of "colorectal malignancy related lesions"**

| Cancer site | Younger age group (< 60 yr) (%) | Older age group (≥ 60 yr) (%) | Total | |
|---|---|---|---|---|
| | | | *n* | % |
| Right-sided | 37.5 | 39.3 | 115 | 38.6 |
| adenoma | 20.3 | 29.1 | 75 | 24.7 |
| cancer | 17.2 | 10.2 | 40 | 13.7 |
| Left-sided | 62.5 | 60.7 | 180 | 61.4 |
| adenoma | 38.7 | 37.1 | 110 | 37.9 |
| cancer | 23.8 | 22.9 | 70 | 23.5 |
| Total (%) | 100 | 100 | - | 100 |
| N | 120 | 175 | 295 | - |

**Table 4 Locations of colorectal malignancy related lesions and indication for colonoscopy in elderly patients**

| Indication | Right-sided lesions (%) | Left-sided lesions (%) | Total | |
|---|---|---|---|---|
| | | | *n* | % |
| Anemia | 20.0 | 18.5 | 34 | 19.1 |
| Abnormal radiography | 4.3 | 0.9 | 4 | 2.2 |
| Bowel habit change | 41.4 | 29.6 | 61 | 34.3 |
| Family history | 5.7 | 1.9 | 6 | 3.4 |
| Stool occult blood positive | 1.4 | 5.6 | 7 | 3.9 |
| Abdominal mass | 2.9 | 6.5 | 9 | 5 |
| Abdominal pain | 2.9 | 6.5 | 9 | 5 |
| Hematochezia | 5.7 | 22.2 | 27 | 15.7 |
| Check-up | 12.9 | 5.6 | 13 | 8.4 |
| Weight loss | 2.9 | 2.8 | 5 | 2.8 |
| Total (%) | 100 | 100 | | 100 |
| N | 69 | 106 | 175 | |

**Table 5 Cancer staging by age groups**

| Cancer staging | Younger age group (< 60 yr) (%) | Older age group (≥ 60 yr) (%) | Total | |
|---|---|---|---|---|
| | | | *n* | % |
| Duke A | 14.3 | 13.6 | 15 | 13.9 |
| Duke B | 46.9 | 40.7 | 47 | 43.5 |
| Duke C | 8.2 | 23.7 | 18 | 16.7 |
| Duke D | 30.6 | 22.0 | 29 | 25.9 |
| Total (%) | 100 | 100 | | 100 |
| N | 50 | 59 | 109 | |

than in the younger age group (12%), ($\chi^2$ = 22.76, DF = 1, $P$ < 0.05). When we classified colorectal malignancy related lesions into "adenoma" and "cancer", we found that adenoma cases were detected significantly more often in the older age group ($\chi^2$ = 26.45, DF = 1, $P$ < 0.05) as well. In addition, tubulous adenoma was the most common cell type detected in both age groups (45.2% and 47.2%). Although colorectal cancers were more often found in the older patients than in the younger patients, this difference was not statistically significant ($\chi^2$ = 3.67, DF = 1, $P$ < 0.06), (Table 2).

There was no statistical difference in sites of lesions found between younger and older age groups ($P$ < 0.05). Left-sided lesions were detected more commonly than right-sided in approximately two-thirds of all cases in both age groups. Additionally, both colorectal adenoma and cancers were detected more often in the left than in the right. When we analyzed subgroups of "adenoma" and "cancer", we found no statistically significant difference between locations of either "adenoma" or "cancer" and age groups (Table 3). There was no patient with synchronous cancer. However, 3 patients with left-sided cancer had small polyps in the same side. In addition, pancolonic polyps were demonstrated only in 7 patients (not included in the Table). In the elderly patients, bowel habit change was the most common presentation for both left- and right-sided lesions. However, there was no statistical difference among locations of lesions for this symptom. Hematochezia showed greater association with left-sided lesions in elderly group ($\chi^2$ = 8.73, DF = 1, $P$ < 0.05). On the other hand, "check-up" was a common indication to find right-sided lesions in this age group ($\chi^2$ = 10.68, DF = 1, $P$ < 0.05). There was also no statistical correlation between anemia and location of the lesions in elderly group ($\chi^2$ = 1.10, DF = 1, $P$ = 0.29). Patients with colorectal cancer had significantly higher proportion of abdominal pain and abdominal mass than those with adenomatous polyps ($\chi^2$ = 13.48, DF=1, $P$ < 0.05). However, there was no significant difference in other symptoms between patients with colorectal cancer and polyps. Other indications, including abnormal radiography, family history of cancer, positive stool occult blood, and weight loss were presented in only a small number of patients (Table 4).

Moderately differentiated adenocarcinoma was the most common cancer cell type detected in both age groups (51.0% and 59.3%). Duke B (40%) and Duke C (24%) were the most common stages found in elderly group (Table 5). However, there was no statistical difference in cancer staging between age groups.

## DISCUSSION

Evidence supports that the prevalence of colorectal neoplasm increases with age and varies with country and lifestyle[7]. In the United States, autopsy studies have shown an overall prevalence of colorectal neoplasm ranges from 30% to 50%, which is increasing with age: 30% at 50 years; 40%-50% at 60 years, and 50%-65% at 70 years[8]. For

patients undergoing colonoscopies, the detection rate of a neoplasm was significantly higher in the older age group. These findings are supported by reports of neoplasm at colonoscopy of higher yield in the older patients[9-11].

The present series has shown that colorectal neoplasm was more likely to be found in older age Thais. This finding was consistent with previous studies in Western countries[12-14]. The hypothesis "with age advances, alteration or change of tissues in natural process of more accumulation of somatic mutation" may be applicable in this finding[15].

There are several factors that influent prognosis of patients with colorectal cancer, including cell types and staging at presentation. Some reports from many countries suggested that younger patients have cancers with less aggressive cell types and staging than older patients[16-18]. However, the present study did not demonstrate the difference of tumor cell types and staging between the age groups. We speculate that the advanced age may not have a significant effect on the severity of colorectal cancer in Thais. In addition, a subgroup analysis of the extremely aged patients (> 75 years) was not done to determine the impact of extreme age as a factor for colonic neoplasm development.

The completeness of colonoscopy is important to determine the quality of the study. Incomplete colonoscopy may cause a falsely low prevalence of right-sided lesion. Our study demonstrated a high rate of complete colonoscopy (96%) which is comparable to others[19-21]. The present series showed that left-sided cancer and polyp were detected more often than the other side. These findings were consistent in both age groups, whereas the evidences in Western countries were different. They showed a rightward shift of colonic neoplasm in elderly population[22-24]. Although this difference may be the result of a small number of subjects with colorectal polyps and cancer found, the characteristics of this tumor in Thais may actually be different from those in Western countries. Epidemiological studies also describe that this variation in the frequencies of right-sided and left-sided lesions can be explained by differences in some environmental factors including dietary and life style, although the underlying etiology is not clear[13,25]. The pattern of external risk factors in Thai population might be different from those of the Western countries. Moreover, part of our study population was a group of referred patients. In addition, our results represented only patients from a single center. Therefore, we need a multi-center study for the exposure of different risk factors among different age groups in the Thai population in order to identify the reason for the discrepancy between the findings in this study and those from Western countries.

When this study specifically looked at the older age subjects, it showed that bowel habit change, anemia and hematochezia were among the most common presentations associated with "colorectal malignancy related lesions". Additionally, this study found that some clinical indications were associated with the location of lesions. For example, hematochezia showed a greater association with left-sided lesions and "check-up" was the most common indication to find right-sided lesions.

This finding was quite similar to those reported in previous studies from Thailand[26-27]. Information from this study helps predict tumor location based on some clinical presentations, which in turn, may guide the choice of investigation, especially in the settings with limited access to gastroenterologists or colonoscopy, such as the rural area of Thailand. For example, hematochezia suggests that lesions were more likely to be found in left-sided colon. Therefore, physicians may choose to first investigate with flexible sigmoidoscopy, which can clearly examine left-sided colon, and barium enema was only done in cases of tumors which are not detected with sigmoidoscopy.

In our series, bowel habit change was the most common presentation of colorectal malignancy related lesions. In addition, it was also more commonly recorded in right-sided lesions. However, the relationship between this symptom and location was not statistically significant. Furthermore, other major clinical presentations recorded in the older age group, including anemia, abdominal pain and mass did not show significant association between locations of colorectal lesions and these symptoms. Therefore, this study demonstrated that other symptomatic clinical presentations except hematochezia tend to be of no benefit as a diagnostic tool for prediction of location in the older patients.

The present series showed no statistical significance between location of tumor and staging in Thai elderly. While Kawazuma *et al*[28] found that right-sided lesions were more commonly associated with higher degree of staging and severity. The reason why patients with right-sided colon cancer had poorer survival than other anatomical sub-sites may be due to lack of symptoms and delayed detection of cancer[28].

Currently, colonoscopy is widely used for detection of colorectal neoplasm among the older age group because of its high diagnostic yield to detect right-sided lesions[14]. From this study, although we did not find more association with right-sided lesions in advanced age, it showed that right-sided neoplasm represented approximately 40% of all subjects. Therefore, it suggests that colonoscopy continues to be the best screening method for colorectal neoplasm detection for Thai elderly because of its capacity to examine the entire colon and rectum. However, it is practically impossible to have this modality available for the whole nation at this moment due to the lack of colonoscopic resources[29]. In addition, double contrast barium enema plus flexible sigmoidoscopy may be an alternative screening method which could also examine entire colon in the mean time when access to colonoscopic resources is limited.

The major limitation of this study was that many patients underwent colonoscopy in the study had been examined at other institutes and were referred to this department because colorectal cancer was suspected. In addition, we are unable to know whether the subjects in this study were representative of the general population in Thailand. Therefore, the results of this study may overestimate the overall prevalence. Additionally, this study was conducted at only one center, which may limit the generalization. Moreover, data collected in this study only included clinical characteristics of colorectal neoplasm

detected by colonoscopy, but did not include information on risk factors, which are necessary to explain findings in this study. Therefore, further studies regarding risk factors such as diet and life style in Thai population is necessary to explain the similarity and differences in the characteristics of colorectal cancer between Thais and those in Western countries.

In conclusion, colorectal neoplasm was more likely to be found in the elderly Thais. Some clinical presentations in older patients may give a clue to the location of lesions that can help choose appropriate investigations. In contrast to Western population that advanced aged patients had more right-sided lesions, left-sided neoplasm still predominated in the symptomatic Thais elderly.

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## COMMENTS

### Background

Colorectal malignancy is one of the major cancers in the West. Recently, there has been a rising incidence of this cancer in Asians. However, data regarding this type of cancer in Asians are still scanty.

### Research frontiers

Elderly Thais had a higher chance of colorectal cancer than the young. The majority of cancers were found to be in the left side of the colon in both elderly and the young.

### Innovations and breakthroughs

This is so far, the largest report of colonoscopic results in Thai elderly. In this report, the location of tumor in elderly Thais was still predominantly in the left side of the colon, whereas there is a rightward shift of tumor location in the western population.

### Applications

The screening for colorectal cancers by colonoscopy is not yet a standard protocol in Thailand. This study shows the benefit of colonoscopy in elderly

Thais. Due to the higher incidence of left-sided tumors, the protocol for screening for Thais may require only a sigmoidoscopy.

### *Peer review*

This study provides the interesting differences in clinical characteristics, presentations and findings of colorectal neoplasm in young and elderly Thais. However, due to the limited data from this retrospective format, there is a need for further prospective studies to address many unanswered questions, especially "what are the most appropriate age and tool for colon cancer screening for Asians?".

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*RAPID COMMUNICATION*

# Non-invasive measurement of pan-colonic pressure over a whole digestive cycle: Clinical applications of a capsule-style manometric system

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## Abstract

**AIM:** To study the prolonged colonic motility under normal conditions with a novel capsule-style micro-system and to assess its clinical significance.

**METHODS:** A single use telemetry capsule (10 mm in diameter, 20 mm in length) embedded with a pressure sensor was ingested by the subjects. The sensor is capable of transmitting colonic pressure wirelessly for more than 130 h. The time of capsule entering the segmental colon was detected by ultrasound. The ultrasonic electrodes were mounted on the surface of the ileocecum and navel and at the junction of the left and rectosigmoid colon of the subjects in sequence, which were identified by abdominal X-rays with radiopaque markers. To verify the accuracy and reliability of ultrasonic detection of telemetry capsules at key points of colon, the segmental colonic transit time was simultaneously recorded by using radiopaque markers.

**RESULTS:** The signal lamp showed that all recorders could receive the radio signal transmitted by the telemetry capsule. The X-rays showed that all telemetry capsules were detected successfully when they were passing through the key points of colon. There was a significant correlation between the transit results obtained by ultrasonic detection or by radiopaque markers. Colorectal recording was obtained from 20 healthy subjects during 613 h (411 h during waking, 202 h during sleep). Compared to waking, the number of pressure contractions and the area under pressure contractions were significantly ($P < 0.05$) decreased during sleep (21 ± 5 $h^{-1}$ *vs* 15 ± 4 $h^{-1}$, 463 ± 54 mmHg·s/min *vs* 342 ± 45 mmHg·s/min). The colonic motility exhibited significant regional variations both in the circadian behavior and in response to waking and meal.

**CONCLUSION:** The capsule-style micro-system is reliable and noninvasive, and may represent a useful tool for the study of physiology and pathology of colonic motor disorders.



**Key words:** Colon; Pan-colonic pressure; Pan-colonic motor activity; Telemetry

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## INTRODUCTION

Colonic motility disorders are frequently encountered in clinical practice. Intraluminal manometry (*via* perfused and solid-state catheters) allows a direct study of colonic contractile activity over prolonged periods. However, catheter studies have generally been performed in the left and rectosigmoid colon, because of the technical difficulties in accessing the right colon[1].

Hagger *et al*[2] have reported a pan-colonic manometry by combined antegrade/retrograde intubation. However, due to sensor failure and/or catheter displacement, they could not assess the amplitude of pressure activities in the right colon.

Recent technological advances facilitate prolonged pan-colonic investigations. The success of Israeli M2A capsule endoscope has led to the research and development of other capsules aimed at monitoring either functional or organic diseases[3]. The American SmartPill capsule is capable of transmitting pressure, temperature and pH data continuously for 72 h, and suitable for diagnosing small intestine motility or delayed emptying of the stomach[4-6].

To satisfy the need of prolonged and noninvasive monitoring colonic pressures, the authors have developed a novel capsule-style manometric micro-system with its

prototype reported[7]. The system was approved by the Food and Drug Administration of China in 2005. The system is composed of a one-off telemetry capsule, a data recorder, an ultrasonic location detector and a computer with analysis software. The capsule-style manometric system is capable of acquiring and transmitting intraluminal pressure data continuously for more than 130 h.

The aim of this study was to evaluate the pan-colonic motor activity under the physiological conditions with a capsule-style manometric system. A study period of a whole digestive cycle (26-53 h) was used to characterize the infrequent patterns of motor activity of the colorectum.

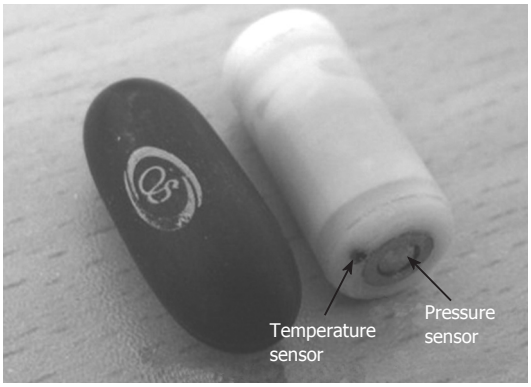


**Figure 1** A vitamin pill (left) and a telemetry capsule (right).

## MATERIALS AND METHODS

### Subjects

Twenty healthy subjects (12 females and 8 males, median age 35 years, range 23-52 years) were studied. The subjects had a normal bowel habit which was defined as a stool frequency of ⩽ 3 stools per day and ⩾ 3 stools per week. The subjects were not on any medication and had no gastrointestinal symptoms and no history of previous gastrointestinal disorder or gastrointestinal surgery. Female subjects had a negative pregnancy test. An informed written consent was obtained from all subjects. The study protocol was approved by the Ethics Committee of Capital Medical University Chaoyang Hospital.

### Capsule-style manometric system

**Telemetry capsule**: The newly-developed telemetry capsule (10 mm in diameter, 20 mm in length, and weighing 2.9 g ) developed by the authors is shown in Figure 1. The effective range could reach 3-5 m between the capsule and the recorder.

The pressure sensor can be used from -60 to 200 mmHg with its error ⩽ ± 1.5 mmHg, and the temperature sensor is sensitive in range of 33-42℃ with its error ⩽ ± 0.25℃. Changes in pressure sensor output due to fluctuations of temperature can be compensated automatically.

**Data recorder:** The data recorder consists of three functional blocks: a wireless receiver module, a microcontroller and an outer pressure transducer. The outer pressure transducer records the pressure changes induced by the surrounding altitude. The timer in the microcontroller records the time of received data. The obtained pressure data, temperature data, ultrasonic location information and relevant time are stored in a flash memory card.

**Ultrasonic detection of capsule location**: When the telemetry capsule passes through the ultrasonic electrodes mounted on the surface of key points of colon, the echo from batteries in the capsule would be much stronger than other echoes. When the amplitude of an echo is higher than the preset threshold, it can be regarded as returning from the capsule. Detailed technical information about the ultrasonic detection of the capsule positions has been reported elsewhere[8].

Colon was divided into three segments by three key points (ileocecum, navel and the junction of the left and rectosigmoid colon), which were identified by an experienced doctor with the help of abdominal X-rays with radiopaque markers.

**Analysis software**: In addition to the novel manometric system, a Matlab-based (The MathWorks, Natick, MA) software program was developed to correct baseline shifts, eliminate artefacts, and calculate the number of pressure contractions, amplitude and area under curve (AUC) in a fully automated way. The software features an intuitive graphical user interface that supports graphical and statistical data analysis. The baseline pressures were determined by identifying the minimum pressure in each 30 min, which was then subtracted from the raw signals. Pressure contractions with an amplitude ⩾ 10 mmHg from the baseline and a duration ⩾ 6 s were included in the analysis.

### Experimental protocol

To verify the accuracy and reliability of ultrasonic detection of telemetry capsules at the key points of colon, the segmental colonic transit time was simultaneously recorded by using radiopaque markers.

After an overnight fast, the subjects ingested 20 radiopaque markers contained in a gelatin capsule (Binhai Hospital, Tianjin, China) at 8:00 AM next morning. A daily abdominal X-ray was taken at the same time on the following consecutive days until the number of remained markers was ⩽ 4. The total and segmental colonic transit time (CTT) was calculated using the following formula: $CTT = 1.2 \times \Sigma n_i$, where 1.2 = time interval (24 h)/number of radiopaque markers (20), i = day of X-rays taken, and $n_i$ = the total number of markers present on a given film sector on day I[9,10].

The subjects took the telemetry capsule an hour after ingestion of the markers. The subjects wore the recorder and checked the signal lamp on the receiver.

To help mounting the ultrasonic electrodes on the surface of the ileocecum of the patients correctly, an abdominal X-ray was taken at 6 h after the telemetry capsule was taken.

The ultrasonic electrodes were first mounted on the surface of the ileocecum by an experienced doctor with the help of radiopaque markers and bony landmarks in the X-ray film. The subjects put on the waistcoat containing an ultrasonic signal processor and an alarm buzzer. When

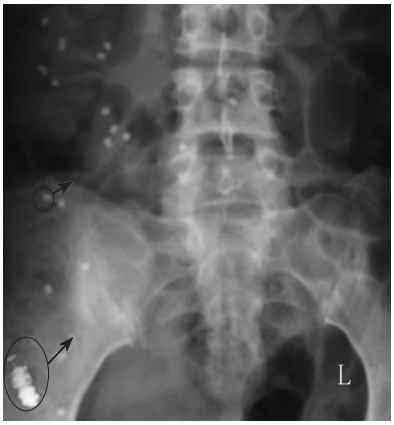


**Figure 2** An X-ray after ultrasonic detection confirming capsule in the ileocecum.

alarming occurred, an abdominal X-ray was taken to confirm the location of telemetry capsules.

After successful detection of the ileocecum, the ultrasonic sensors were then mounted on the surface of the navel and at the junction of the left and rectosigmoid colon in sequence.

The subjects stayed totally ambulatory during inspecting, but drastic sport activities were prohibited. The subjects ate 1000 kcal standard meals. Ingestion of alcohol and caffeine containing drinks was prohibited.

After defecation, the signal lamp on the receiver would indicate whether the capsule was still in body or not. All capsules were recovered to confirm the discharge.

### *Statistical analysis*

The data were expressed as mean ± SD unless otherwise stated**.** High-amplitude contraction (HAC) analysis was made by visual inspection of the pressure trace. The HAC was defined as a pressure contraction with an amplitude ⩾ 50 mmHg from the baseline, a duration ⩾ 20 s and a peak-to-peak time interval ⩾ 40 s.

The diurnal and postprandial differences in AUC and the number of contractions were compared using repeated measures of ANOVA. $P < 0.05$ was considered statistically significant in all analyses.

## RESULTS

The signal lamp on recorders showed that all recorders could receive the radio signal transmitted by the telemetry capsule in the subjects. No discomfort was reported by any volunteers during the experiment.

The telemetry capsules passed through the gastrointestinal (GI) tract without any difficulties. All capsules were recovered with the guidance of the signal lamp.

### *Capsule location and transit results*

All telemetry capsules could be detected successfully when they were passing through the three key points of the colon. Additional X-rays confirmed the capsule location (Figure 2). The location of capsules could be recognized with the help of bony landmarks and radiopaque markers.

The total transit time and segmental colonic transit time obtained by ultrasonic detection and radiopaque markers are shown in Table 1. There was a significant correlation between the two methods for the detection of transit results. It verified the reliability and accuracy of ultrasonic detection for the segmental transit time.

**Table 1 Transit time detected by markers and ultrasound (mean ± SD)**

| Transit time (h) | Total colon | Right colon | Left colon | Rectosigmoid |
|---|---|---|---|---|
| Markers | 31.5 ± 6.8 | 8.6 ± 3.3 | 11.4 ± 3.6 | 11.5 ± 3.3 |
| Ultrasonic | 29.7 ± 5.9 | 9.3 ± 3.5 | 10.8 ± 3.1 | 9.7 ± 2.9 |
| Correlation | 0.92 | 0.91 | 0.93 | 0.86 |

**Table 2 Colonic motility response to waking and meal (mean ± SD)**

| | Waking | | Meal Ingestion | |
|---|---|---|---|---|
| | 1 h before | 1 h after | 2 h before | 2 h after |
| No. of Contrctions ($h^{-1}$) | | | | |
| Right colon | 14 ± 4[a] | 45 ± 6[a] | 21 ± 6 | 44 ± 8[b] |
| Left colon | 13 ± 3[a] | 39 ± 4 | 22 ± 5 | 38 ± 6 |
| Rectosigmoid | 19 ± 5[a] | 29 ± 4[a] | 20 ± 6 | 32 ± 7[b] |
| AUC (mmHg·s·$min^{-1}$) | | | | |
| Right colon | 322 ± 44 | 984 ± 91[a] | 471 ± 59 | 862 ± 79 |
| Left colon | 307 ± 41[b] | 835 ± 82 | 465 ± 52 | 812 ± 74 |
| Rectosigmoid | 431 ± 52[b] | 725 ± 64[a] | 453 ± 52 | 773 ± 71 |

[a]$P < 0.05$, [b]$P < 0.01$ *vs* segmental colons.

### *Colonic motility*

Colorectal recording was obtained from the 20 healthy subjects for a total of 613 h (411 h during waking, 202 h during sleep). In this study, the authors laid more emphasis on the colorectal motility response to meal ingestion and morning waking. During the colorectal pressure recording period, 26 subjects were waking in the morning and 78 had meal ingestions (1.3 and 3.9 per subject respectively).

Compared to waking, the number of pressure contractions and the AUC of pressure contractions during sleep were significantly decreased (21 ± 5 $h^{-1}$ *vs* 15 ± 4 $h^{-1}$, 463 ± 54 mmHg·s·$min^{-1}$ *vs* 342 ± 45 mmHg·s·$min^{-1}$, $P < 0.05$).

After waking and meal ingestion, both the number of pressure contractions and the AUC of pressure contractions were significantly increased. The contractile response to waking was significantly higher in the left/right colon than in the rectosigmoid colon (Figure 3), whereas the waking response was lower in the left colon than in the right colon, but was not significantly different. There were no significant differences in segmental colon motility response to meal ingestion. The details are shown in Table 2.

Regional variations in colonic contractile activity were apparent. During the daytime, the contractile frequency was significantly higher in the right colon than in the left and rectosigmoid colon. However, the contractile frequency was significantly higher in the rectosigmoid colon than in the left/right colon during the night. The HAC was recorded in all of the subjects, with 13.5 ± 6.1

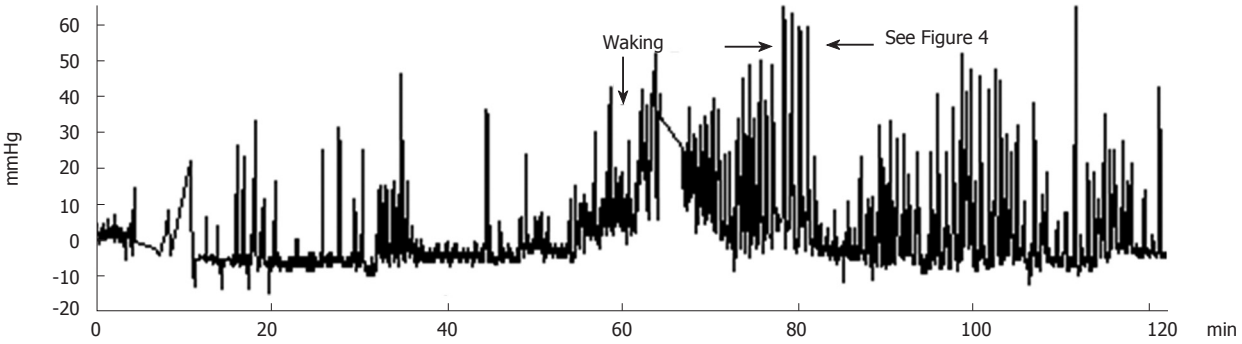


**Figure 3** Pressure curve 1 h before and after waking in the right colon of a healthy subject.

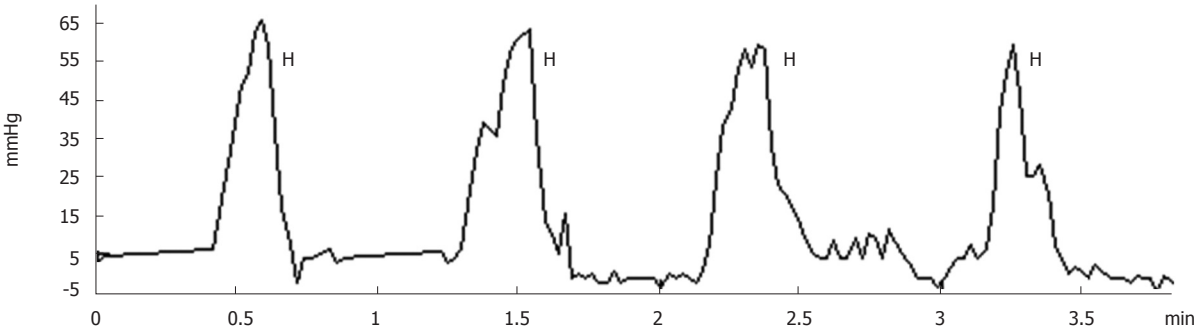


**Figure 4** Occurrence of 4 consecutive HACs 1 h after waking in the right colon.

times per subject (Figure 4). The regional variation was strongly associated with the incidence of HACs. The incidence of HACs was significantly higher in the right and rectosigmoid colon than in the left colon (39%, 44%, and 17%, respectively).

## DISCUSSION

This study introduced a new technique for examining pan-colonic pressures under physiological conditions. Human studies demonstrated that the capsule-style manometric system was safe and easy to handle. The single-use telemetry capsule is encased in inert. The bio-compatible, medical-grade polycarbonate makes it safe for human consumption and easy to swallow. The sensors are surface mounted at the ends of the outer shell. All the other parts are sealed in the shell by medical silicone. The solid-state pressure transducer with a stainless cover is robust and biocompatible.

Location detection of telemetry capsules is a critical issue, since it can move freely through the digestive tract. Some telemetry capsules, such as M2A and Smartpill, can be localize at the capsule's position in the upper digestive tract by detecting the angle and intensity of radio signal[11,12]. Radio signal detection can trace the capsule in real time, yet the precision is limited to 6-10 cm.

In this study, the authors verified that ultrasonic detection of telemetry capsules at the key points of colon was simple and accurate, suggesting that it is suitable for detection of telemetry capsules and segmental transit time in colon. However, it failed to trace the capsule in real time, indicating that a real-time tracing method based on magnetic field mapping should be developed.

The major advantages of telemetry capsule include noninvasiveness, long battery lifespan and low cost. During the process of inspecting, subjects even cannot feel the existence of the telemetry capsule which ensures the examination under physiological conditions. The lifespan of batteries in capsule and a memory capacity of 128 MB of recorder make it feasible to record telemetry capsules and segmental transit time in colon for more than 130 h. The cost of a single-use capsule is also relatively inexpensive.

The major disadvantage of the capsule-style system is hard to judge the propagation and direction of pressure contractions with a single recording point, which will be tested by a multi-capsule method in following studies.

Water-perfused and solid-state catheters are currently available for intraluminal manometry. Water-perfused catheters have the advantages of simplicity, relatively inexpensive components and applicability to the measurement of colon motor activity in several regions. Importantly, they are fully autoclavable, enabling simple sterilization. The major disadvantage of the system is that the catheter is linked to a pneumohydraulic infusion pump and a recording sensor, which almost precludes any ambulatory study.

Solid-state catheters allow measurements over a prolonged period from totally ambulant subjects. However, the transducers in solid state catheters are expensive, rather fragile and hard to be sterilized, and the maximum number of recording sensors available is notably reduced.

Compared to catheter manometry, capsule-style manometry has become a necessary supplement due to its

noninvasiveness and long battery life.

Our results have confirmed in general the circadian behavior of the colonic motility, as well as its response to waking and meals[13-18].

There is no currently accepted gold standard for catheter manometry, because of the differences in catheter type and configurations, and methodology. As a result, the values of some parameters are significantly different. For example, it was reported that the amplitude threshold of HAC in catheter manometry ranges from 50 mmHg to 135 mmHg[19].

It was difficult to compare our results with previous results. However, the average amplitude of pressure contractions in colon obtained with the capsule in this study was lower than that obtained with water-perfused or solid-state catheters[20-25]. This may reflect the stimulation of catheters on colon.

In the present study, the colon also exhibited regional variations in the circadian behavior of the colonic motility, as well as its response to waking and meals, emphasizing the importance of studying the pancolonic motility activities.

In summary, the capsule-style manometric system is reliable and safe for the study of pancolonic motor activity under physiological conditions, representing a useful tool for the study of physiology and pathology of colonic motor disorders.

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*RAPID COMMUNICATION*

# Expression of ATP7B in human gastric cardiac carcinomas in comparison with distal gastric carcinomas

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## Abstract

**AIM:** To analyze expression of ATP7B in gastric cardiac adenocarcinomas, its clinicopathologic significance, in comparison with distal gastric adenocarcinomas.

**METHODS:** Immunohistochemical avidin-biotin peroxidase complex method was applied to detect the expression of ATP7B in 49 cases of cardiac carcinomas, the corresponding adjacent non-neoplastic epithelium and 55 cases of distal gastric carcinomas.

**RESULTS:** The proportion of ATP7B positive samples in gastric cardiac carcinomas (51.0%, 25 of 49) was significantly higher than that in the corresponding adjacent non-neoplastic epithelium (22.4%, 11 of 49) ($P$ = 0.003). ATP7B expression in poorly differentiated gastric cardiac carcinomas was significantly higher than that in well/moderately differentiated gastric cardiac carcinomas ($P$ = 0.030). ATP7B expression in gastric cardiac carcinomas was independent of age, tumor size, nodal stage and metastasis status. ATP7B protein was detected in 30.9% (17/55 cases) of distal gastric carcinomas, markedly lower than that in gastric cardiac carcinomas ($P$ = 0.037).

**CONCLUSION:** ATP7B protein is frequently overexpressed in gastric cardiac carcinomas, and correlated with the differentiation of cardiac carcinoma. ATP7B expression in gastric cardiac carcinomas is significantly higher than that in distal gastric carcinomas, which might partially explain the difference of chemotherapy response and prognosis between these two gastric carcinomas.



**Key words:** ATP7B; Platinum; Distal gastric carcinoma; Gastric cardiac carcinoma; Resistance

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## INTRODUCTION

Gastric carcinoma can be subdivided into distal gastric carcinoma and proximal cardiac carcinoma[1]. Recent studies have suggested that distal gastric carcinoma and proximal cardiac carcinoma have distinct epidemiological, biological and clinical characteristics[1]. For instance, the incidence of carcinoma of the gastric cardia was reported to have increased in most countries during the past few decades, while that of carcinoma of the distal stomach has decreased steadily[2-5]. Carcinoma of the cardia has been reported to form a specific category distinct from carcinoma of the rest of the stomach[6,7].

Drug resistance is a major obstacle of cancer chemotherapy[8,9]. The presence of drug resistance may be prior to exposure to chemotherapy (primary resistance), or may be induced after exposure to chemotherapy (secondary resistance)[8,9]. Platinum containing compounds, such as cisplatin, carboplatin, oxaliplatin, satraplatin, are among the most effective chemotherapeutic agents for cancer. However, their clinical efficacy is often limited by primary or secondary resistance[10]. Several mechanisms have been implicated in cisplatin resistance, including reduced drug accumulation, increased cellular thiol/folate levels and increased DNA repair[10,11]. Among them, reduced intracellular accumulation of cisplatin is a determinant of cisplatin resistance[11]. However, it was not until recently that the mechanism of decrease of cisplatin accumulation in cisplatin-resistant tumor cells was elucidated[11,12]. In 2000, Akiyama and co-workers demonstrated that a copper-transporting P-type adenosine triphosphatase (ATP7B) was associated with cisplatin resistance *in vitro*[12]. The ATP7B gene was induced by exposure to cisplatin in human prostate cells and ATP7B-transfected cells showed a dramatic decrease in cisplatin accumulation[12]. The above study was supported by subsequent reports[13,14]. ATP7B protein is a member of a class of heavy metal-transporting P-type ATPases that pump copper, cadmium, zinc, silver or lead[15]. ATP7B has been found to be expressed in

certain breast carcinomas[16], endometrial carcinomas[17], esophageal carcinomas[18], gastric carcinomas[19], oral squamous cell carcinomas[20] and ovarian carcinomas[21]. Moreover, ATP7B expression status in some carcinomas was correlated with prognosis[17,21] and sensitivity of chemotherapy[18,20].

The aim of this study was to investigate the expression of ATP7B in human gastric cardiac carcinomas, and its clinicopathologic significance, in comparison with distal gastric carcinomas.

## MATERIALS AND METHODS

### Patients

It included 49 patients with adenocarcinomas of the gastric cardia, 27 males and 22 females with an average age of 55 ± 12 years, and 55 patients with adenocarcinomas of the distal stomach, 30 males and 25 females with an average age of 58 ± 9.7 years. The 49 patients with adenocarcinomas of the gastric cardia underwent surgery at the Esophageal Carcinoma Hospital of Linzhou in Henan Province, and the 55 patients with distal gastric carcinomas underwent surgery at the First Hospital of Jiaxing in Zhejiang Province. None of the patients had received chemotherapy or radiotherapy before surgery.

### Tissue processing

All specimens were fixed with formalin and embedded in paraffin. Each block was sectioned serially at 5 μm, one of which was stained with hematoxylin and eosin for histopathological analysis by two pathologists and the others were used for immunostaining[22].

### Histopathological analysis

Histopathological diagnoses were made according to previous reports[22].

### Immunohistochemical staining

Anti-ATP7B antibody was a rabbit polyclonal antibody against ATP7B(Boster, China). Avidin-biotin-peroxidase complex (ABC) method was used for ATP7B immunostaining. In brief, after dewaxing of sections, endogenous peroxidase activity was quenched with 3% $H_2O_2$, and cross-reactivity was blocked with normal serum. The tissues were incubated overnight at 4℃ with primary antibodies (1:100 for anti-ATP7B antibody). Localization of the primary antibodies was achieved by subsequent use of a biotinylated anti-primary antibody, an avidin-biotin complex conjugated with horseradish peroxidase, and 3',5'-diaminobenzidine (Vectasitain Elite Kit). Normal serum blocking and omission of the primary antibody were used as negative controls[22].

### Evaluation of immunostaining

The criterion for a positive reaction was clear cytoplasm and cell membrane staining. If more than 10% of the tumor cells were stained, the samples were considered to be ATP7B-positive carcinomas[21].

### Statistical analysis

Pearson Chi-Square and Fisher's exact tests were performed. The statistical analyses were performed using SPSS 12.0 software. Two-sided *P* values were calculated and a difference was considered significant if the *P* value was less than 0.05.

## RESULTS

### ATP7B expression in human cardiac carcinomas and corresponding adjacent non-neoplastic epithelium

To examine the expression of ATP7B at protein level in human gastric cardia carcinomas, we performed immunohistochemical analysis using a polyclonal antibody against ATP7B. As shown in Figure 1, ATP7B expression was observed in the cytoplasm of gastric cardiac adenocarcinoma cells. A negative control did not reveal ATP7B protein. We used 49 primary gastric cardiac carcinoma specimens for the detection of ATP7B by immunohistochemistry and found that various degrees of cytoplasmic staining of tumor cells were observed in 51.0% (25/49) of the analyzed tumors. We also determined ATP7B expression of the corresponding adjacent non-neoplastic epithelium of all the gastric cardiac carcinomas. ATP7B was identified in 11 of 49 (22.4%) of the examined cases, which was markedly lower than that in gastric cardiac carcinomas ($P = 0.003$). ATP7B was localized close to the cell membrane of adjacent non-neoplastic epithelium, mainly concentrated at the pit pole of the gastric epithelium[23].

### Clinicopathologic significance of ATP7B protein expression in human gastric cardiac carcinoma

We examined the relationship between clinicopathologic variables and ATP7B expression in human gastric cardiac carcinomas. No significant association was found between ATP7B expression and age, tumor size, nodal stage and metastasis status. Regarding histopathologic type, ATP7B protein positivity in poorly differentiated carcinomas was significantly higher than that in well/moderately differentiated carcinomas ($P = 0.030$) (Table 1).

### Comparison of expression of ATP7B in human gastric cardiac carcinomas and distal gastric carcinomas

As shown in Figure 2, ATP7B expression was also observed in the cytoplasm of distal gastric carcinoma cells. We used 55 primary distal gastric carcinoma specimens for the detection of ATP7B by immunohistochemistry and found that various degrees of cytoplasmic staining were observed in 30.9% (17/55) of the analyzed tumors, markedly lower than that in gastric cardiac carcinomas ($P = 0.037$).

## DISCUSSION

Recent research has identified that ATP7B, an energy-dependent copper transporter, is a new resistance marker of tumor cells to platinum containing anticancer drugs[12-14]. The present study provides direct evidence of frequent ATP7B expression in gastric cardiac carcinomas, suggesting the importance of ATP7B protein in primary resistance of gastric cardiac carcinoma cells to platinum containing anticancer drugs. Furthermore,

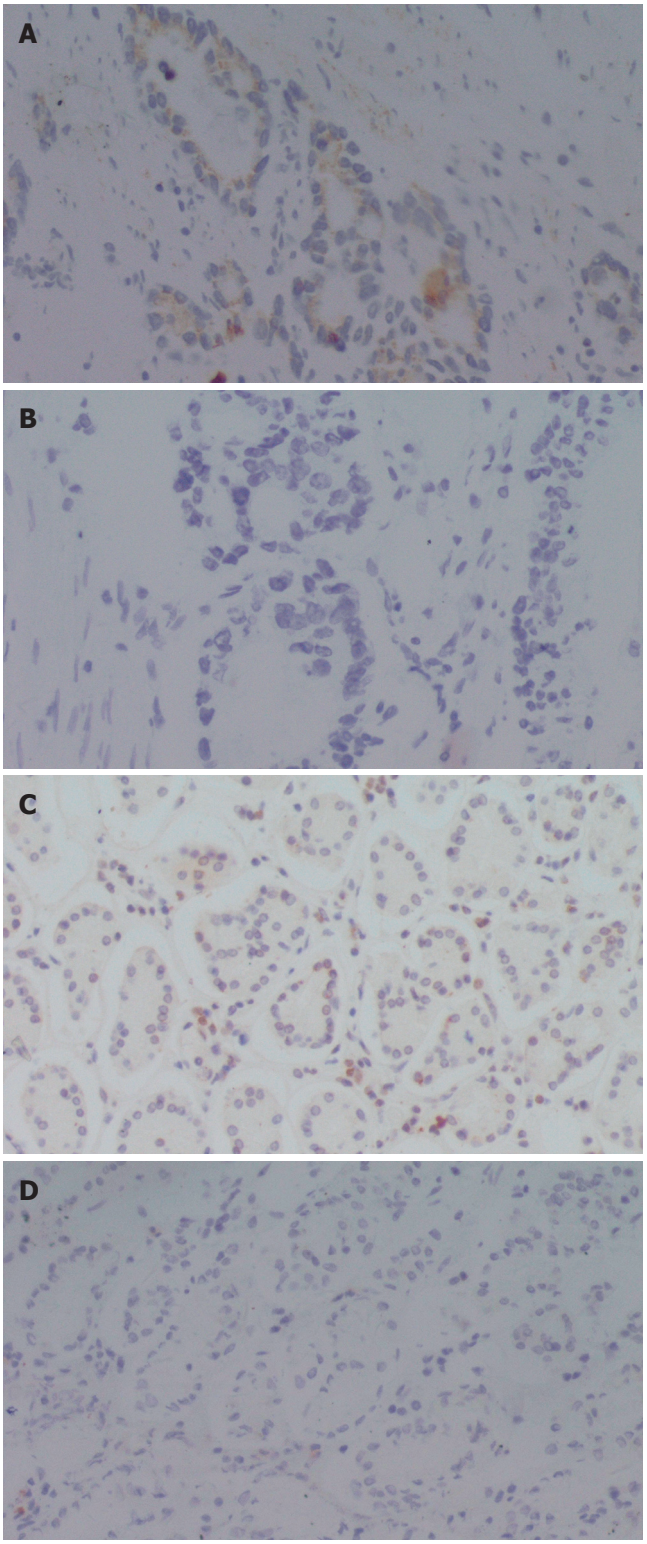


**Figure 1** Immunohistochemical staining of gastric cardiac adenocarcinomas and the corresponding adjacent non-neoplastic epithelium using antibody to ATP7B. **A:** ATP7B-positive gastric cardiac adenocarcinoma; **B:** ATP7B-negative gastric cardiac adenocarcinoma; **C:** ATP7B-positive adjacent non-neoplastic epithelium; **D:** ATP7B-negative adjacent non-neoplastic epithelium.

**Table 1 Relationship between ATP7B expression and clinicopathological variables in gastric cardiac carcinomas**

| Variables | *n* | ATP7B | | Significance[1] |
|---|---|---|---|---|
| | | Negative | Positive | |
| Total | 49 | 24 | 25 (51.0%) | |
| Median age (yr) | | 54 | 56 | NS |
| Degree of differentiation | | | | 0.030 |
| Well/moderately differentiated | | 13 | 6 | |
| Poorly differentiated | | 11 | 19 | |
| Tumor size | | | | NS |
| $T_1 + T_2$ | | 10 | 7 | |
| $T_3 + T_4$ | | 14 | 18 | |
| Nodal stage | | | | NS |
| N (-) | | 3 | 5 | |
| N (+) | | 21 | 20 | |
| Metastasis status | | | | NS |
| $M_0$ | | 21 | 19 | |
| $M_1$ | | 3 | 6 | |

[1]Pearson Chi-Square and Fisher's exact tests were used; NS: Not significant.

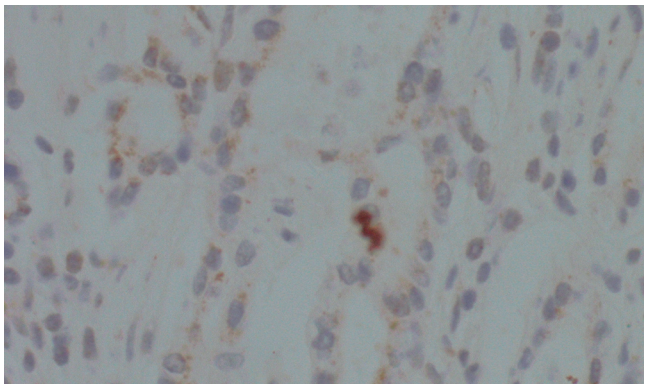

**Figure 2** ATP7B-positive immunohistochemical staining of distal gastric carcinoma using antibody to ATP7B.

ATP7B expression in poorly differentiated gastric cardiac carcinomas was more frequent than in well/moderately differentiated carcinomas, indicating that ATP7B expression might serve as an independent prognostic factor in these patients.

Several study groups detected no ATP7B protein expression in adjacent non-neoplastic epithelium[16-18]. However, our results are somewhat different from the previous reports. We found that ATP7B protein expression was also seen in some of adjacent non-neoplastic epithelium, although it was much lower than in gastric cardiac carcinomas. This suggests that ATP7B protein in gastrointestinal tract may have physiological function. We suppose that ATP7B might protect gastric cardiac epithelium cells from the harmful heavy metals.

It is well known that gastric cardiac carcinoma is more resistant to chemotherapy and has worse prognosis compared with distal gastric carcinoma, although the underlying mechanisms have not been elucidated[1,24]. Our observations demonstrated that ATP7B expression in gastric cardiac carcinomas was significantly higher than that in distal gastric carcinomas. This might partially explain the difference of chemotherapy response and prognosis between gastric cardiac carcinoma and distal gastric carcinoma. Combined determination of ATP7B and other drug-resistant molecular markers[8], such as Pgp, MRP, LRP, BCRP, could be useful for selection of chemotherapy regimens.

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*RAPID COMMUNICATION*

# Myoelectric activity and motility of the Roux limb after cut or uncut Roux-en-Y gastrojejunostomy

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## Abstract

**AIM:** To explore the mechanisms of uncut Roux-en-Y gastrojejunostomy, which is used to decrease the occurrence of Roux stasis syndrome.

**METHODS:** The changes of myoelectric activity, mechanic motility and interstitial cells of Cajal (ICC) of the Roux limb after cut or uncut Roux-en-Y gastrojejunostomy were observed.

**RESULTS:** When compared with the cut group, the amplitude (1.15 ± 0.15 mV *vs* 0.48 ± 0.06 mV, $P < 0.05$) and frequency (14.4 ± 1.9 cpm *vs* 9.5 ± 1.1 cpm, $P < 0.01$) of slow waves and the incidence (98.2% ± 10.4% *vs* 56.6% ± 6.4%, $P < 0.05$) and amplitude (0.58 ± 0.08 mV *vs* 0.23 ± 0.06 mV, $P < 0.01$) of spike potential of the Roux limb in the uncut group were significantly higher. The migrating myoelectric complexes (MMC) phase Ⅲ duration in the uncut group was significantly prolonged (6.5 ± 1.1 min *vs* 4.4 ± 0.8 min, $P < 0.05$), while the MMC cycle obviously shortened (42.5 ± 6.8 *vs* 55.3 ± 8.2 min, $P < 0.05$). Both gastric emptying rate (65.5% ± 7.9% *vs* 49.3% ± 6.8%, $P < 0.01$) and intestinal impelling ratio (53.4% ± 7.4% *vs* 32.2% ± 5.4%, $P < 0.01$) in the uncut group were significantly increased. The contractile force index of the isolated jejunal segment in the uncut group was significantly higher (36.8 ± 5.1 *vs* 15.3 ± 2.2, $P < 0.01$), and the expression of *c-kit* mRNA was significantly increased in the uncut group (0.82 ± 0.11 *vs* 0.35 ± 0.06, $P < 0.01$).

**CONCLUSION:** Uncut Roux-en-Y gastrojejunostomy may lessen the effects of operation on myoelectric activity such as slow waves, spike potential, and MMC, decrease the impairment of gastrointestinal motility, and remarkably increase the expression of *c-kit* mRNA.



**Key words:** Roux-en-Y anastomosis; Electromyography; Gastrointestinal motility; *c-kit*

Zhang YM, Liu XL, Xue DB, Wei YW, Yun XG. Myoelectric activity and motility of the Roux limb after cut or uncut Roux-en-Y gastrojejunostomy. *World J Gastroenterol* 2006; 12(47): 7699-7704

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## INTRODUCTION

Roux-en-Y anastomosis is a commonly used surgical procedure in gastroenterological surgery; however, more than one third of the patients who experience such an operation suffer Roux stasis syndrome[1,2]. It has been proposed that the occurrence of Roux stasis syndrome is related to the blockage of electro-conduction caused by cut of the jejunum[3]. Therefore, some researchers designed an uncut Roux-en-Y gastrojejunostomy[4], which was based on Billroth Ⅱ subtotal gastrectomy, combined with jejunojejunostomy (Braun's anastomosis) and occluded jejunal-gastric pathway. Theoretically, this surgical procedure can maintain the integrity of the intestinal canal and normal conduction of impulses, as well as make gastric contents to be drained directly into the intestinal tract along the movement direction of the alimentary tract. Due to blockade of gastric pathway, digestive fluids, such as bile, pancreatic juice, are unable to enter the stomach, and can merely be evacuated through the Braun's anastomosis. Clinical practice has proved that this surgical procedure decreases the occurrence of Roux stasis syndrome[5,6]. In order to explore the underlying mechanisms, we observed the changes of myoelectric activity, mechanic motility and interstitial cells of Cajal (ICC) of the Roux limb after cut or uncut Roux-en-Y gastrojejunostomy.

## MATERIALS AND METHODS

### *Experimental protocol*

Male Wistar rats (250 ± 20 g) were provided by the

Animal Research Center of the First Clinical College of Harbin Medical University, Harbin, China. Fourteen rats were divided into two groups randomly. After the rats were anaesthetized with intraperitoneal injection of pentobarbital sodium (40 mg/kg body mass), resection of the distal stomach and reconstruction of the gastrointestinal tract were performed.

For the cut Roux-en-Y gastrojejunostomy, a standard Roux-en-Y gastrojejunostomy was undertaken (Figure 1). In brief, the jejunum was cut 1.0 cm distal to the ligament of Treitz, and the remaining gastric pouch was anastomosed to the aborad portion of the jejunum by an end-to-side procedure. The orad portion of the jejunum was anastomosed to the mid jejunum by an end-to-side procedure 2.0 cm distal to the gastrojejunostomy.

For the uncut Roux-en-Y gastrojejunostomy (Figure 1), an end-to-side gastrojejunostomy was constructed approximately 4.0 cm distal to the ligament of Treitz. The jejunal lumen was occluded 0.5 cm proximal to this anastomosis, and a side-to-side Braun's jejunojejunostomy was made approximately 2.0 cm distal to this anastomosis.

One month after the operation, electric activity of the Roux limb was studied. Then the rats were sacrificed and gastrointestinal motility and levels of *c-kit* mRNA of the Roux limb were detected.

### Myoelectrical studies

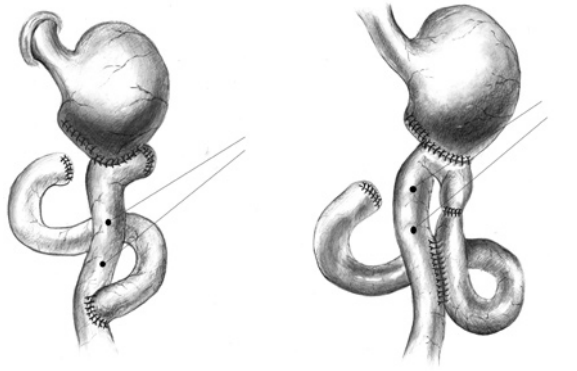
As shown in Figure 1, a couple of monopolar silver-silver chloride electrodes were implanted on the seromuscular layer of the Roux limb, which was spaced evenly 1.0 cm apart. The electrodes were connected by insulated leads to a multipinned socket contained in a plastic cannula, the outer end of which was exteriorizd and the inner end anchored to the left lower quadrant of the abdominal wall. One month after the operation, electric activity was recorded during fasting conditions. The electrodes were connected to a 3802 biological signal amplifier system and the amplified analogue signals were converted to digital signals using NSA4 data acquisition system, sampled at 100 Hz, and displayed in real time on a VGA monitor while simultaneously stored on magnetic media for later analysis using Pclab software (Version 2.2.0).

The amplitude and frequency of slow waves, as well as incidence and amplitude of spike potential of jejunal smooth muscle were recorded. Amplitude of spike potential was represented by the maximum value of spike potential cluster.

Incidence of spike potential (%) = (number of spike potential cluster/number of slow wave) × 100%.

### Determination of gastrointestinal motility in vivo

Each rat took orally 0.3 mL of the mixture of Arabian gel and active charcoal powders. Thirty minutes later, the rats were sacrificed and a laparotomy was performed. Then the stomach from proventriculus to the gastrointestinal stoma was cut and its gross and net mass were weighed. In addition, intact small intestines (from gastrointestinal stoma to ileocecal junction) were took out, and placed on paper without pull strength. Then the lengths from gastrointestinal stoma to the proximal end of charcoal paste reach and to ileocecal junction were measured.



Cut Roux-en-Y gastrojejunostomy Uncut Roux-en-Y gastrojejunostomy

**Figure 1** Distal gastrectomy, cut or uncut Roux-en-Y gastrojejunostomy, placement of electrodes.

Gastric emptying rate (%) = [1- (gastric gross mass-gastric net mass)/mass of charcoal paste] × 100%

Small intestinal impelling ratio (%) = (distance of charcoal paste reach/length of total intestine) × 100%

### Detection of motility of isolated jejunal segment[7]

About 2.0 cm Roux limb was took off and put into 10 mL Tyrode's solution, which had been pre-saturated by a mixed gas (95% $O_2$ + 5% $CO_2$). One end of the jejunal segment was tied on a specimen fixation hook in the water-bath, and the other end was connected with a tonotransducer, then the tonotransducer was linked with the CH1 input interface of BL-New Century bio-signal collecting and processing system. The motility changes of the jejunal segment were recorded. During the experiment, the temperature of Tyrode's solution should be kept at 37.0℃ ± 0.5℃, and continuously ventilated at a constant speed. Contractile force index = contraction amplitude × contraction frequency.

### Measurement of c-kit mRNA by RT-PCR

Total RNA was isolated from one part of the Roux limb using Trizol reagent (Invitrogen, USA) and was reversely transcribed into cDNA according to the instruction of the kit (Promega, USA). The resulting cDNA was used as a template for subsequent polymerase chain reaction (PCR). Samples were then heated to 94℃ for 5 min and cycled 40 times at 94℃ for 15 s, 48℃ for 60 s, and 72℃ for 90 s, and finally extended at 72℃ for 10 min. The primer set designed for PCR amplification of rat *c-kit* mRNA was 5'-AGCAAGAGTTAACGATTCCGGAG-3' and 5'-CCAGAAAGGTGTAAGTGCCTCCT-3', and the predicted size of the PCR product was 344 bp according to the reported sequence of rat *c-kit* mRNA. The primer of housekeeping gene β-actin (348 bp) was 5'CATCAC-CATTGGCAATGAGCG 3' and 5'CTAGAAGCATTT-GCGGTCGGAC 3'. The PCR products were measured using Quantity One software.

### Statistical analysis

Data were expressed as mean ± SD. Comparison between groups was performed with analysis of variance (ANOVA)

**Table 1 Myoelectric activity of the Roux limb in the two groups**

| Group ($n$ = 7) | Frequency of slow wave (cpm) | Amplitude of slow wave (mV) | Incidence of spike potential (%) | Amplitude of spike potential (mV) | MMC phase Ⅲ duration (min) | MMC cycle (min) |
|---|---|---|---|---|---|---|
| Cut group | 9.5 ± 1.1 | 0.48 ± 0.06 | 56.6 ± 6.4 | 0.23 ± 0.06 | 4.4 ± 0.8 | 55.3 ± 8.2 |
| Uncut group | 14.4 ± 1.9[a] | 1.15 ± 0.15[b] | 98.2 ± 10.4[a] | 0.58 ± 0.08[b] | 6.5 ± 1.1[a] | 42.5 ± 6.8[a] |

[a]$P < 0.05$, [b]$P < 0.01$ *vs* cut group.

**Table 2 Analytical data of gastrointestinal motility of the Roux limb and expression *c-kit* mRNA**

| Group ($n$ = 7) | Gastric emptying rate (%) | Small intestinal impelling ratio (%) | Contractile force index | *c-kit*/β-actin ratio |
|---|---|---|---|---|
| Cut group | 49.3 ± 6.8 | 32.2 ± 5.4 | 15.3 ± 2.2 | 0.35 ± 0.06 |
| Uncut group | 65.5 ± 7.9[b] | 53.4 ± 7.4[b] | 36.8 ± 5.1[b] | 0.82 ± 0.11[b] |

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

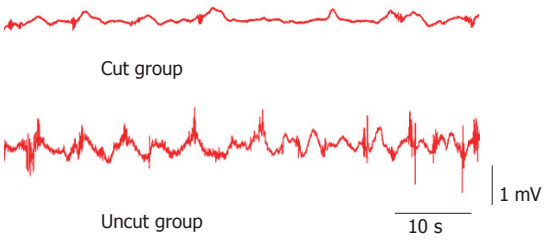



**Figure 2** Original manometric tracings showing the myoelectric activity of the Roux limb.

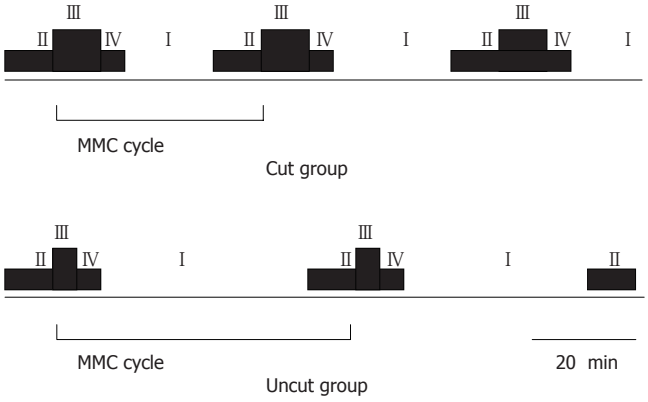



**Figure 3** Diagram of MMC of the Roux limb.

and Student-Newman-Keuls test (q test). Statistical significance was assumed at $P < 0.05$.

## RESULTS

### *Myoelectricity of Roux limb smooth muscle*

Slow waves were found in both the uncut group and cut group as shown in Figure 2, which were composed of an initial three phase complex potential and a secondary isopotentiality. When compared with the cut group, the amplitude (1.15 ± 0.15 mV *vs* 0.48 ± 0.06 mV, $P < 0.05$) and frequency (14.4 ± 1.9 cpm *vs* 9.5 ± 1.1 cpm, $P < 0.01$) of slow waves were significantly higher in the uncut group. Spike potential was overlapped with slow wave potential and emerged following the three phase complex potential. The incidence (98.2 ± 10.4% *vs* 56.6 ± 6.4%, $P < 0.05$) and amplitude (0.58 ± 0.08 mV *vs* 0.23 ± 0.06 mV, $P < 0.01$) of spike potential in the uncut group were significantly higher than the cut group (Table 1).

### *Changes of migrating myoelectric complexes (MMC)*

Slow waves were occasionally loaded with spike potential. According to the spike potential loading situation, the periodicity of electric activity could be observed, namely, MMC. At the phase Ⅲ of MMC, large-amplitude and clustered spike potentials almost covered all slow waves, in which the lasting time was defined as MMC phase Ⅲ duration and the interval time between two MMC phase Ⅲ was defined as an MMC cycle. MMC phase Ⅲ duration in the uncut group was significantly prolonged when compared with the cut group (6.5 ± 1.1 min *vs* 4.4 ± 0.8 min, $P < 0.05$), while the MMC cycle obviously shortened (42.5 ± 6.8 *vs* 55.3 ± 8.2 min, $P < 0.05$) (Figure 3 and Table 1).

### *Influence on gastrointestinal motility of rats*

Gastric emptying and food-transmitting rate of rats were reflected by gastric emptying rate and intestinal impelling ratio, respectively. A lower relative charcoal residual rate represents a higher gastric emptying rate, which means a better gastric emptying capacity. On the other hand, the higher the intestinal impelling ratio, the faster the intestinal transmission rate. When compared with the cut group, both gastric emptying rate (65.5 ± 7.9% *vs* 49.3 ± 6.8%, $P < 0.01$) and intestinal impelling ratio (53.4 ± 7.4% *vs* 32.2 ± 5.4%, $P < 0.01$) in the uncut group were significantly increased, suggesting that gastrointestinal motility was impaired more seriously in the cut group (Table 2).

### *Changes of isolated jejunal segment movement*

Spontaneous contraction could be observed in isolated jejunal segment as shown in Figure 4. The contractile force index in the uncut group was significantly higher than that in the cut group (36.8 ± 5.1 *vs* 15.3 ± 2.2, $P < 0.01$) (Table 2).

### Expression of *c-kit* mRNA

To evaluate the quantity and function of ICC, *c-kit* mRNA expression was examined using semi-quantitive RT-PCR.

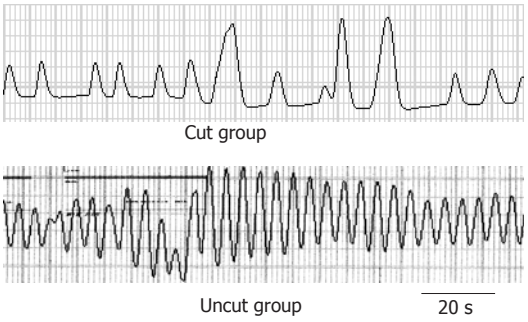



**Figure 4** Motility of isolated jejunal segment.

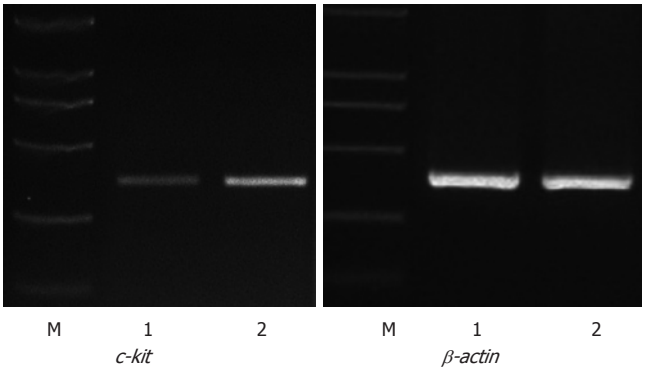



**Figure 5** Expression of *c-kit* mRNA in jejunal segment. L1: Cut group; L2: Uncut group; M: DL-2000 molecular weight markers.

The 348 bp band of β-actin confirmed the integrity of the cDNA obtained from each sample. For *c-kit* a 344 bp band was detectable at variable levels (Figure 5). The expression of *c-kit* mRNA was significantly increased in the uncut group compared with that of the cut group ($0.82 \pm 0.11$ *vs* $0.35 \pm 0.06$, $P < 0.01$) (Table 2).

## DISCUSSION

Patients undergoing Roux-en-Y anastomosis complain of upper abdominal fullness and distension, abdominal pain, nausea or vomiting, and these symptoms are aggravated after meals. Such a syndrome is known as Roux Stasis Syndrome. Van der[8] reported that 26 of 37 (70%) patients experienced such a syndrome. The high incidence has attracted much attention in the research circle. Schippers' findings showed that intestinal movement of patients with Roux stasis syndrome is disordered and lacks regular MMC in interdigestive phase, suggesting that the small intestine may lose the stimulation of pacemaker potential initiated from the duodenum due to destruction of the continuity of the small intestine[9]. Thus, it may be helpful to relieve Roux stasis syndrome by keeping the integrity of the nerves and muscles between duodenal pacemaker potential and the Roux limb. First, back flow of the bile and pancreatic juice through ascending Roux limb should be prevented; second, the integrity of muscular layer of Roux limb should be maintained. Uncut Roux-en-Y gastrojejunostomy could meet these requirements. Chen[10] showed that the reconstitution of the gastrointestinal tract with Roux-en-Y gastrojejunostomy is more effective than with Billroth Ⅱ operation on gastric emptying. Vogel[11] carried out a re-operation to reestablish the continuity of the small intestine for patients with Roux stasis syndrome. As a result, these patients could take food normally post operation without biliary vomiting or burning epigastric pain. In order to explore the underlying mechanisms, we carried out a comparative study between uncut Roux-en-Y gastrojejunostomy and traditional cut Roux-en-Y gastrojejunostomy, and observed the changes of myoelectric activity and motility.

Slow waves, also known as basic or pacemaker potential, are the basis of various electric activities of the gastrointestinal tract, of which the major function is to enhance the excitability of smooth muscles. Cells in each part of the gastrointestinal tract have spontaneous electric activity, and the slow waves attenuate along the gastrointestinal tract; the slow wave of the orad portion with a higher frequency has the inherent effect to drive the lower frequency of the aborad portion, which makes the rhythm of the aborad portion to keep pace with that of the orad portion. After Roux-en-Y gastrojejunostomy, the jejunum is cut off, which causes the frequency of slow waves diminished due to intervention of normal transmission of slow waves. This viewpoint was proved by the current study. Because the intestinal canal was severed in the cut group, the frequency of slow waves was lower compared with the uncut group ($P < 0.05$). It indicated that the excitability of smooth muscles in the uncut group was higher than that in the cut group.

Spike or active potentials are able to trigger contractions of smooth muscles, and the contraction intensity of intestinal smooth muscles has a positive relationship with the incidence of spike potential. This study showed that the incidence of spike potential in the uncut group was higher than that in the cut group ($P < 0.01$). It suggests that the application of uncut Roux-en-Y gastrojejunostomy can decrease the influence on the incidence of spike potential and impairment of the contraction activity of the intestine.

Occurred periodically, MMC is clustered, large-amplitude spike potential activity, which can migrate from the duodenum to terminal ileum. MMC consists of four phases: phase I is characterized by slow waves without spike potential; phase Ⅱ and IV are characterized by a small quantity of spike potential loaded on some slow waves; phase Ⅲ shows large-amplitude, clustered spike potential loaded on all slow waves. Phase Ⅲ electric activity corresponds to a series of propagated contractions, which produce intensive impelling action for small intestinal contents, and periodically scavenge gastrointestinal contents between meals. Deficiency or shortened MMC phase Ⅲ would lead to accumulation of remnant digestive products, cast-off cell, and secretory juice, and facilitate the propagation of bacteria in the small intestine[12]. Le Blanc-Louvry[13] reported that traditional Roux-en-Y operation may shorten MMC phase Ⅲ duration. In the current study, the duration of MMC phase Ⅲ significantly prolonged in the uncut group ($P < 0.01$). Previous findings have shown that uncut Roux-en-Y surgical procedure can relieve Roux stasis syndrome by increasing the frequency of MMC occurrence, prolonging the duration of MMC phase Ⅲ, and

enhancing the clearance of interdigestive remnants. Some studies on gastrointestinal dynamics such as gastric emptying and small intestine impelling ratio also showed that the gastrointestinal motility of experimental animals was significantly improved in the uncut Roux-en-Y group, which is consistent with the studies of Ward[14].

When eliminating the influence of organism adjustment factors such as nerves, and body fluids, we found that there was still some difference in intestinal motility between the two groups. To explore this inner mechanism, we detected the ICC changes of different operations with RT-PCR technique.

ICC is a kind of special interstitial cell in gastrointestinal tract, which has a close relationship with the nervous system. The cell can produce slow waves itself, and thus it has been thought as the pacemaker cell of the gastrointestinal tract[15,16]. It has been shown that ICC plays an important role in the gastrointestinal electrophysiology, dynamo-development and dyskinesis[17,18]. In addition, abnormal expression of ICC has been discovered in some gastrointestinal tract diseases such as gastroparesis[19], chronic idiopathic intestinal pseudo-obstruction[20], slow transit constipation, ulcerative colitis[21] and Crohn's disease[22]. Won[23] observed that the expression of ICC decreased in the animal model of bowel obstruction. *c-kit* is an important marker to identify ICC. It is a kind of transmembrane protein with tyrosine kinase function. During embryonic development period, *c-kit* signal can make ICC precursor develop to myenteric ICC, and finally to be the pacemaker cell of the intestinal tract; whereas those non-connected with *c-kit* signal develop to longitudinal muscle layer[24]. With respect to the long-term maintenance of ICC function, *c-kit* signal is of great importance. Chronic loss or deficiency of this signal transduction may cause blockage or deprivation as well as functional impairment of ICC network[25,26]. Animals with *c-kit* mutant defect have difficulties to produce complete, regular spontaneous slow waves by gastrointestinal tissue[27]. Therefore, *c-kit* reflects the quantity and function of ICC[28]. In this study, *c-kit* mRNA in the uncut group was significantly higher than that in the cut group, which may result from decreased stimulation of the high-frequency electric activity of the orad portion in the cut group.

In summary, compared with traditional cut Roux-en-Y gastrojejunostomy, uncut Roux-en-Y gastrojejunostomy may lessen the effects of operation on myoelectric activity such as slow waves, spike potential, and MMC, decrease the impairment of gastrointestinal motility, and remarkably improve the ICC quantity and function.

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## COMMENTS

### Background

Roux-en-Y anastomosis is a commonly used surgical procedure in gastroenterological surgery; however, more than one third of the patients who experience such an operation suffer Roux stasis syndrome. Therefore, it is important to develop a moderate surgical procedure to control these kinds of complications.

### Research frontiers

The etiology and treatment of Roux stasis syndrome are yet to be clarified.

### Innovations and breakthroughs

We studied the myoelectric activity, gastrointestinal motility, and *c-kit*, which represent the ICC quantity and function.

### Applications

Uncut Roux-en-Y gastrojejunostomy has already been used in clinical practice, and this article will provide more rationale for its application.

### Terminology

**Uncut Roux-en-Y gastrojejunostomy** is based on Billroth Ⅱ subtotal gastrectomy, combined with Braun's jejunojejunostomy and occluded jejunal-gastric pathway. Theoretically, this surgical procedure can maintain the integrity of intestinal canal and normal conduction of impulse, as well as make gastric contents to be drained directly into the intestinal tract along the movement direction of the alimentary tract.

### Peer review

The authors performed an experimental study in rats on myoelectric activity and motility of the Roux limb following cut versus uncut Roux-en-Y gastrojejunostomy.

The research background of the study is clearly defined. The presentation of the results and their discussion are adequate. Results provide sufficient evidence for the conclusions drawn. Overall, the content of the manuscript is interesting and could be valuable for a broad readership.

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*RAPID COMMUNICATION*

# Ligustrazine alleviates acute renal injury in a rat model of acute necrotizing pancreatitis

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**Key words:** Pancreatitis; Microcirculation; Ligustrazine; Renal injury

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## Abstract

**AIM:** To evaluate the effect of ligustrazine, a traditional Chinese medicine, on renal injury in a rat model of acute necrotizing pancreatitis (ANP).

**METHODS:** A total of 192 rats were randomly divided into three groups: control (C group), ANP without treatment (P group), and ANP treated with ligustrazine (T group). Each group was further divided into 0.5, 2, 6, 12 h subgroups. All rats were anesthetized with an intraperitoneal injection of sodium pentobarbital. Sodium taurocholate was infused through the pancreatic membrane to induce ANP. T group was infused sodium taurocholate as above, and 0.6% ligustrazine was then administered *via* the femoral vein. Serum urea nitrogen (BUN) and creatinine (Cr) concentrations were measured for the evaluation of renal function. The effects of ligustrazine on the severity of renal injury were assessed by renal function, $TXA_2/PGI_2$ and histopathological changes. Renal blood flow was determined by the radioactive microsphere technique (RMT).

**RESULTS:** Compared with control group, the renal blood flow in P group was decreased significantly. Serious renal and pancreatic damages were found in P group, the BUN and Cr levels were elevated significantly, and the ratio of $TXA_2$ to $PGI_2$ was increased at 2, 6 and 12 h. Compared with P group, the blood flow of kidney was elevated significantly at 6 and 12 h after induction of ANP, the renal and pancreatic damages were attenuated, and the BUN and Cr levels were decreased significantly, and the ratio of $TXA_2$ to $PGI_2$ was decreased at 6 and 12 h in T group.

**CONCLUSION:** Microcirculatory disorder (MCD) is an important factor for renal injury in ANP. Ligustrazine can ameliorate the condition of MCD and the damage of pancreas and kidney.

## INTRODUCTION

Acute pancreatitis complicated by multiple organ dysfunctions is still a life-threatening disease[1,2], although the precise mechanism by which such local inflammation in the pancreas progresses to systemic illness is still unclear. Recently, this systemic inflammatory response syndrome (SIRS) has become a widely accepted disease state[3], which could lead to the failure of distant organ systems, such as the lungs, intestine, stomach and kidneys[4-6].

Acute pancreatitis (AP) is often complicated by renal injury. However, its pathogenesis remains unclear. Recent studies indicate that during the pathogenesis of acute necrotizing pancreatitis (ANP), the change of microcirculation plays an important role in the worsening of pancreatitis[7].

Pharmacologic studies have demonstrated that ligustrazine, an intravenous drug made from traditional Chinese herbs, is able to inhibit release of intracellular calcium and to scavenge oxygen free radicals[8,9]. Ligustrazine has been widely applied in the treatment of vascular diseases in China due to its significant efficacy on cerebral ischemia and reperfusion injury. However, its role and mechanism in treatment of renal injury have not been extensively studied. The effect of ligustrazine on renal injury was observed in this study based on the established model of ANP.

## MATERIALS AND METHODS

### *Animals*

One hundred and ninety-two adult Sprague-Dawley rats (250-300 g) were provided by the Laboratory Animal Center of Jiangsu University, China. The animals were kept in rooms at 21 ± 1℃ in a 12 h light/dark cycle for 1 wk to acclimate to the surrounding with free access to water and standard laboratory chow. Prior to experiment, the rats

were fasted overnight with access to water.

### Experimental design

The animals were randomly divided into three groups: control ($n$ = 64, C group), ANP without treatment ($n$ = 64, P group), and ANP treated with ligustrazine ($n$ = 64, T group). Each group was further divided into 0.5, 2, 6, 12 h subgroups. All rats were anesthetized with an intraperitoneal injection of sodium pentobarbital (50 mL/kg). Sodium taurocholate (50 g/L, 4 mL/kg, Ward Blen Kinsop CO., UK) was infused through the pancreatic membrane to induce ANP as previously described[10]. After 5-10 min, pancreatic edema and dotted bleeding occurred. T group was infused sodium taurocholate as above, 6 g/L ligustrazine (Seventh Pharmaceutical Factory, Wuxi, China; batch number: 0008241) was then administered *via* the femoral vein (10 mL/kg) as previously described[11,12]. C group received isovolumetric infusion of 9 g/L physiological saline solution using the same method. The abdominal wounds were closed and all the rats were sent back to their cages. Half of the animals in each subgroup were sacrificed at 0.5, 2, 6 and 12 h after infusion for further examination. Left kindeys were removed immediately and fixed in paraformaldehyde solution for 12-24 h and paraffin-embedded for routine histopathologic analysis. The histopathologist was blinded to routine histopathologic analysis. The blood was obtained from eight rats in each group *via* superior mesenteric vein for determination of serum blood urea nitrogen (BUN), creatinine (Cr), $TXA_2$ and $PGI_2$. The remaining rats in each group were used for kidney blood flow determination by the radioactive microsphere technique (RMT). The left kidney was also removed and weighed at 0.5, 2, 6 and 12 h after infusion for subsequent radioactive measurement.

### Blood flow measurements

At 0.5, 2, 6 and 12 h after the infusion, renal blood perfusion values were determined by RMT as previously described[10]. $^{99}Tc^m$-labeled microspheres ($^{99}Mo$-$^{99}Tc^m$ generator preparation was provided by Chinese Institute of Nuclear Power) with a specific activity of 74 MBq/Ml were used for measurement of blood flow. The right carotid artery was catheterized with placement of the tip of the tubing in the left ventricle for infusion of $^{99}Tc^m$-labeled microspheres. One milliliter $^{99}Tc^m$ radioactive microspheres (approximately 500 000 microspheres) was injected for 10 s *via* the catheter with its tip in the aortic ventricle of the heart. A reference blood sample was obtained from the femoral artery catheter for 60 s at a constant rate of 1 mL/min with a continuous-withdrawal pump. The animals injected microspheres were killed by intra-arterial injection of 2 mL 100 g/L KCL. The whole left kidney was removed, weighed, cut into small pieces and placed in a γ-counter (GC-1200 Gamma Radioimmunoassay Counter, USTC Chuangxin CO. Ltd., China) to determine the radioactivity (cpm).

The blood flow values were calculated according to the following formula:

$$Q_{org}\ [mL/(min \cdot g)] = \frac{Q_{ref}\ (mL/min) \times N_{org}\ (cpm)}{N_{ref}\ (cpm) \times weighing\ (g)}$$

Where $Q_{org}$ denotes organ blood flow (mL/min.g), $Q_{ref}$ is withdrawal rate of the reference sample (mL/min), $N_{org}$ is the number of microspheres in the organ (count/min) and $N_{ref}$ is the number of microspheres in the reference sample.

**Table 1 Grading of histological injury using microscopic injury score**

| Score | Findings |
|---|---|
| O | Normal |
| I | Notable cloudy swelling of tubular epithelial cells |
| II | Swelling denaturation of renal tubular epithelial cells, interstitial congestion, edema and infiltration of inflammatory cells |
| III | Diffuse coagulation necrosis in tubular epithelial cells |

### Detection of serum BUN, Cr, $TXA_2$ and $PGI_2$

The blood from the superior mesenteric vein was collected into a tube. The tube was immediately centrifuged at 3500 r/min for 15 min. Collected plasma was stored at -40℃ until use. BUN and Cr levels were assayed using an automatic biochemistry analyzer (CL-7300; SHIMADZU Corporation, Kyoto, Japan) with assay kits. The level of $TXA_2$ and $PGI_2$ was detected according to the manufacturer's instructions (Science and Technology Development Center, General Hospital of PLA, Beijing, China).

### Histological examination

Renal and pancreas specimens were harvested and fixed in 10 g/L formalin for histological examination. The tissues were dehydrated, embedded in paraffin wax, cut into 5-μm sections, and mounted. After removed from the paraffin, the tissues were stained with hematoxylin and eosin. The severities of renal injury were quantified using a histological scoring system as previously described[13]. Histopathologic analysis of renal specimens was performed and scored as 0-Ⅲ (Table 1). Twenty fields per kidney were examined, a mean of the total score was compared between the groups. The renal sections were also analyzed with a HPIAS-1000 multimedia color analysis system (Huahai Co., Shanghai). Five fields (0.265 mm × 0.2 mm) of each section were read. Average values of neutrophil infiltration were calculated and recorded. All examinations were performed in a blind fashion by an experienced pathologist.

### Statistical analysis

All data were analyzed by the SPSS 11.0 software. The results were expressed as mean ± SD except for data on the grading of renal lesions. Differences in grading of renal lesion were determined using the non-parametric Mann-Whitney test. Statistical analysis was performed with post-hoc test. $P < 0.05$ was considered statistically significant.

## RESULTS

### Renal blood flow

Blood flow in the P group was significantly lower than that in the C group. It began to decrease at 0.5 h and became

**Table 2 Renal blood flow in groups C, P and T [mL/(min•g)] (mean ± SD, *n* = 8)**

| Group | 0.5 h | 2 h | 6 h | 12 h |
|---|---|---|---|---|
| C | 7.33 ± 0.35 | 7.63 ± 0.43 | 7.46 ± 0.67 | 7.55 ± 0.67 |
| P | 5.67 ± 0.51[b] | 6.64 ± 0.68[b] | 5.81 ± 0.67[b] | 5.16 ± 0.72[b] |
| T | 7.17 ± 0.72 | 7.22 ± 0.82 | 7.22 ± 0.73[d] | 7.31 ± 1.12[d] |

[b]$P < 0.01$ *vs* C group; [d]$P < 0.01$ *vs* P group.

**Table 3 Serum BUN level in groups C, P and T (mean ± SD, mmol/L)**

| Group | 0.5 h | 2 h | 6 h | 12 h |
|---|---|---|---|---|
| C | 9.80 ± 1.36 | 10.31 ± 1.50 | 10.05 ± 0.87 | 10.21 ± 1.33 |
| P | 11.99 ± 2.08[a] | 12.73 ± 1.72[a] | 14.71 ± 2.08[b] | 15.16 ± 2.73[b] |
| T | 10.12 ± 1.23[c] | 10.53 ± 2.25[c] | 11.52 ± 2.21[d] | 11.71 ± 1.31[d] |

[a]$P < 0.05$, [b]$P < 0.01$ *vs* C group; [c]$P < 0.05$, [d]$P < 0.01$ *vs* P group.

**Table 4 Serum Cr level in groups C, P and T (mean ± SD, mmol/L)**

| Group | 0.5 h | 2 h | 6 h | 12 h |
|---|---|---|---|---|
| C | 21.12 ± 1.67 | 21.06 ± 1.58 | 21.50 ± 1.78 | 21.62 ± 1.75 |
| P | 24.97 ± 3.40[a] | 25.36 ± 3.11[b] | 30.40 ± 1.93[b] | 30.60 ± 2.04[b] |
| T | 22.13 ± 1.57[c] | 23.5 ± 2.61[a,c] | 23.58 ± 2.61[a,d] | 24.73 ± 1.01[b,d] |

[a]$P < 0.05$, [b]$P < 0.01$ *vs* C group; [c]$P < 0.05$, [d]$P < 0.01$ *vs* P group.

**Table 5 Serum level of $TXA_2/PGI_2$ in groups C, P and T (mean ± SD)**

| Group | 0.5 h | 2 h | 6 h | 12 h |
|---|---|---|---|---|
| C | 1.18 ± 0.15 | 1.22 ± 0.11 | 1.24 ± 0.15 | 1.23 ± 0.16 |
| P | 1.23 ± 0.16 | 1.50 ± 0.21[b] | 1.61 ± 0.19[b] | 1.86 ± 0.28[b] |
| T | 1.19 ± 0.14 | 1.31 ± 0.14 | 1.31 ± 0.17[a,c] | 1.45 ± 0.24[a,d] |

[a]$P < 0.05$, [b]$P < 0.01$ *vs* C group; [c]$P < 0.05$, [d]$P < 0.01$ *vs* P group.

**Table 6 Renal tissue injury in groups C, P and T**

| Group | 0.5 h | | | | 2 h | | | | 6 h | | | | 12 h | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 0 | I | II | III | 0 | I | II | III | 0 | I | II | III | 0 | I | II | III |
| C | 8 | 0 | 0 | 0 | 8 | 0 | 0 | 0 | 8 | 0 | 0 | 0 | 8 | 0 | 0 | 0 |
| P | 0 | 1 | 3 | 4[b] | 0 | 0 | 4 | 4[b] | 0 | 0 | 3 | 5[b] | 0 | 0 | 0 | 8[b] |
| T | 4 | 4 | 0 | 0[d] | 0 | 3 | 5 | 0[d] | 1 | 2 | 2 | 3[d] | 0 | 1 | 3 | 4[c] |

[b]$P < 0.01$ *vs* C group; [c]$P < 0.05$, [d]$P < 0.01$ *vs* P group.

**Table 7 Renal tissue neutrophil infiltration in groups C, P and T (mean ± SD)**

| Group | 0.5 h | 2 h | 6 h | 12 h |
|---|---|---|---|---|
| C | 5.5 ± 1.2 | 6.5 ± 1.2 | 6.7 ± 1.3 | 6.9 ± 1.4 |
| P | 13.0 ± 1.6[b] | 15.0 ± 1.9[b] | 18.0 ± 1.7[b] | 21.1 ± 3.0[b] |
| T | 6.9 ± 1.4 | 10.8 ± 1.4[b,d] | 13.0 ± 1.6[b,d] | 15.0 ± 2.0[b,d] |

[b]$P < 0.01$ *vs* C group; [d]$P < 0.01$ *vs* P group.

the lowest at 12 h. However, the blood flow in the T group was significantly higher than that in the P group at 6 and 12 h, showing no significant difference from the C group (Table 2).

### *Serum levels of BUN, Cr, $TXA_2$ and $PGI_2$*

Compared with the control group, the BUN and Cr levels in the P group were elevated significantly ($P < 0.01$ or $P < 0.05$), and the ratio of $TXA_2$ to $PGI_2$ was increased at 2, 6 and 12 h ($P < 0.01$). Compared with the P group, the BUN and Cr levels were decreased significantly ($P < 0.01$ or $P < 0.05$), and the ratio of $TXA_2$ to $PGI_2$ was decreased at 6 and 12 h in the T group ($P < 0.01$ or $P < 0.05$) (Table 3, Table 4, Table 5).

### *Pathological examination*

After induction of ANP model, the pancreas showed mild edema and congestion. At 0.5 h, typical pathological changes of ANP were found, such as a large number of inflammatory cells, necrosis of the adjacent fat tissues, interstitial edema, parenchyma hemorrhage and necrosis, large amount of ascites. The changes became severer with the prolongation of time. The renal pathological changes were aggravated significantly in the P group. Histopathologic scores were higher in the P group than in the C group throughout the experiment ($P < 0.01$) and lower in the T group than in the P group (Table 6). Under the light microscope, different swelling denaturation and necrosis of renal tubular epithelial cells were observed. Simultaneously, interstitial congestion, edema and infiltration of inflammatory cells were also observed. While in the ligustrazine-treated group, the outward appearance of the kidney was normal. The mean number of neutrophils infiltrated in × 400 field increased from 0.5 h in the T group, while decreased significantly from 2 h in the P group (Table 7).

### *Correlated analysis*

Correlated analysis showed that there was a negative correlation between renal blood flow and serum Cr ($r = -0.931$, $P < 0.01$) and $TXA_2/PGI_2$ ($r = -0.977$, $P < 0.05$), as well as between renal blood flow and pathologic score($r = -0.948$, $P < 0.05$).

## DISCUSSION

Acute renal injury is a major cause of morbidity in ANP. Our experimental study in rats demonstrated that microcirculatory disorder (MCD) of rats resulted in a sequence of events that ultimately caused renal injury. Although the renal injury occurring in ANP has been well described, the underlying mechanism remains unclear. The rat model of renal injury in this study resulted in a dramatic decrease in renal blood flow as evidenced by

RMT and renal histological changes.

Microcirculatory disturbances are important early pathophysiological events in various organs during AP[14-18]. Microcirculatory change is an important factor during the development of ANP. It can damage the pancreas and extrapancreatic vital organs[19,20]. The possible contributory mechanisms involved in the development of this disease include increased vascular permeability, reduced blood flow, leukocyte-endothelia interactions and development of intravascular thrombi[21,22]. The radioactive microsphere technique can efficiently estimate blood flow to various organs in the body. With the availability of different radioactive labeled microspheres, it is possible to measure regional blood flow repeatedly[23]. In our study, at the early stage of ANP, the renal blood flow began to decrease significantly at 0.5, 2, 6 and 12 h after infusion as compared with the C group. The renal injury was possibly due to ANP and the release of inflammatory mediators. A series of changes in the nerve endocrine system led to the redistribution of visceral blood flow, thus producing a sharp decrease of renal blood flow, suggesting that microcirculation disturbances may contribute to renal injury under conditions such as ischemia/reperfusion at the early stage of ANP. Therapeutic agents that improve pancreatic blood flow might be valuable in the treatment of acute pancreatitis[24]. In this study, ligustrazine improved microcirculation and reduced acute renal injury in rats with ANP.

In the present study, the protective effect of ligustrazine against renal injury was investigated in rats with ANP. Scrum levels of BUN and Cr were used as indicators of renal protection. $TXA_2$ is both a vasoconstrictor and a potent stimulus for platelet aggregation. Its effect is antagonized by prostacylin, which is released from vascular endothelial cells. Prostacylin exerts a variety of effects on the cardiovascular system, including a decrease in blood pressure associated with a decrease in systemic vascular resistance. Feng *et al*[25] demonstrated that $TXA_2$ may be involved in the pathogenesis of acute pancreatitis at its early stage. The ratio of $TXA_2$ to $PGI_2$ was significantly lower after reperfusion than before reperfusion, indicating that the disorder of $TXA_2$ and $PGI_2$ might also be involved in the circulation disorders during ANP. Although the precise mechanism remains unknown, they may play a role in the pathobiology of ANP.

The second prominent feature in this experimental model is the development of granulocytosis and accumulation of neutrophils in the microvasculature of renal capillaries. Because neutrophils are known to be required for the development of renal injury in ANP, interventional studies were undertaken to assess whether intravenous administration of ligustrazine would result in decreased neutrophil accumulation in renal tissues. Our results showed that neutrophil accumulation was markedly elevated after induction of pancreatitis, which was significantly reduced by ligustrazine.

Ligustrazine is widely used in traditional Chinese medicine, often in combination with other herbs. It was used traditionally to treat a diversity of ailments, particularly cardiac (heart) and vascular disorders such as atherosclerosis or blood clotting abnormalities. Ligustrazine could intervene in hemorheological events, such as blood flow, erythrocyte deformation, leukocyte adhesion, platelet aggregation and thrombolysis[26]. It was reported that ligustrazine could inhibit pulmonary hypertension by decreasing the mRNA expression of endothelin-1, oxygen free radical level, lipid peroxidation and adjusting $TXA_2/PGI_2$ imbalance in pulmonary arterioles[27,28].

To investigate the protective effects of ligustrazine against renal injury, the influence of ligustrazine injection on BUN, Cr and $TXA_2/PGI_2$, as well as changes of morphology of renal tubules, were studied in a rat kidney model during ANP. Ligustrazine improved renal microcirculation, suggesting that the protective effects of ligustrazine against renal injury may be attributable to improving microcirculation and further preventing accumulation of neutrophils.

In conclusion, MCD plays an important role in the development of renal injury. The early use of ligustrazine seems to be effective. This provides further evidence for ligustrazine as a therapeutic strategy against renal injury during ANP.

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## COMMENTS

### Background

Acute pancreatitis (AP) is often complicated by renal injury. However, its pathogenesis remains unclear. The significant efficacy of ligustrazine on cerebral ischemia and reperfusion injury was confirmed in this study. However, the role and mechanisms of ligustrazine in treatment of renal injury have not been extensively studied.

### Research frontiers

To investigate the protective effects of ligustrazine against renal injury, the influence of ligustrazine injection on BUN, Cr, and $TXA_2/PGI_2$, as well as changes of morphology of renal tubules, were studied in a rat kidney model during ANP.

### Innovations and breakthroughs

The effect of ligustrazine on renal injury was observed in this study based on the established model of ANP. The radioactive microsphere technique was used to analyze the blood flow. Although it is not commonly used and has major disadvantages, it can analyze the blood flow in the pancreas and extrapancreatic vital organs simultaneously.

### Applications

In the present study, ligustrazine improved renal microcirculation, suggesting that ligustrazine can protect against renal injury by improving microcirculation and further preventing accumulation of neutrophils. The early use of ligustrazine seems to be effective. This provides further evidence for ligustrazine as a therapeutic strategy against renal injury during ANP.

### Terminology

Radioactive microsphere technique (RMT): Microspheres containing radioactive substances are infused into the circulatory system to measure perfusion rates in tumors and normal tissues, cerebral blood flow, tissue oxygenation, cardiovascular function, regional vascular resistance, and the effect of various drugs on these parameters. In the present study, $^{99}Tc^{m}$-labeled microspheres were used for measurement of blood flow.

### Peer review

This is a very interesting study demonstrating the effects of ligustrzineon on the pathophysiology of acute pancreatitis. However, some limitations and additional data should be provided.

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*CASE REPORT*

# Analysis of ileal sodium/bile acid cotransporter and related nuclear receptor genes in a family with multiple cases of idiopathic bile acid malabsorption

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## Abstract

The etiology of most cases of idiopathic bile acid malabsorption (IBAM) is unknown. In this study, a Swedish family with bile acid malabsorption in three consecutive generations was screened for mutations in the ileal apical sodium-bile acid cotransporter gene (ASBT; gene symbol, SLC10A2) and in the genes for several of the nuclear receptors known to be important for ASBT expression: the farnesoid X receptor (FXR) and peroxisome proliferator activated receptor alpha (PPARα). The patients presented with a clinical history of idiopathic chronic watery diarrhea, which was responsive to cholestyramine treatment and consistent with IBAM. Bile acid absorption was determined using $^{75}$Se-homocholic acid taurine (SeHCAT); bile acid synthesis was estimated by measuring the plasma levels of 7α-hydroxy-4-cholesten-3-one (C4). The ASBT, FXR, and PPARα genes in the affected and unaffected family members were analyzed using single stranded conformation polymorphism (SSCP), denaturing HPLC, and direct sequencing. No ASBT mutations were identified and the ASBT gene did not segregate with the bile acid malabsorption phenotype. Similarly, no mutations or polymorphisms were identified in the FXR or PPARα genes associated with the bile acid malabsorption phenotype. These studies indicate that the intestinal bile acid malabsorption in these patients cannot be attributed to defects in ASBT. In the absence of apparent ileal disease, alternative explanations such as accelerated transit through the small intestine may be responsible for the IBAM.



**Key words**: Bile acid malabsorption; Diarrhea; Genetics; $^{75}$Se-homocholic acid taurine test; Nuclear receptors

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## INTRODUCTION

Bile acids are synthesized from cholesterol in the liver and secreted into the small intestine, where they facilitate absorption of fat, fat-soluble vitamins and cholesterol[1]. The bile acids are then reabsorbed from the intestine and returned to the liver *via* the portal venous circulation. The enterohepatic cycling of bile acids is an extremely efficient process, and less than 5% of the intestinal bile acids escape reabsorption and are eliminated in the feces. The ileal apical sodium/bile acid cotransporter (ASBT)[1] mediates the first step in the active uptake of bile acids from the intestine, and defects in ileal ASBT function may be responsible for bile acid malabsorption associated with watery diarrhea. Impaired ileal uptake of bile acids has been documented in several patients[2] and inherited ASBT mutations were demonstrated in congenital primary bile acid malabsorption (PBAM)[3]. However, ASBT mutations are not found in most patients with adult-onset bile acid malabsorption, chronic diarrhea, and a morphological and

functionally normal ileum[4], a more common condition termed idiopathic bile acid malabsorption (IBAM)[5-7]. In this study we examined the association between IBAM and inherited mutations affecting the ASBT and several of the nuclear receptors known to be important for ASBT expression, the farnesoid X receptor (FXR)[8] and peroxisome proliferator activated receptor A (PPARα)[9], in a Swedish family with three generations of bile acid malabsorption.

## CASE REPORT

Three family members (subjects 1, 10, and 11) reported chronic diarrhea, occurring especially after meals (Figure 1). Fasting blood samples were obtained from each family member. Informed consent to participate in the study was obtained from each subject and the protocol was approved by the Ethics Committee of Karolinska University Hospital Huddinge. Bile acid absorption was determined using $^{75}$Sehomocholic acid taurine (SeHCAT), a synthetic analog of taurocholic acid, as previously described[10]. Briefly, a capsule containing 10 μCi of $^{75}$SeHCAT was given orally and retained activity was measured after 3 h and 7 d using an uncollimated gamma counter. Retention of less than 10% of the administered radiolabeled bile acid was considered abnormal. The plasma level of 7α-hydroxy-4-cholesten-3-one (C4) (normal < 19 ng/mL), an intermediate product in the synthesis of bile acids, was measured as described[11]. C4 is a reliable marker for the activity of hepatic cholesterol 7α-hydroxylase, the rate-determining enzyme in bile acid synthesis[12,13].

Patient 11 had a history of diarrhea since adulthood with 15 to 20 watery bowel movements per day over the past 10 years. Clinical history was unremarkable except for a cholecystectomy at age 24. Patient 10 had a history of frequent watery diarrhea since her teenage years. Patient 1 reported frequent bowel movements following a meal. In all three patients, celiac disease was excluded; lactose tolerance tests, vitamin B12 absorption, and routine laboratory blood tests including hemoglobin, sedimentation rate and liver function tests were normal. Barium contrast gastrointestinal exams and ileocolonoscopy with mucosal biopsies (patients 10 and 11) were normal. SeHCAT tests (patients 10 and 11) and plasma levels of C4 (patients 1, 10 and 11) were markedly abnormal (Figure 1). Treatment of patients 10 and 11 with cholestyramine (Questran, Bristol-Myers) reduced the stool frequency and improved the stool consistency.

Dysfunctional mutations in the ASBT gene were previously identified in a subject with PBAM[3]. To determine if similar mutations in ASBT are associated with bile acid malabsorption in this family, we employed simple sequence length polymorphism (SSLP) analysis using a dinucleotide repeat linked to the ASBT gene and SSCP analysis to screen for mutations in the ASBT coding and proximal promoter regions. The SSCP primers designed for ASBT intron or exon sequences and PCR amplification conditions have been described previously[3,14]. PCR amplification products were resolved using three different gel electrophoresis conditions, gels contained 10% glycerol, 1 × TBE buffer, and 6% acrylamide, 10% acrylamide (acrylamide: N, N'-methylenebisacrylamide ratio 50:1), or 0.4 × MDE (Mutation Detection Enhancement acrylamide; FMC Bioproducts, Rockland, Maine), in order to increase the assay sensitivity[15,16], and the nucleotide sequence changes responsible for the SSCP band shifts were subsequently identified by PCR amplification and sequencing. No ASBT mutations or polymorphisms were found in patients 1 or 10, whereas patient 11 was heterozygous for two common polymorphisms that do not affect ASBT function[14], a G-to-T transversion in exon 3 that causes an alanine to serine substitution at position 171 (A171S) and an intronic A-to-G transition located 20 bp upstream of exon 6 (int 5). Following SSCP analysis, the two ASBT alleles could then be distinguished in the original proband (patient 11) using a combination of the linked dinucleotide repeat marker and the single nucleotide polymorphisms (A171S and int 5). The affected individuals shared only one ASBT allele, and four unaffected individuals (subjects 2, 3, 6 and 8) also inherited this allele.

Since the nuclear receptor, FXR, is an important regulator of ASBT expression and bile acid metabolism, the coding region of the FXR gene, exons 3 to 11[17], was also analyzed in patients 1, 10, and 11 using SSCP conditions described by Lind *et al*[18]. Sequences for SSCP primers designed for FXR intron and exon sequences are shown in Table 1. Briefly, exons 3-11 were amplified by PCR from genomic DNA, generating fragments varying from 200 to 250 bp in length, except exon 10 (315 bp) and exon 11 (385 bp). Due to its larger size, exon 4 was PCR-amplified using two sets of primers that yielded products of 240 and 300 bp. The fragments were separated on precast polyacrylamide gels visualized by silver staining (GenePhor DNA Separation System, Amersham Bioscience, Uppsala, Sweden). This analysis detected no mutations or polymorphisms in the human FXR gene of these patients.

**Table 1 Sequences for the primers for SSCP on human FXR**

| Exon | | Primer |
|---|---|---|
| 3 | Forward | 5′-CATTCCCACAGTCACAAACTATTTA-3′ |
| | Reverse | 5′-GTAGTTTGTTCTTATTGATATTCAAATG-3′ |
| 4 proximal | Forward | 5′-GATGACATTCATTCCAGTTTTGTTGTC-3′ |
| | Reverse | 5′-AGCTGGCATACGCCTGAGTTCATAT-3′ |
| 4 distant | Forward | 5′-TCATCCTATTATTCCAACCTGGGTTTC-3′ |
| | Reverse | 5′-AGTAAAACCTGAAGGAGAAAACTGCC-3′ |
| 5 | Forward | 5′-GAGGACTTTTTACACTTTTCAGTGT-3′ |
| | Reverse | 5′-AATGTAATTGCTTGAAGTGAATACC-3′ |
| 6 | Forward | 5′-GTACTTTCTGTGATTGGTGAAGTCTC-3′ |
| | Reverse | 5′-AAACTCAGTTCCTGCCAGTCTTGGC-3′ |
| 7 | Forward | 5′-GATGAATGCACATATAGAAAGAAGGC-3′ |
| | Reverse | 5′-CTCCAGAAAATATTAACTTTAAACCCACAT-3′ |
| 8 | Forward | 5′-CAAAGATCTGAGAAATAGTAAGATGG-3′ |
| | Reverse | 5′-AGGTAATCTAATCTGTGGGCAC-3′ |
| 9 | Forward | 5′-GTTACTCCTTGATACCAATTTGATTATC-3′ |
| | Reverse | 5′-CTCCTAGAAACAAACTCTTTTACCAT-3′ |
| 10 | Forward | 5′-CTAGTTTTACACTGTTTAGTCACTC-3′ |
| | Reverse | 5′-ATTTGGATAGCAGAATTATAGGCTAC-3′ |
| 11 | Forward | 5′-CTTACACTTCAAAATAGTTAACGTTGC-3′ |
| | Reverse | 5′-GCTCCTTTTTCTCTCTCATATTAATC-3′ |

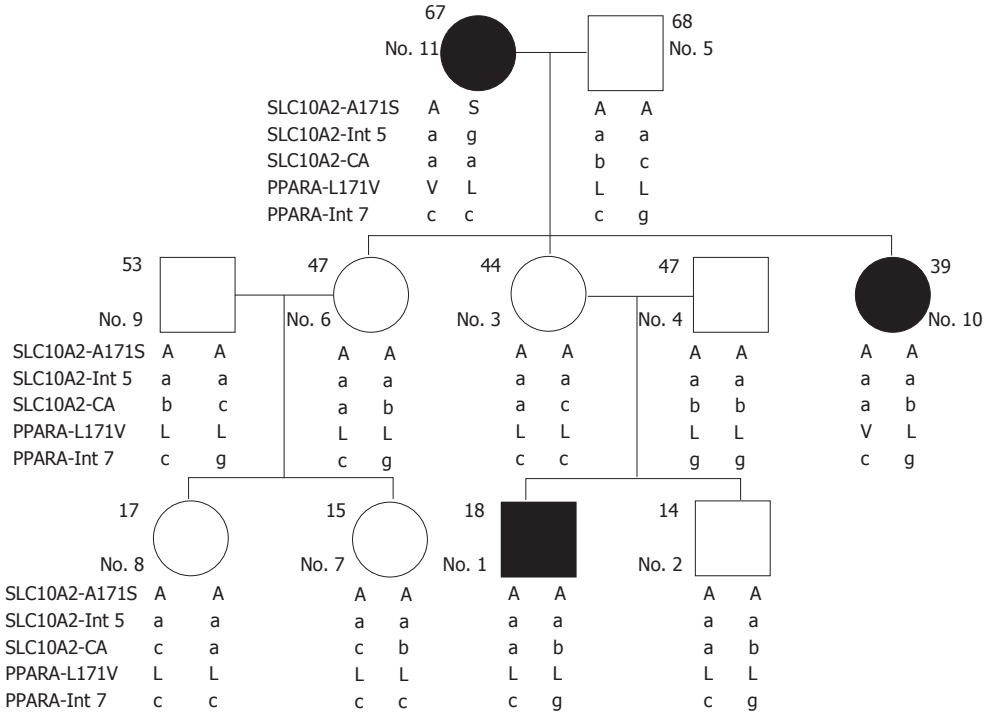


**Figure 1** Family pedigree and genetic analysis. Individuals with bile acid malabsorption are indicated by the shaded symbols. The haplotype for each subject is provided below the symbol in the pedigree. The affected subjects' number, age, BMI, SeHCAT results, and C4 results are indicated below the pedigree.

| Subject No. | 11 | 10 | 1 |
|---|---|---|---|
| Age (yr) | 67 | 39 | 18 |
| BMI (kg/m$^2$) | 23.7 | 36.6 | 22.0 |
| Cholesterol (mmol/L) | 4.4 | 3.6 | 4.0 |
| LDL cholesterol (mmol/L) | 2.5 | 2.5 | 2.2 |
| Triglyceride (mmol) | 0.8 | 1.5 | 0.6 |
| SeHCAT (% retention) | 1 | 4 | ND |
| C4, times normal | 2.5 | 2.0 | 2.5 |

BMI: Body mass index; ND: Not determined.

Polymorphisms in the intronic and exonic regions of the PPARα gene (PPARA) have been previously described[19,20], and PPARα is a known regulator of ASBT gene expression[21]. We analyzed two well-characterized polymorphic regions of PPARA, exon 5 and intron 7, in the PBAM family in order to determine if a mutation in this gene could be associated with the disease. Specific primers were employed for PCR amplification of exon 5 (forward: 5'-AGTAAAGCAAGTGCGCTGGT-3'; reverse: 5'-AAGGAAGGGGAACTGAGGAA-3') and intron 7 (forward: 5'-CCTCCCGAGTATCTGGGATT-3'; reverse: 5'-TGAGCTGCCTTTAGATATTGTCA-3'). The PCR products were analyzed for polymorphisms or mutations by denaturing HPLC (D-HPLC) (Transgenomic Wave, Transgenomic, Omaha, Nebraska) and automatic sequencing (automatic sequencer CEQ$^{TM}$8000 XL, Beckman Coulter Inc., Fullerton. CA). PPARA gene analysis did not show any new mutations. Analysis of the L162V polymorphism of exon 5 and G > C polymorphism of intron 7 revealed that PPARA alleles did not segregate with the bile acid malabsorption symptoms (Figure 1).

## DISCUSSION

The enterohepatic circulation efficiently conserves bile acids, thereby maintaining bile flow and adequate intraluminal bile acid concentrations for micellular solubilization and absorption of lipids[22]. Defective small intestinal absorption leads to increased concentrations of dihydroxy bile acids reaching the colon, where they alter water and electrolyte movement leading to secretory diarrhea[23,24]. Three types of intestinal bile acid malabsorption are generally recognized[25]. Type Ⅰ bile acid malabsorption is the most common form and is caused by ileal resection, ileal disease such as Crohn's disease, ileal bypass, and radiation enteritis[26,27]. Type Ⅲ bile acid malabsorption is associated with conditions such as cholecystectomy, peptic ulcer surgery, chronic pancreatitis, celiac disease, diabetes mellitus, cystic fibrosis, and the use of various drugs[28].

In contrast to types Ⅰ and Ⅲ, type Ⅱ bile acid malabsorption (also called primary or idiopathic bile acid malabsorption) is not associated with obvious ileal disease. A very rare congenital form of type Ⅱ bile acid malabsorption (primary bile acid malabsorption) exhibiting refractory infantile diarrhea, steatorrhea, and growth failure[2,25] was found to be associated with inherited mutations in the ASBT gene[3]. However, most patients with adult-onset idiopathic bile acid malabsorption appear to have a normal ASBT gene[4] and the etiology is still obscure. The identification of a family with idiopathic bile acid malabsorption in three consecutive generations offered a rare occasion to further evaluate association of this syndrome with inherited mutations affecting

the ASBT. These patients were diagnosed with IBAM on the basis of clinical presentation, low SeHCAT test values, increased bile acid synthesis, and response to cholestyramine treatment. Analysis of these individuals and unaffected family members conclusively demonstrated that the intestinal bile acid malabsorption in these subjects is not due to inherited defects in the ASBT gene. In addition, we also looked for polymorphisms of PPARα and FXR, two nuclear receptors known to be important for the regulation of the ASBT. To our knowledge, polymorphism analysis of the human FXR has not been described previously and no mutations of the FXR gene were found in the present study. Likewise, no association between PPARA and IBAM was found in this family.

There is increasing evidence emerging in support of IBAM etiologies other than defective ileal uptake of bile acids. Earlier studies had provided evidence for an increased ileal uptake of bile acids[29] as well as an expanded bile acid pool in some patients with type Ⅱ bile acid malabsorption[28]. Very recently, Bajor *et al*[30] demonstrated elevated *in vitro* bile acid uptake and ASBT protein expression in ileal biopsies from patients with bile acid malabsorption, abnormal SeHCAT-retention values, and elevated plasma C4 levels. This apparent increase in ASBT activity and expression could be explained by accelerated small bowel transit in IBAM patients, thereby reducing the contact time between the luminal contents and the mucosa. In support of this hypothesis, a more rapid small bowel transit has been reported for patients with IBAM[31]. The etiology of the postulated accelerated small bowel transit in these patients is not clear. However, more rapid small bowel transit has been noted in subjects with elevated BMI[31,32] and in subjects consuming high fat diets[33], suggesting dysregulation of gut motility under these conditions. The rapid small bowel transit is predicted to reduce the opportunity for ileal absorption, leading to decreased levels of bile acids in the ileal enterocytes and increased ASBT expression. Previous *in vitro* studies have shown that the human ASBT promoter is negatively regulated by bile acids through an FXR dependent mechanism[8]. The decreased enterocyte levels of bile acids are also predicted to reduce the FXR-dependent induction of FGF19 expression, thereby increasing hepatic bile acid synthesis and plasma C4 levels. FGF19 is an ileal enterocyte derived factor that mediates repression of the hepatic cholesterol 7α-hydroxylase gene and bile acid synthesis[34,35].

In conclusion, the present findings further argue against defective ileal uptake of bile acids as the direct cause of IBAM and support the exploration of alternative explanations such as reduced contact time with the ileal mucosa due to changes in small intestinal motility.

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*CASE REPORT*

# Alpha-fetoprotein-producing colon cancer with atypical bulky lymph node metastasis

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## Abstract

Alpha-fetoprotein (AFP)-producing colorectal cancer is extremely rarely reported until now. All of the reported cases harboring synchronous hematogenous spread including liver and/or lung metastasis had a poor prognosis and died within 12 mo. We here describe a 71-year old man with AFP-producing colon cancer who presented with an unusual bulky lymph node metastasis instead of hematogenous spread. He underwent adjuvant chemotherapy in addition to curative surgical resection, which prolonged his survival.



**Key words**: Alpha-fetoprotein; Colon cancer; Bulky lymph node metastasis; Computed tomography; Colonoscopy

Fu K, Kobayashi A, Saito N, Sano Y, Kato S, Ikematsu H, Fujimori T, Kaji Y, Yoshida S. Alpha-fetoprotein-producing colon cancer with atypical bulky lymph node metastasis. *World J Gastroenterol* 2006; 12(47): 7715-7716

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## INTRODUCTION

Alpha-fetoprotein (AFP)-producing colorectal cancer is extremely rare, and only ten cases have been described in the English literature[1]. All reported cases presented with lung and/or liver metastasis and had a very poor prognosis. We here report the first case of AFP-producing colon cancer with bulky lymph node metastasis. This patient had characteristics such as harboring synchronous bulky nodal involvement but not hematogenous spread. Interestingly, he could be curatively operated and survived a long time after adjuvant chemotherapy in addition to surgical resection.

## CASE REPORT

A 71-year old man visited our hospital for positive fecal occult blood and mild anemia. Physical examination disclosed a painless abdominal mass, approximately 50 mm in diameter, in the right lower quadrant of abdomen. Colonoscopy revealed an ulcerative lesion suggestive of an advanced colon cancer in the cecum (Figure 1). Biopsy specimens revealed a moderately-differentiated adenocarcinoma. Additionally, preoperative computed tomography of the abdomen and pelvis showed a bulky mass around the cecal cancer, which suggested nodal involvement corresponding to the palpated abdominal mass on physical examination (Figure 2). However, no distant hematogenous spread, including liver or lung metastasis, was detected. The serum AFP level was high (318.9 μg/L before operation). Right hemicolectomy including lymph node dissection was performed uneventfully, and histological examination of the surgical specimen revealed a subserosally invasive poorly-differentiated adenocarcinoma with nodal involvement. Immunohistochemically, both the primary tumor and the bulky lymph node metastasis showed strong expression of AFP. The serum AFP level became normal after adjuvant chemotherapy in addition to surgery. The patient was in good condition at the time of our report and had no sign of recurrence in the past 5 years.

## DISCUSSION

Elevated levels of AFP, commonly associated with hepatocellular carcinoma or embryonic cell carcinoma, have been reported in neoplasms of several other organs, such as pancreas, gallbladder and gastrointestinal tract. However, AFP-producing colorectal carcinomas are extremely rare. The reported colorectal carcinomas have generally occurred in middle-aged to older men with the

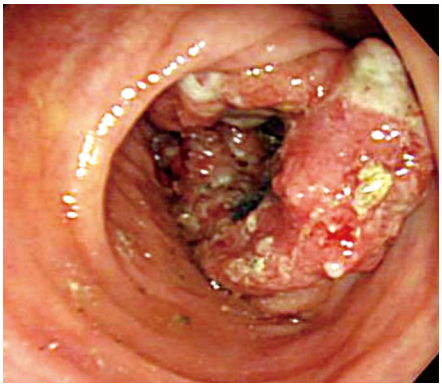

**Figure 1** Colonoscopy showing a circumferentially advanced colon cancer in the cecum.

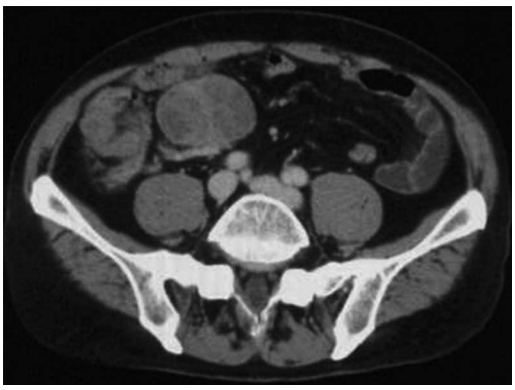

**Figure 2** Abdominal computed tomography showing a bulky mass approximately 50 mm in diameter, suggestive of nodal involvement around the primary cecal cancer.

rectum most commonly affected, the serum AFP level is usually as high as several-thousand nanograms per milliliter[1-5]. Moreover, AFP appears to be a potential marker for tumor activity, as the serum level of AFP is higher in patients with liver metastasis than in those without liver metastasis[6]. AFP-producing colorectal carcinoma generally has a poor prognosis because of the frequent occurrence of blood-borne metastases. All the reported cases have extensive liver and/or lung metastases at the time of diagnosis and a very poor prognosis[1]. Our patient was unusual in comparison with the reported cases, as his AFP-producing colon cancer was located in the cecum, and showed only synchronous lymphogenous spread but not synchronous or metachronous blood-borne metastasis during a long time of follow-up. Furthermore, he was a long-time survivor, and was able to undergo curative surgical resection in addition to adjuvant chemotherapy. Clinical evaluation of a large number of patients is necessary to clarify whether systemic adjuvant chemotherapy is associated with a favorable prognosis for this kind of patients.

In summary, patients with AFP-producing colon cancer and synchronous bulky lymph node metastasis can survive a long time after adjuvant chemotherapy in addition to surgical resection.

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*CASE REPORT*

# Laparoscopic cholecystectomy in a patient with situs inversus totalis

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## Abstract

Currently, laparoscopic cholecystectomy is an undoubtfully optimal treatment of cholelithiasis. What about performing this procedure on a patient with situs inversus totalis and what are the difficulties of this operation for a right-handed surgeon? We presented a 35-year-old man with unknown situs inversus totalis who was admitted with epigastric pain and digestive problems. Ultrasonography and computed tomography of the abdomen confirmed the diagnosis of a gallstone. Besides, the liver and gallbladder were on the left side and the spleen was on the right. All systems were left-right reversal as mirror image in all diagnostic studies. Laparoscopic cholecystectomy was safely performed, despite of difficulties of situs inversus. The patient was discharged on postoperative day 1. It should be considered that existence of other anomalies may easily cause uninvited injuries. In the patients with situs inversus, laparoscopic cholecystectomy can be safely managed by an experienced surgeon through laparoscopy, and also hepatobiliary surgery.



**Key words:** Situs inversus totalis; Laparoscopic cholecystectomy; Cholelithiasis

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## INTRODUCTION

Situs inversus totalis (SIT) is a very uncommon entity. It was first reported by Fabricius in 1600[1]. The incidence is thought to be in the range of 1:10 000 to 1:20 000[2]. Transposition of the organs may also affect thoracic organs, besides abdominal organs. It can be associated with Kartegener triad or some other cardiac anomalies. There is no evidence for increased incidence of cholelithiasis in SIT[3]. Since Mouret first performed it in 1987, laparoscopic cholecystectomy (LC) has become the standard operative procedure for cholelithiasis[4].

In the present study, the new technique and subsequently possible difficulties of LC in a patient with SIT was reported.

## CASE REPORT

A 35-year-old male was admitted to the Department of General Surgery, Ege University School of Medicine, with a 2-year history of intermittent epigastric pain and concomitant digestive problems. The patient had not been diagnosed as situs inversus totalis before. Apex beat was found in the right hemithorax on physical examination. An electrocardiography showed right axis deviation and right ventricular hypertrophy, in keeping with dextrocardia. Laboratory data showed no abnormality of liver function tests, or white blood cell count. An ultrasonography identified SIT and multiple stones, which were millimetric in size in the gallbladder. The common bile duct was also noted as dilated. A thoracoabdominal computed tomography (CT) revealed the situs inversus totalis abnormality and a suspicious opacity located at the neck of the gallbladder (Figure 1). Endoscopic retrograde cholangiopancreatography was performed and a stone was extracted from the common bile duct (Figure 2). The patient then underwent LC.

### *Operation techniques*

The operation team and the laparoscopic devices were located in the theater as a mirror image configuration of normal LC. Dissection of the Calot's triangle is one of the major problems for a right-handed surgeon in case of situs inversus totalis abnormality. Four ports were used: optical (10 mm), one 5 mm and one 10 mm operating, and one 5 mm assisting port (Figure 3). The optical port was near the umbilicus and a 30° laparoscope was used. Because of previously unoperated abdomen, the closed technique was employed by the insertion of a Veress needle through the subumbilical area. Carbon dioxide ($CO_2$) pneumoperitoneum was then created with a pressure of 12 mmHg. Two 10-mm trocars were inserted into the abdominal cavity, one in the position of the Veress needle for laparoscope and the other one in the sub-xiphoid

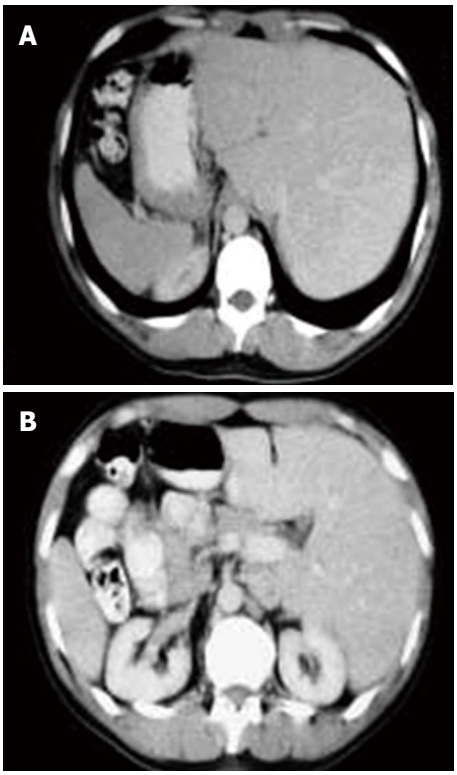


**Figure 1** Computed tomography of the patient. **A**: The liver is on the left side; **B**: The spleen and stomach are on the right side of the patient.

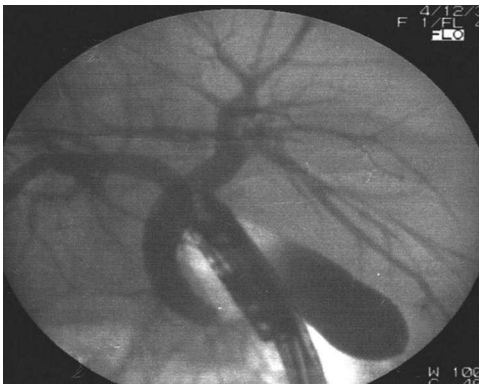

**Figure 2** Endoscopic retrograde cholangiography of the patient. A stone was extracted from the main duct. The gallbladder can be observed on the left side.

location. A 5-mm trocar was inserted midline at the midpoint of xiphoid-umbilicus line and a second 5-mm trocar was inserted into the abdominal cavity in the left mid-axillary line under the view of laparoscope. Fundus of the gallbladder was grasped and retracted by the assistant using a clinch, which was inserted through the 5-mm trocar in the left mid-axillary region. Traction of the Hartmann's pouch was performed by the left hand of the surgeon using a grasper inserted through the trocar located at the subxiphoid region. Dissection of Calot's triangle was carried out with a posterior approach by using a forceps that was inserted through the trocar located in the midline. The cystic duct and cystic artery were first divided by blunt dissection and separated. Dissection was performed above the plane of Rouviere's sulcus to avoid any injury. Both of them were clipped (double proximal, single distal

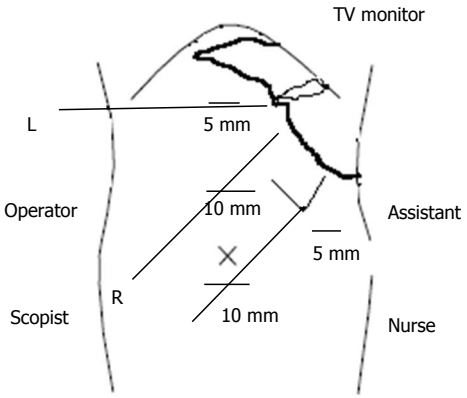


**Figure 3** Port sites in new technique. L: left hand; R: right hand.

10-mm titanium clips) and then divided by hook scissors. After division of all peritoneal reflection on either side, the gallbladder was retrogradely separated from the liver bed by using electrocautery. It was then extracted through the 10-mm operating port. Fascial closure of large ports was sutured with absorbable material (polydiaxonon) to prevent herniation.

## DISCUSSION

Situs inversus viscerum is considered to have a genetic predisposition that is autosomal recessive[5,6] and may be associated with several syndromes[7,8]. Physical examination of the patient with situs inversus can be misleading. As the central nervous system may not share the general transposition, pain may be felt in the epigastrium alone or in the right-upper quadrant and in the mid-scapular region[9-11]. In patients of acute cholecystitis with situs inversus, pain can be felt in the left upper quadrant due to irritation of the peritoneum[11,12]. Intrahepatic biliary tree, arterial and venous systems are in the mirror-image configuration of the normal anatomy. The hepatoduodenal ligament is the same as seen in the orthotopic patient[13]. Arterial system anomalies are in the mirror-image configuration of the orthotopic patients[14]. Although there are many reports of patients with situs inversus and cholelithiasis, there is no evidence that the incidence of cholelithiasis is greater in these patients[15]. In the preoperative period CT may be useful in determining the anomalies[12]. Twenty cases of LC in SIT were reported in the literature[16]. The most challenging factor for performing LC in patients with situs inversus is the mirror image anatomy. This uninvited condition may lead to some problems in orientation and dissection during the procedure since at least two thirds of the surgeons are right handed[16]. To overcome this issue, several alternative modifications were proposed: (1) retraction of Hartmann pouch by the first assistant, (2) surgeon standing between the two abducted lower limbs of the patient[1,16].

We propose that it helps by changing the position of grasper with the dissector and dissecting the Calot's triangle from the lateral side, taking advantage of using the right hand. To use the midclavicular trocar in dissection, which is used only for traction in traditional technique, the view angle should be increased and crossing of the scope and dissector

should be prevented. To achieve this, the midclavicular 5 mm trocar is placed at the midline and a 30º viewing scope is used *via* the subumbilical trocar. By using this technique, an extra 45º is added to the 30º viewing of the scope. Careful dissection of Calot's triangle can be managed by performing it from the lateral side with the help of this totally 75º. Working in two dimensions is the biggest difficulty in laparoscopic surgery. In addition to this, mirror-image that is seen in situs inversus increases the difficulty. As a result, this may lead to high risk of iatrogenic injuries. In those cases as Thomas B Hugh mentioned in his article; in traditional cholecystectomy technique, the start point is fundus of the gallbladder but this is not convenient for laparoscopic method[16]. In laparoscopic cholecystectomy, the sulcus, which is called Rouviere's sulcus that runs to the right of the hilum of the liver can be an alternative reference point. This sulcus is open in 78% of patients and its site is recognizable in more than 90% of the patients[16]. Dissection should be safely carried out close to the gallbladder in the triangle that is described in this article. Reviewing the operative field regularly and releasing the traction of the gallbladder may help you know where you are. Withdrawing the camera will help to obtain a large field of view. With the help of these, overall relationship in the hepatobiliary triangle can be evaluated. The findings during the operation should be checked with other members of the operating team. Care must be taken for the concentration of the operative team and arrangement of the equipment setup in the operation room.

In conclusion, we suggest that LC procedure in these patients with SIT should be performed by surgeons who are experienced in laparoscopy and hepatobiliary surgery as well.

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# Meetings

## MAJOR MEETINGS COMING UP

First Biennial Congress of the Asian-Pacific Hepato-Pancreato-Biliary Association
March, 2007
Fukuoka, Japan
http://www.congre.co.jp/1st-aphpba

American College of Gastroenterology Annual Scientific
20-25 October 2006
Las Vegas, NV

14th United European Gastroenterology Week, UEGW
21-25 October 2006
Berlin, Germany

APDW 2006: Asian Pacific Digestive Week 2006
26-29 November 2006
Lahug Cebu City, Philippines

## EVENTS AND MEETINGS IN THE UPCOMING 6 MONTHS

Falk Symposium 151: Emerging Issues in Inflammatory Bowel Diseases
24-25 March 2006
Sydney - NSW
Falk Foundation e.V.
symposia@falkfoundation.de

10th International Congress of Obesity
3-8 September 2006
Sydney
Event Planners Australia
enquiries@ico2006.com
www.ico2006.com

Easl 2006 - the 41st annual
26-30 April 2006
Vienna, Austria
Kenes International

Prague hepatology 2006
14-16 September 2006
Prague
Foundation of the Czech Society of Hepatology
veronika.revicka@congressprague.cz
www.czech-hepatology.cz/phm2006

12th International Symposium on Viral Hepatitis and Liver Disease
1-5 July 2006
Paris
MCI France
isvhld2006@mci-group.com
www.isvhld2006.com

Falk Symposium 152: Intestinal Disease Part I, Endoscopy 2006 - Update and Live Demonstration
4-5 May 2006
Berlin
Falk Foundation e.V.
symposia@falkfoundation.de

Falk Symposium 153: Intestinal Disease Part II, Immunoregulation in Inflammatory Bowel Disease - Current Understanding and Innovation
6-7 May 2006
Berlin
Falk Foundation e.V.
symposia@falkfoundation.de

ILTS 12th Annual International Congress
3-6 May 2006
Milan
ILTS
www.ilts.org

Internal Medicine: Gastroenterology
22 July 2006-1 August 2006
Amsterdam
Continuing Education Inc
jbarnhart@continuingeducation.net
6th Annual Gastroenterology And Hepatology
15-18 March 2006
Rio Grande
Office of Continuing Medical Education
cmenet@jhmi.edu
www.hopkinscme.net

World Congress on Gastrointestinal Cancer
28 June 2006-1 July 2006
Barcelona, Spain
c.chase@imedex.com

International Conference on Surgical Infections, ICSI2006
6-8 September 2006
Stockholm
European Society of Clinical Microbiology and Infectious Diseases
icsi2006@stocon.se
www.icsi2006.se/9/23312.asp

7th World Congress of the International Hepato-Pancreato-Biliary Association
3-7 September 2006
Edinburgh
Edinburgh Convention Bureau
convention@edinburgh.org
www.edinburgh.org/conference

Society of American Gastrointestinal Endoscopic Surgeons
26-29 April 2006
Dallas - TX
www.sages.org

Digestive Disease Week 2006
20-25 May 2006
Los Angeles
www.ddw.org

Annual Postgraduate Course
25-26 May 2006
Los Angeles, CA
American Society of Gastrointestinal Endoscopy
www.asge.org/education

American Society of Colon and Rectal Surgeons
3-7 June 2006
Seatlle - Washington
www.fascrs.org

## EVENTS AND MEETINGS IN 2006

10th World Congress of the International Society for Diseases of the Esophagus
22-25 February 2006
Adelaide
isde@sapmea.asn.au
www.isde.net

Falk Symposium 151: Emerging Issues in Inflammatory Bowel Diseases
24-25 March 2006
Sydney - NSW
Falk Foundation e.V.
symposia@falkfoundation.de

10th International Congress of Obesity
3-8 September 2006
Sydney
Event Planners Australia
enquiries@ico2006.com
www.ico2006.com

Easl 2006 - the 41st annual
26-30 April 2006
Vienna, Austria
Kenes International

VII Brazilian Digestive Disease Week
19-23 November 2006
www.gastro2006.com.br

International Gastrointestinal Fellows Initiative
22-24 February 2006
Banff, Alberta
Canadian Association of Gastroenterology
cagoffice@cag-acg.org
www.cag-acg.org

Canadian Digestive Disease Week
24-27 February 2006
Banff, Alberta
Digestive Disease Week Administration
cagoffice@cag-acg.org
www.cag-acg.org

Prague Hepatology 2006
14-16 September 2006
Prague
Foundation of the Czech Society of Hepatology
veronika.revicka@congressprague.cz
www.czech-hepatology.cz/phm2006

12th International Symposium on Viral Hepatitis and Liver Disease
1-5 July 2006
Paris
MCI France
isvhld2006@mci-group.com
www.isvhld2006.com/

Falk Seminar: XI Gastroenterology Seminar Week
4-8 February 2006
Titisee
Falk Foundation e.V.
symposia@falkfoundation.de

European Multidisciplinary Colorectal Cancer Congress 2006
12-14 February 2006
Berlin
Congresscare
info@congresscare.com
www.colorectal2006.org

Falk Symposium 152: Intestinal Disease Part I, Endoscopy 2006 - Update and Live Demonstration
4-5 May 2006
Berlin
Falk Foundation e.V.
symposia@falkfoundation.de

Falk Symposium 153: Intestinal Disease Part II, Immunoregulation in Inflammatory Bowel Disease - Current Understanding and Innovation
6-7 May 2006
Berlin
Falk Foundation e.V.
symposia@falkfoundation.de

14th United European Gastroenterology Week
21-25 October 2006
Berlin
United European Gastroenterology Federation
www.uegw2006.de

World Congress on Controversies in Obesity, Diabetes and Hypertension
25-28 October 2006
Berlin
comtec international
codhy@codhy.com
www.codhy.com

Asia Pacific Obesity Conclave
1-5 March 2006
New Delhi
info@apoc06.com
www.apoc06.com/

ILTS 12th Annual International Congress
3-6 May 2006
Milan
ILTS
www.ilts.org

XXX Panamerican Congress of Gastroenterology
11-16 November 2006
Cancun
www.panamericano2006.org.mx

Internal Medicine: Gastroenterology
22 July 2006-1 August 2006
Amsterdam
Continuing Education Inc
jbarnhart@continuingeducation.net

6th Annual Gastroenterology And Hepatology
15-18 March 2006
Rio Grande
Office of Continuing Medical Education
cmenet@jhmi.edu
www.hopkinscme.net

Hepatitis 2006
25 February 2006-5 March 2006
Dakar
hepatitis2006@mangosee.com
mangosee.com/mangosteen/hepatitis2006/hepatitis2006.htm

World Congress on Gastrointestinal Cancer
28 June 2006-1 July 2006
Barcelona, Spain
c.chase@imedex.com

International Conference on Surgical Infections, ICSI2006
6-8 September 2006
Stockholm
European Society of Clinical Microbiology and Infectious Diseases
icsi2006@stocon.se
www.icsi2006.se/9/23312.asp

5th International Congress of The African Middle East Association of Gastroenterology
24-26 February 2006
Sharjah
InfoMed Events
infoevent@infomedweb.com
www.infomedweb.com

7th World Congress of the International Hepato-Pancreato-Biliary Association
3-7 September 2006
Edinburgh
Edinburgh Convention Bureau
convention@edinburgh.org
www.edinburgh.org/conference

13th International Symposium on Pancreatic & Biliary Endoscopy
20-23 January 2006
Los Angeles - CA
laner@cshs.org

2006 Gastrointestinal Cancers Symposium
26-28 January 2006
San Francisco - CA
Gastrointestinal Cancers Symposium
Registration Center
giregistration@jspargo.com

Society of American Gastrointestinal Endoscopic Surgeons
26-29 April 2006
Dallas - TX
www.sages.org

Digestive Disease Week 2006
20-25 May 2006
Los Angeles
www.ddw.org

Annual Postgraduate Course
25-26 May 2006
Los Angeles, CA
American Society of Gastrointestinal Endoscopy
www.asge.org/education

American Society of Colon and Rectal Surgeons
3-7 June 2006
Seatlle - Washington
www.fascrs.org

71st ACG Annual Scientific and Postgraduate Course
20-25 October 2006
Venetian Hotel, Las Vegas, Neveda
The American College of Gastroenterology

AASLD 57th Annual - The Liver Meeting™
27-31 October 2006
Boston, MA
AASLD

New York Society for Gastrointestinal Endoscopy
13-16 December 2006
New York
www.nysge.org

## EVENTS AND MEETINGS IN 2007

9th World Congress on Gastrointestinal Cancer
20-23 June 2007
Barcelona
Imedex
meetings@imedex.com

***Gastro 2009, World Congress of Gastroenterology and Endoscopy London, United Kingdom 2009***

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# Instructions to authors

## GENERAL INFORMATION

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Manuscripts should be typed double-spaced on A4 (297 mm × 210 mm) white paper with outer margins of 2.5 cm. Number all pages consecutively, and start each of the following sections on a new page: Title Page , Abstract, Introduction, Materials and Methods, Results, Discussion, acknowledgements, References, Tables, Figures and Figure Legends. Neither the editors nor the Publisher is responsible for the opinions expressed by contributors. Manuscripts formally accepted for publication become the permanent property of The WJG Press, and may not be reproduced by any means, in whole or in part without the written permission of both the authors and the Publisher. We reserve the right to put onto our website and copy-edit accepted manuscripts. Authors should also follow the guidelines for the care and use of laboratory animals of their institution or national animal welfare committee.

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All contributions should be written in English. All articles must be submitted using a word-processing software. All submissions must be typed in 1.5 line spacing and in word size 12 with ample margins. The letter font is Tahoma. For authors from China, one copy of the Chinese translation of the manuscript is also required (excluding references). Style should conform to our house format. Required information for each of the manuscript sections is as follows:

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Full manuscript title, running title, all author(s) name(s), affiliations, institution(s) and/or department(s) where the work was accomplished, disclosure of any financial support for the research, and the name, full address, telephone and fax numbers and email address of the corresponding author should be included. Titles should be concise and informative (removing all unnecessary words), emphasize what is new, and avoid abbreviations. A short running title of less than 40 letters should be provided. List the author(s)' name(s) as follows: initial and/or first name, middle name or initial(s) and full family name.

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An informative, structured abstract of no more than 250 words should accompany each manuscript. Abstracts for original contributions should be structured into the following sections: AIM: Only the purpose should be included. METHODS: The materials, techniques, instruments and equipments, and the experimental procedures should be included. RESULTS: The observatory and experimental results, including data, effects, outcome, *etc*. should be included. Authors should present *P* value where necessary, and the significant data should accompany. CONCLUSION: Accurate view and the value of the results should be included.

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### *Tables*

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Data that are not statistically significant should not be noted. $^{a}P<0.05$, $^{b}P<0.01$ should be noted ($P>0.05$ should not be noted). If there are other series of *P* values, $^{c}P<0.05$ and $^{d}P<0.01$ are used. Third series of *P* values can be expressed as $^{e}P<0.05$ and $^{f}P<0.01$. Other notes in tables or under

illustrations should be expressed as $^1F$, $^2F$, $^3F$; or some other symbols with a superscript (Arabic numerals) in the upper left corner. In a multi-curve illustration, each curve should be labeled with ●, ○, ■, □, ▲, △, *etc.* in a certain sequence.

### *Acknowledgments*

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

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When the authors code the references, please ensure that the order in text is the same as in reference part and also insure the spelling accuracy of the first author's name. Do not code the same citation twice.

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PMID roots in the abstract serial number indexed by PubMed (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=PubMed). The author should supply the PMID for journal citation. For those references that have not been indexed by PubMed, a printed copy of the first page of the full reference should be submitted.

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### *Style for journal references*

Authors: the first author should be typed in bold-faced letter. The surname of all authors should be typed with the initial letter capitalized and followed by their name in abbreviation (For example, Lian-Sheng Ma is abbreviated as Ma LS, Bo-Rong Pan as Pan BR). Title of the cited article and italicized journal title (Journal title should be in its abbreviation form as shown in PubMed), publication date, volume number (in black), start page, and end page [PMID: 11819634]

Note: The author should test the references through reference testing system (http://www.wjgnet.com/cgi-bin/index.pl)

### *Style for book references*

Authors: the first author should be typed in bold-faced letter. The surname of all authors should be typed with the initial letter capitalized and followed by their name in abbreviation (For example, Lian-Sheng Ma is abbreviated as Ma LS, Bo-Rong Pan as Pan BR) Book title. Publication number. Publication place: Publication press, Year: start page and end page.

### *Format*

**Journals**

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

1 **Grover VP**, Dresner MA, Forton DM, Counsell S, Larkman DJ, Patel N, Thomas HC, Taylor-Robinson SD. Current and future applications of magnetic resonance imaging and spectroscopy of the brain in hepatic encephalopathy. *World J Gastroenterol* 2006; **12**: 2969-2978 [PMID: 16718775]

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

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

*In press*

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

*Organization as author*

4 **Diabetes Prevention Program Research Group**. Hypertension, insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension* 2002; **40**: 679-686 [ PMID: 12411462 ]

*Both personal authors and an organization as author*

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

*No author given*

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

*Volume with supplement*

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

*Issue with no volume*

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

*No volume or issue*

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

**Books**

*Personal author(s)*

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

*Chapter in a book (list all authors)*

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

*Author(s) and editor(s)*

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

*Conference proceedings*

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

*Conference paper*

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

**Electronic journal** (list all authors)

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

**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

### *Inappropriate references*

Authors should always cite references that are relevant to their article, and avoid any inappropriate references. Inappropriate references include those that are linked with a hyphen and the difference between the two numbers at two sides of the hyphen is more than 5. For example, [1-6], [2-14] and [1, 3, 4-10, 22] are all considered as inappropriate references. Authors should not cite their own unrelated published articles.

### *Statistical data*

Present 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 $\chi^2$ (in Greek), related coefficient as $r$ (in italics), degree of freedom as $\gamma$ (in Greek), sample number as $n$ (in italics), and probability as $P$ (in italics).

### *Units*

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

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

# New insights into the coagulopathy of liver disease and liver transplantation

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## Abstract

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



**Key words**: Coagulation; Cirrhosis; Liver transplantation; Bleeding

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## INTRODUCTION

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

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

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

## HEMOSTATIC FACTORS

### *Procoagulant factors*

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

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

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

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

### *vWf*

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

### *Fibrinogen*

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

### *Platelets*

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

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

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

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

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

**Table 1 Hemostatic abnormalities associated with liver disease**

| Favoring hemorrage | Favoring thrombosis |
|---|---|
| Low platelet count | |
| Impaired platelet function and platelet-vessel wall interaction | Elevated levels of factors Ⅷ and vWf |
| Enhanced platelet inhibition by nitric oxide (NO) and prostacyclin | Decreased levels of protein C, protein S, antithrormbin |
| Ⅲ, $\alpha_2$-antiplasmin | |
| Decreased levels coagulation factors (Ⅱ, Ⅴ, Ⅶ, Ⅸ, Ⅹ, Ⅺ) | Macroglobulin |
| Quantitative and qualitative abnormalities of fibrinogen | Heparin cofactor Ⅱ elevated |
| Low level of $\alpha_2$-antiplasmin, TAFI, histidine-rich-glycoprotein | Decreased levels of plasminogen |
| levels of tPA, with small increase of PAI-1 levels | |

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

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

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

## ANTICOAGULANT FACTORS

### *Antithrombin Ⅲ*

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

### *Protein C and protein S*

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

## DISORDERS OF THE FIBRINOLYTIC SYSTEM

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

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

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

### *Disseminated DIC and accelerated intravascular coagulation (AIC)*

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

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

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

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

## PROGNOSTIC VALUE OF COAGULATION FACTORS

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

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

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

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

## ASSESSMENT OF THE RISK OF THROMBOSIS AND ANTICOAGULATION

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

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

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

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

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

## ASSESSMENT OF THE RISK OF BLEEDING

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

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

## INVASIVE PROCEDURES

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

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

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

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

## COAGULATION DURING INFECTION AND SEPSIS

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

Using TEG, 20 cirrhotic patients who experienced early

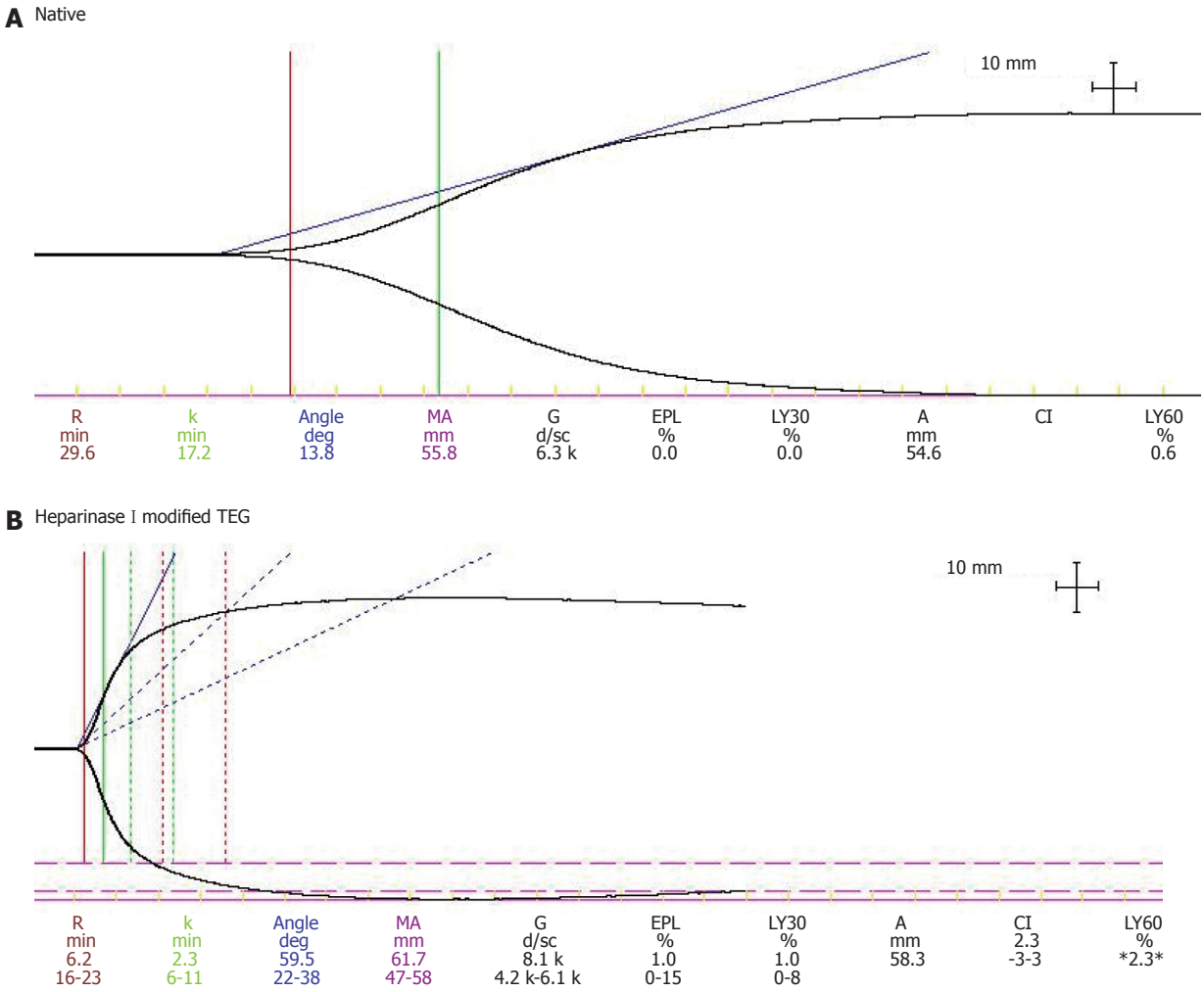


**Figure 1** Native-TEG (**A**) and heparinase I -TEG (**B**) on sample collected at the onset of spontaneous bacterial peritonitis in a patient with liver liver cirrhosis. (**A**) significant heparin-like effect found revealed by the slowed rate of coagulation. (**B**) treatment of the sample with heparinase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

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

## THERAPY OF HEMOSTATIC ABNORMALITIES IN LIVER DISEASE

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

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

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

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

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

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

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

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

## LIVER TRANSPLANTATION

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

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

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

### *Pre-anhepatic stage*

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

### *Anhepatic phase*

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

### *Reperfusion and post reperfusion phase*

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

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

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

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

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

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

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

### *Postoperative period*

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

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*EDITORIAL*

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

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## Abstract

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

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

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

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

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



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

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## INTRODUCTION

### *Hepatic encephalopathy*

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

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

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

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

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

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

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

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

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

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

## INTELLECTUAL AND MOTOR FUNCTIONS ARE IMPAIRED IN HE

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

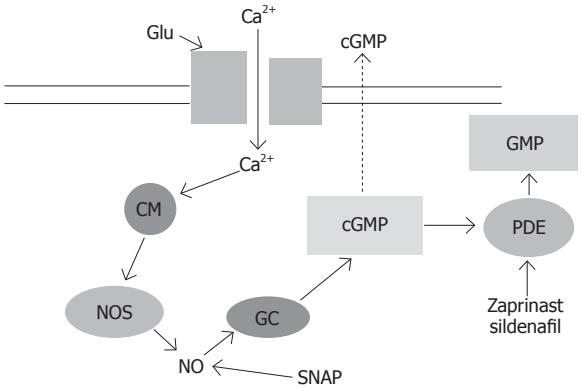


**Figure 1** Glutamate-nitric oxide-cyclic GMP pathway. Activation of ionotropic (mainly NMDA) glutamate receptors leads to increased intracellular calcium ($Ca^{2+}$) which after binding to calmodulin (CM), activates nitric oxide synthase (NOS), leading to increased production of nitric oxide (NO),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

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

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

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

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

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

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

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

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

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

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

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

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

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

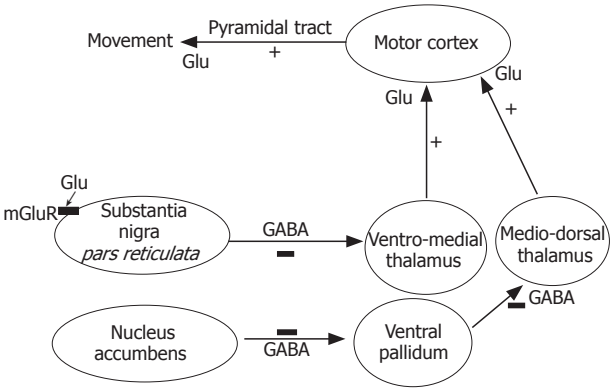


**Figure 2** Hypolocomotion in animal models of HE is due to increased extracellular glutamate and activation of metabotropic glutamate receptors in substantia nigra pars reticulata. Activation of metabotropic glutamate receptors (mGluRs) in nucleus accumbens induces motor activity by activating a neuronal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

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

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

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

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

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

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

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

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

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*REVIEW*

# Food allergy in gastroenterologic diseases: Review of literature

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## Abstract

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



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

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## INTRODUCTION

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

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

## EPIDEMIOLOGY AND PATHOGENESIS

### *Epidemiology*

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

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

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

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

### *Pathogenesis*

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

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

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

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

**Table 1 Pathophysiologic classification of adverse reactions to food**

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

**Table 2 Clinical classification of allergic disorders induced by food**

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

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

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

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

## CLINICAL CLASSIFICATION

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

### *Gastrointestinal food-induced allergic disorders*

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

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

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

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

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

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

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

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

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

### *Cutaneous and respiratory food-induced allergic disorders*

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

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

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

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

### *Systemic food-induced allergic disorders*

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

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

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

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

## DIAGNOSIS

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

### *Skin prick test*

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

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

### *Detection of serum food-specific IgE*

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

### *Other laboratory tests*

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

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

### *Oral food challenge*

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

## MANAGEMENT

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

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

### *Medications*

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

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

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

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

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

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

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

### *Prevention*

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

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

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*REVIEW*

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

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## Abstract

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



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

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## INTRODUCTION

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

## GRKs STRUCTURE AND DISTRIBUTION

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

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

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

## G-PROTEIN-COUPLED RECEPTOR ENDOCYTOSIS: DESENSITIZATION AND SIGNALING

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

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

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

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

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

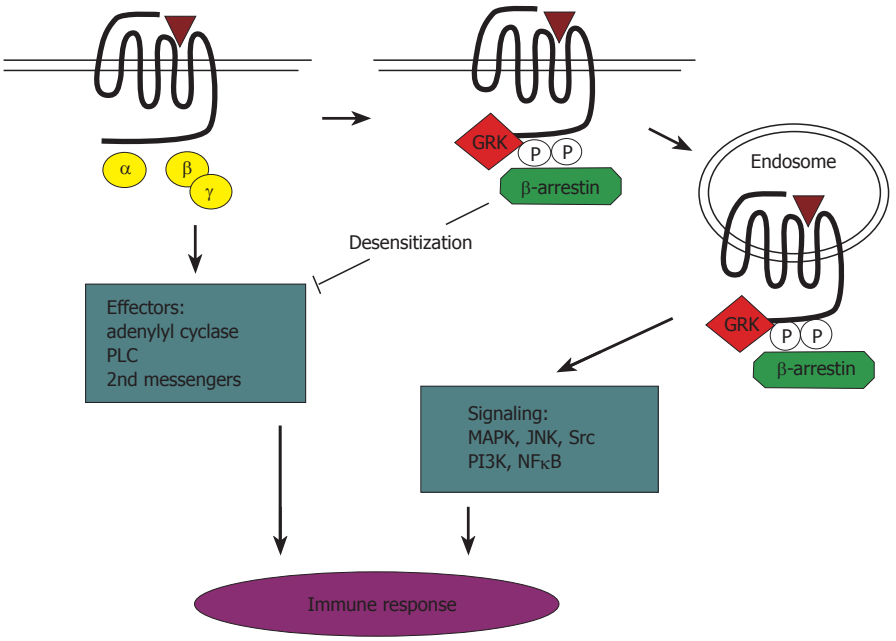


**Figure 1** Schematic summary of the role of GRKs/arrestins in activation, signaling, and desensitization of GPCRs in the immune system. Agonist-activated GPCRs are phosphorylated rapidly by GRKs, leading to recruitment of arrestins. This process, called homologous desensitization, prevents further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

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

## G-PROTEIN-COUPLED RECEPTOR INTERNALIZATION

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

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

## G-PROTEIN-COUPLED RECEPTOR KINASES AND DISEASES

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

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

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

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

## CONCLUDING REMARKS

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

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*ESOPHAGEAL CANCER*

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

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## Abstract

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

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

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

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



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

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## INTRODUCTION

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

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

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

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

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

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

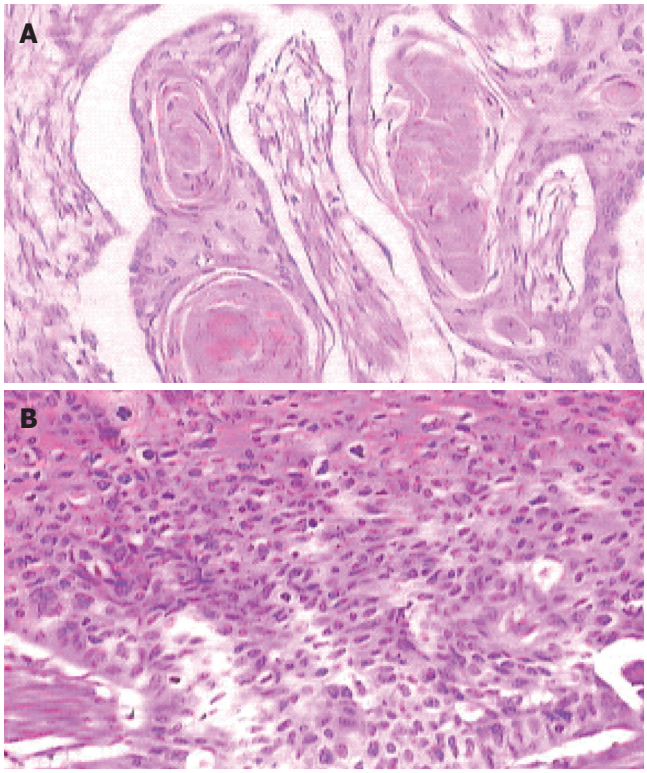


**Figure 1** Histological types of primary ESCC (HE × 400). **A:** Well-differentiated ESCC; **B:** Poorly-differentiated ESCC.

## MATERIALS AND METHODS

### *Blood and tissue*

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

### *DNA extraction and GSTM1 genotyping*

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

### *Statistical analysis*

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

## RESULTS

### *Histological types of primary ESCC*

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

### *GSTM1 genetic polymorphisms in ESCC of Kazakh*

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

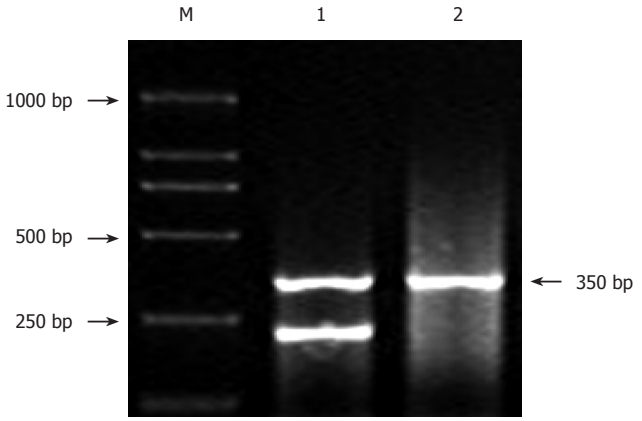


**Figure 2** PCR of the GSTM1 genes. Lane M: DL 2000 DNA molecular weight marker; lane 1: GSTM1 genotype present; lane 2: homozygous deletion of GSTM1.

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

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

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

**Table 1 Frequencies of GSTM1 polymorphisms in three ethnics**

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

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

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

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

## DISCUSSION

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

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

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

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*GASTRIC CANCER*

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

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## Abstract

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

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

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

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



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

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## INTRODUCTION

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

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

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

## MATERIALS AND METHODS

### Subjects

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

### Diagnosis of H pylori infection

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

### ELISA for IgG anti-CagA

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

### HLA-DQ typing

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

### Amplification of genomic DNA

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

### Dot blot hybridization

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

### Oligonucleotide probes

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

### Statistical analysis

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

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

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

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

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

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

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

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

## RESULTS

### Subjects

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

### HLA genotyping in patients with chronic gastritis

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

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

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

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

### HLA genotyping in patients with gastric diffuse-type adenocarcinoma

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

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

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

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

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

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

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

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

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

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

## DISCUSSION

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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## COMMENTS

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

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

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

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

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

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

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*VIRAL HEPATITIS*

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

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## Abstract

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

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

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

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



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

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## INTRODUCTION

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

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

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

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

## MATERIALS AND METHODS

### Patients

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

**Table 1 Characteristics of patients without SVR**

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

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

**Table 2 Characteristics of patients with SVR**

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

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

### Liver stiffness measurement

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

### Serum markers of fibrosis

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

**Table 3 Results of biochemical examination of patients**

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

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

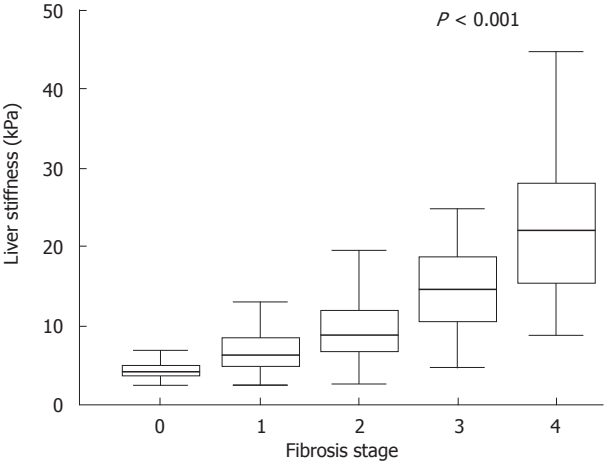


**Figure 1** Liver stiffness measurements for each fibrosis stage. Fibrosis stage 0 is offered to the control group. The top and bottom of the boxes are the 1st and 3rd quartiles. The length of the box thus represents the interquartile range (IQR) within which 50% of values are located. The lines through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

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

### Statistical analysis

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

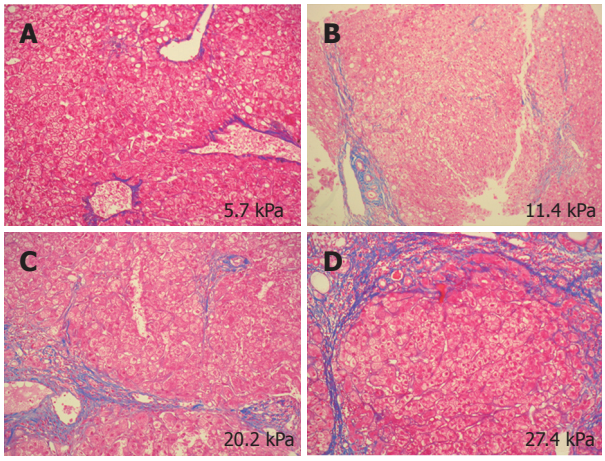


**Figure 2** Azan-Mallory-stained liver tissue and liver stiffness measurement. **A** and **B:** stage 1 liver tissue samples, showing that the grade of fibrosis and elasticity was higher for **B** than for **A; C**: stage 3 liver tissue sample; **D**: stage 4 liver tissue sample. The elasticity thus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

## RESULTS

### Biochemistry

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

### Relationship between the histological stage and the liver stiffness

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

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

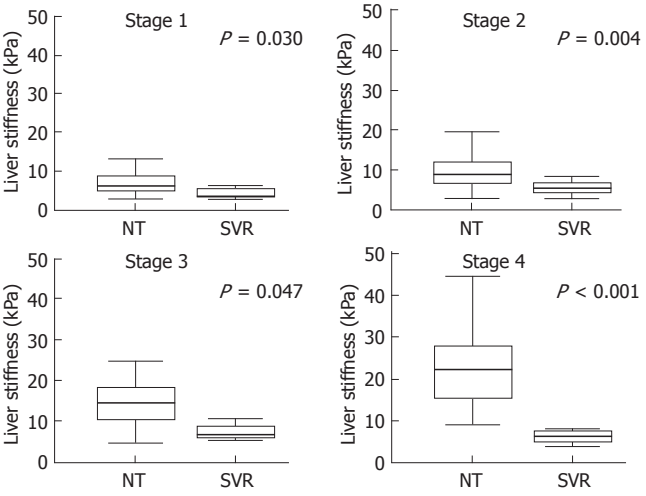


**Figure 3** Liver stiffness measurements for each fibrosis stage in the patients not treated with IFN and the patients with sustained response for IFN therapy. Fibrosis stage 0 indicates the control group. The top and bottom of the boxes are the 1st and 3rd quartiles. The length of the box thus represents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

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

### *Correlations with serum markers of fibrosis*

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

## DISCUSSION

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

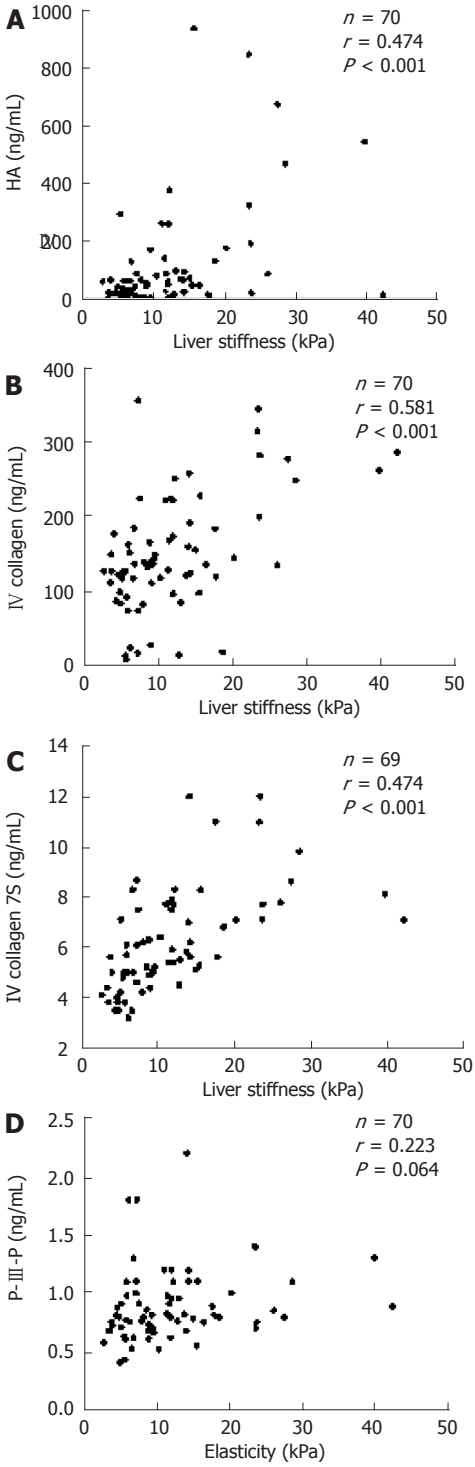


**Figure 4** Correlation between liver stiffness and serum fibrosis markers. Correlations between elasticity measured by the Fibroscan502 and HA (**A**), type Ⅳ collagen (**B**), Ⅳ collagen 7S (**C**), and P-Ⅲ-P (**D**) are indicated. **HA: Hyaluronic acid; P-Ⅲ-P: Type Ⅲ procollagen N peptide.**

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

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

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

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

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## Abstract

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

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

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

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



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

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## INTRODUCTION

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

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

## MATERIALS AND METHODS

### Materials

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

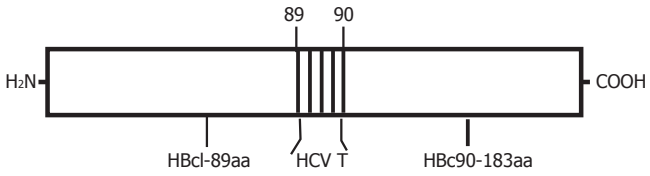


**Figure 1** Hybrid HBcAg-T protein showing HCV T epitope (hatched box) inserted between HBcAg aa 79 and 80, a region located at the tip of core particle surface spikes.

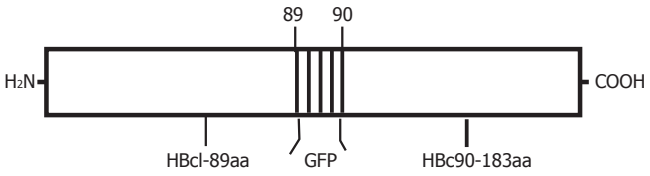


**Figure 3** Hybrid HBcAg-GFP protein showing the GFP protein (hatched box) inserted between HBcAg aa 79 and 80.

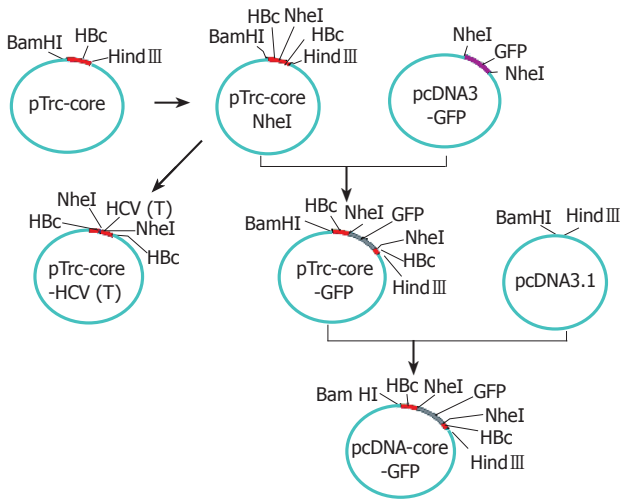


**Figure 2** The construction of plasmids pTrc-core-HCV (T) and pcDNA3.1-core-GFP.

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

### Construction of expression vectors

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

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

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

### Expression and purification of recombinant particles

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

### Immunogenicity

BALB/c mice were obtained from Jilin University. Mice

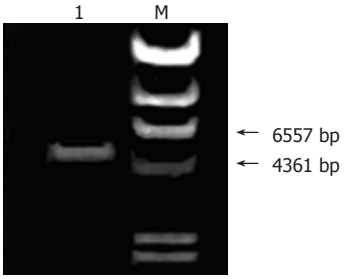


**Figure 4** Plasmid pTrc-core$^{NheI}$ identified by restriction endonuclease NheI cleavage. Land 1: pTrc-core$^{NheI}$ digest; M: λ-Hind Ⅲ digest.

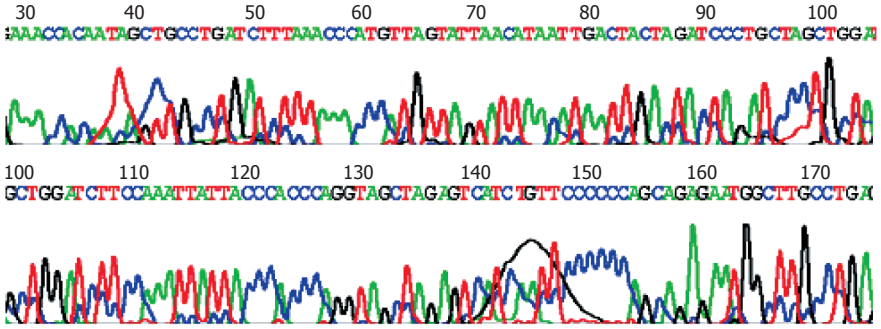


**Figure 5** The sequencing of plasmid pTrc-core$^{NheI}$ PCR product.

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

### *Serological assays*

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

### *T lymphocyte subgroup* assays

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

### *Cellular proliferation assays*

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

## RESULTS

### *Identification of plasmid pTrc-core$^{NheI}$*

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

### *GFP expression*

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

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

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

### *Assay of recombination protein*

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

### *Results of tumor regression trial*

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

### *Results of HBcAb titer*

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

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

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

### *Results of MTT*

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

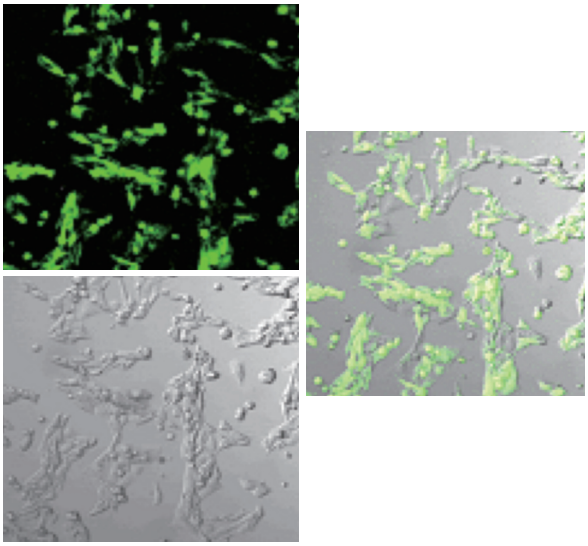

**Figure 6** pcDNA3.1-HBc-GFP expression in Hela cell line (confocal microscopy, 300 ×).

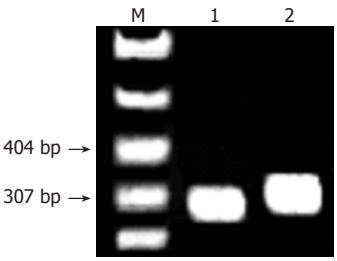


**Figure 7** PCR products of pTrc-core-HCV(T). Lanes 1-2: pTrc-core$^{NheI}$ or pTrc-core-HCV (T) PCR product; M: pBR322 DNA-Msp I digest.

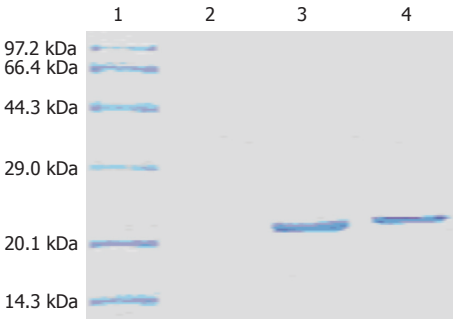


**Figure 8** The results of HBcAg-T and HBcAg by SDS-PAGE. 1: Marker; 2: Negative; 3: HBcAg; 4: HBcAg-T.


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

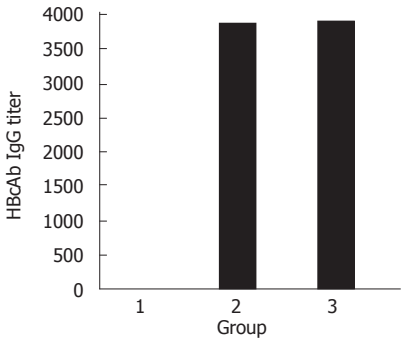


**Figure 10** The titer of HBcAb. 1: Vacuity team; 2: HBcAg immunized team; 3: HBcAg-T immunized team.

## DISCUSSION

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

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

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

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

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

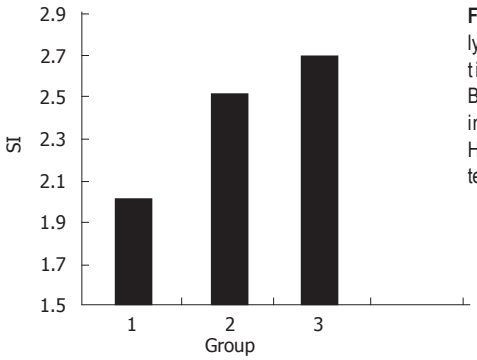


**Figure 11** Nonspecific lymphocytes proliferative response. 1: Blank team; 2: HBcAg immunized team; 3: HBcAg-T immunized team.

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

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

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*CLINICAL RESEARCH*

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

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## Abstract

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

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

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

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



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

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

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## INTRODUCTION

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

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

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

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

## MATERIALS AND METHODS

### The RUGBE initiative and data collection

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

### Patient population

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

### Study variables

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

### Validation of the Rockall scoring system in the Canadian population

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

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

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

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

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

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

## RESULTS

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

### *Calibration of the Rockall scoring system*

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

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

### *Discriminative ability of the Rockall scoring system*

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

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

## DISCUSSION

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

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

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

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

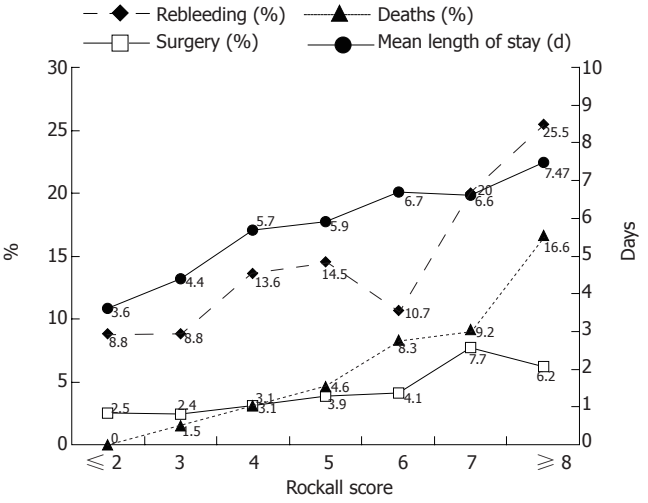


**Figure 1** Clinical outcomes by level of the Rockall risk scoring system.

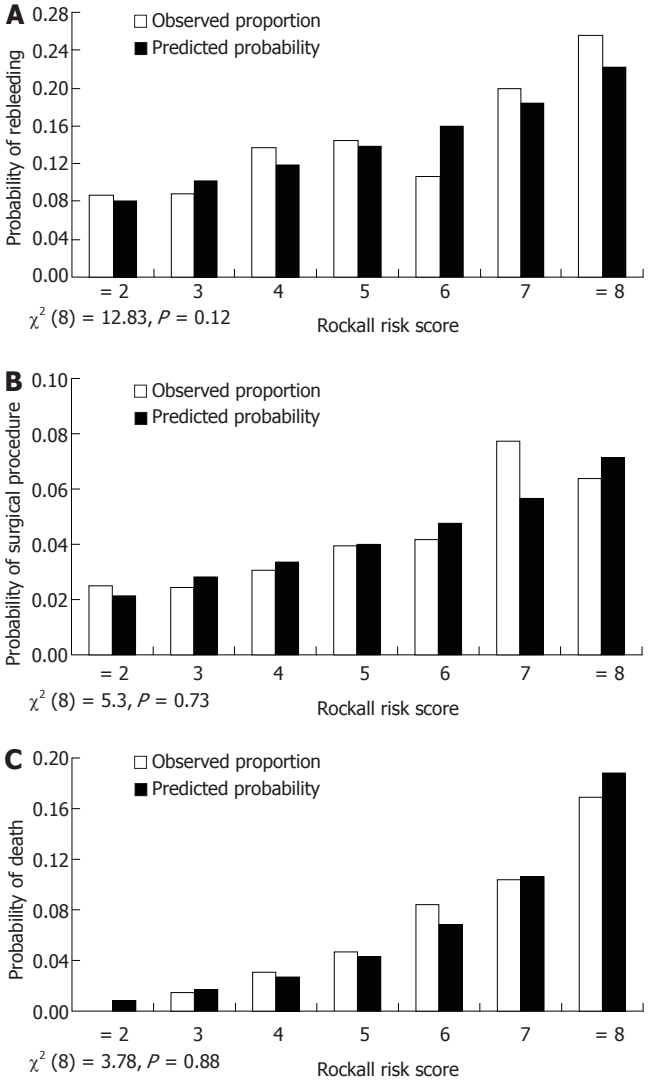


**Figure 2** Predicted versus observed outcomes by Rockall risk core. **A**: Rebleeding; **B**: Surgical procedures; **C**: Death.

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

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

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

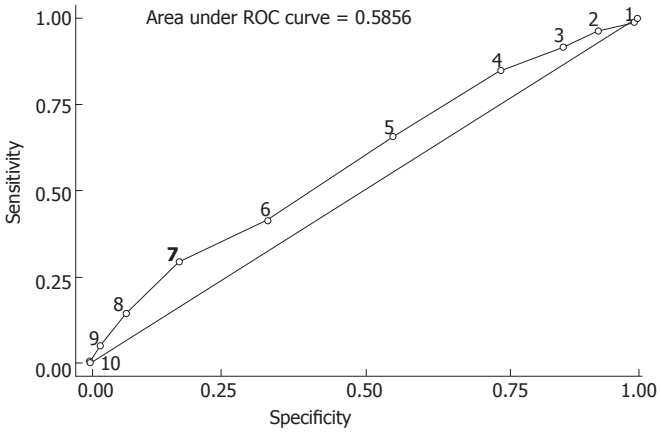


**Figure 3** Receiver operating characteristic (ROC) curve for outcome of rebleeding. Numbers along the curve indicate Rockall risk score cutoff values; The optimal threshold is a Rockall score of 7 (in bold).

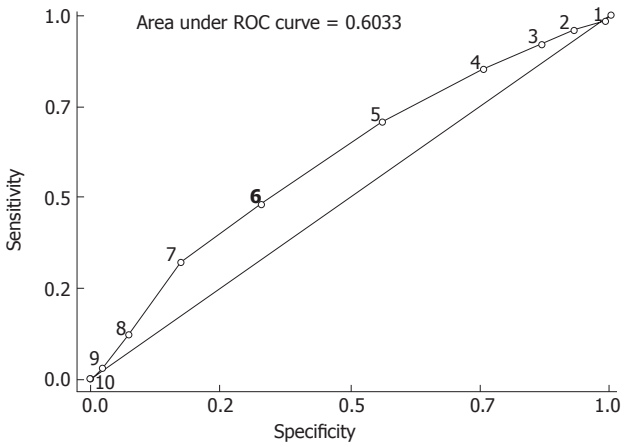


**Figure 4** Receiver operating characteristic (ROC) curve for outcome of surgical procedure. Numbers along the curve indicate Rockall risk score cutoff values; The optimal threshold is a Rockall score of 6 (in bold).

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

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

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

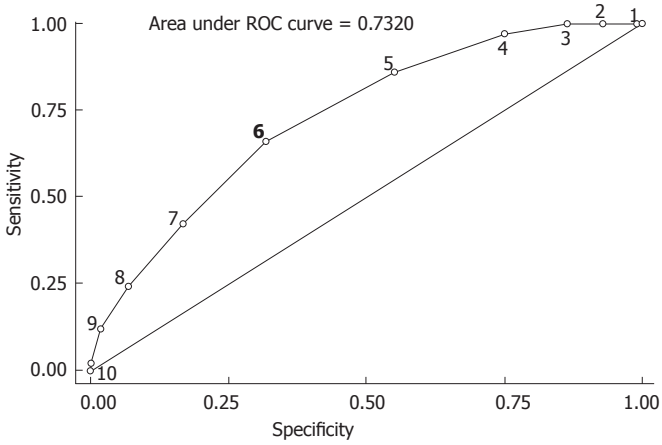


**Figure 5** Receiver operating characteristic (ROC) curve for outcome of death. Numbers along the curve indicate Rockall risk score cutoff values; The optimal threshold is a Rockall score of 6 (in bold).

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

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

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

contribute to the extended length of hospital stay.

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

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

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

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*CLINICAL RESEARCH*

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

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## Abstract

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

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

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

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



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

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## INTRODUCTION

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

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

## MATERIALS AND METHODS

### Study design and population

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

### Data collection

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

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

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

### Statistical analysis

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

## RESULTS

### Characteristics of the study population

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

**Table 1 Baseline data of chronic liver disease and normal groups**

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

[1]Incomplete data.

**Table 2 Variables by severity of the liver diseases**

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

[1]Incomplete data.

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

### *Association of variables and disease severity*

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

### *The effect of disease severity on HRQL by univariate analysis*

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

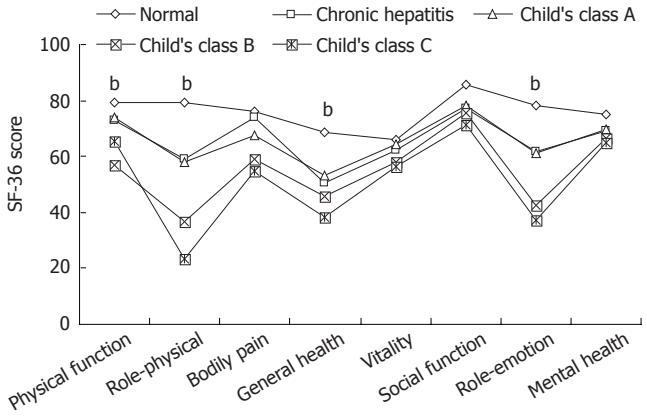


**Figure 1** The domain scores of short-form 36 (SF-36) by disease severity. $^{b}P < 0.001$ *vs* normal group.

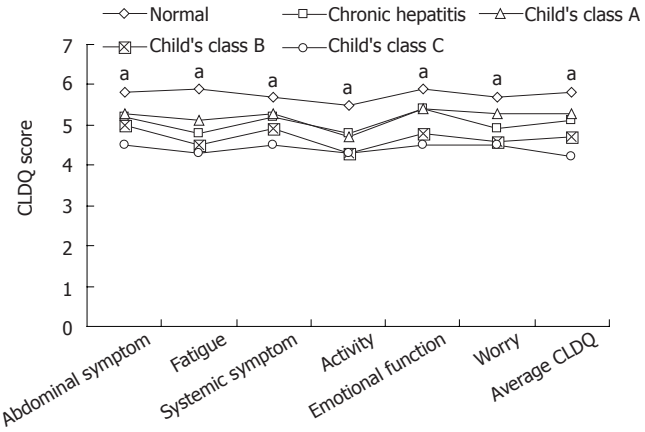


**Figure 2** The domain scores of chronic liver disease questionnaire (CLDQ) by disease severity. $^{a}P < 0.03$ *vs* normal group.

**Table 3 Variables affecting SF-36 domains[1]**

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

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

**Table 4 Variables affecting CLDQ domains[1]**

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

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

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

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

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

## DISCUSSION

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

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

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## COMMENTS

### *Background*

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

### *Research frontiers*

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

### *Innovations and breakthroughs*

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

### *Applications*

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

### *Terminology*

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

### *Peer review*

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

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*CLINICAL RESEARCH*

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

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## Abstract

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

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

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

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

## INTRODUCTION

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

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

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

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

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

## MATERIALS AND METHODS

### Patients

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

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

### Methods

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

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

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

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

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

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

## RESULTS

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

### QOL in liver cirrhosis patients and in the control group

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

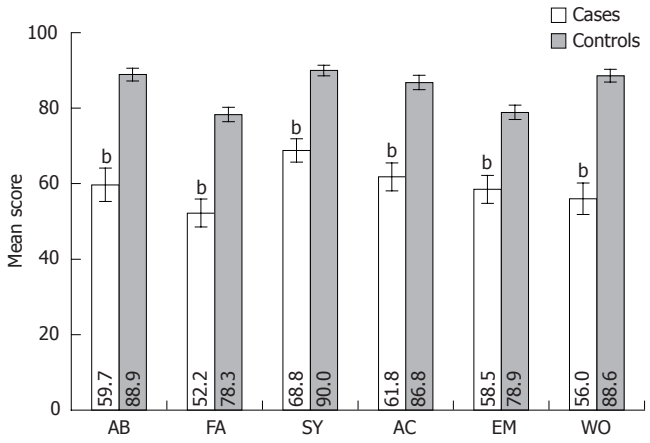


**Figure 1** Chronic liver disease questionnaire (CLDQ) scale score differences in six quality of life (QOL) domains in case and control groups. Error bars indicate 95% confidence interval. AB: Abdominal symptoms; FA: Fatigue; SY: Systemic symptoms; AC: Activity; EM: Emotional function; WO: Worry. NS: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

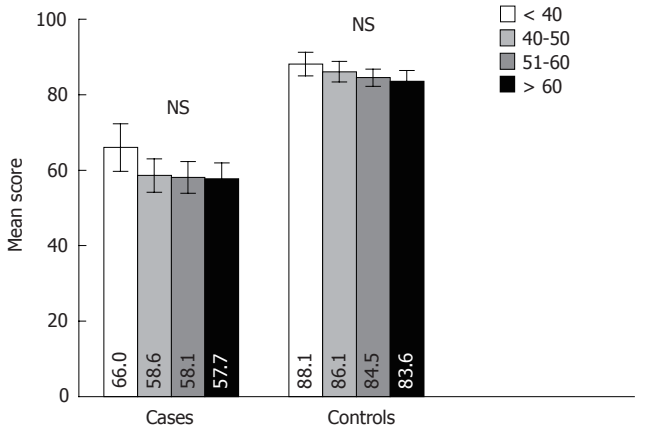


**Figure 2** Chronic liver disease questionnaire (CLDQ) scale score comparison between the age groups in case and control groups. Error bars indicate 95% confidence interval. NS: Not significant.

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

### *Age and QOL*

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

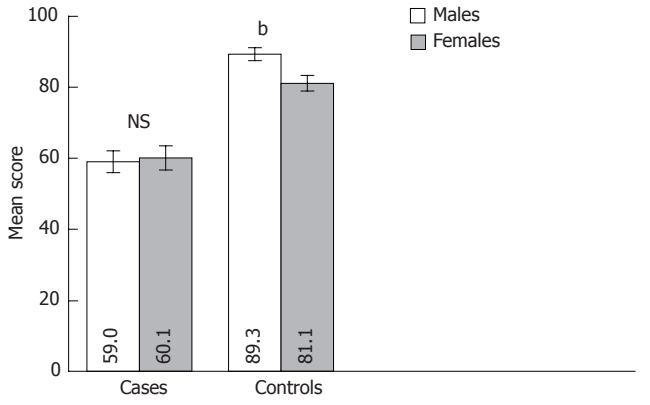


**Figure 3** Chronic liver disease questionnaire (CLDQ) scale score comparison between the genders in case and control groups. Error bars indicate 95% confidence interval. NS: Not significant. $^{b}P < 0.001$ *vs* females in control group.

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

### *Gender and QOL*

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

### *Type of liver cirrhosis and QOL*

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

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

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

## DISCUSSION

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

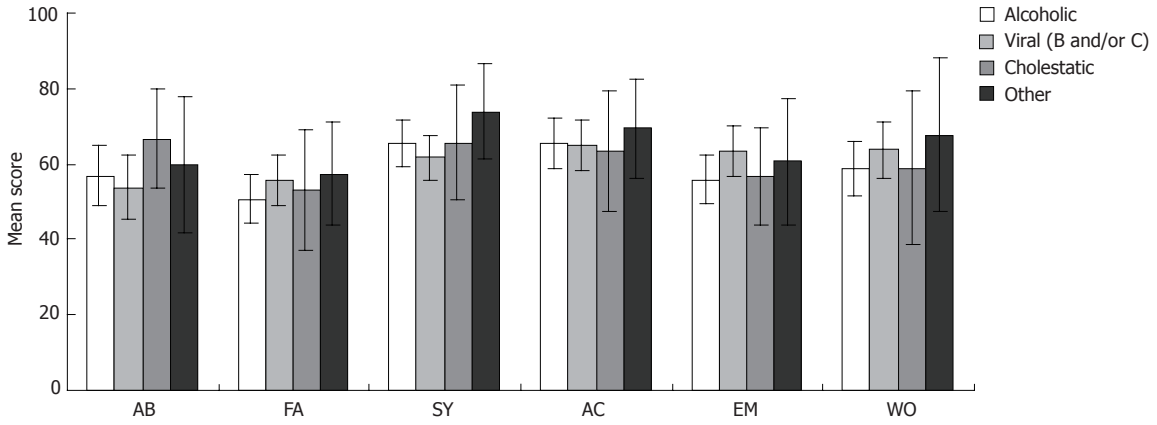


**Figure 4** Chronic liver disease questionnaire (CLDQ) scale score comparison in six quality of life (QOL) domains by the etiology in liver cirrhosis patients. Error bars indicate 95% confidence interval. AB: Abdominal symptoms; FA: Fatigue; SY: Systemic symptoms; AC: Activity; EM: Emotional function;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

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

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

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

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

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

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

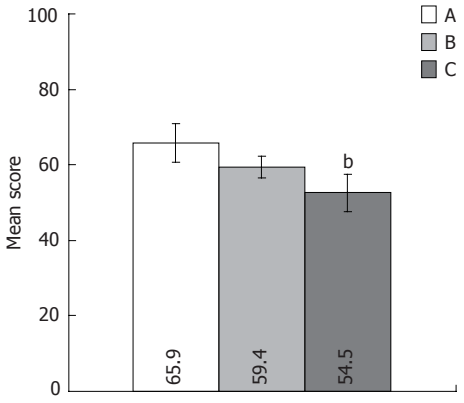


**Figure 5** Chronic liver disease questionnaire (CLDQ) scale score comparison according the disease severity in the case group. Error bars indicate 95% confidence interval. A, B and C represent Child-Pugh class A, B and C, respectively. $^{b}P < 0.01$ *vs* A Child-Pugh class.

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

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

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

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

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

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

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*CLINICAL RESEARCH*

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

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## Abstract

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

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

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

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



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

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## INTRODUCTION

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

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

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

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

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

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

## MATERIALS AND METHODS

### *Clinical material*

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

### *Diagnostic criteria for refractory cirrhotic ascites*

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

### *Exclusion criteria*

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

### *Therapeutic methods*

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

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

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

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

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

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

### Group treatment

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

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

### Treatment procedures

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

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

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

### Observational methods

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

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

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

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

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

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

**Table 2 Changes of azotemia (mean ± SD)**

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

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

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

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

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

### *Efficacy evaluation standards*

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

### *Statistic analysis*

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

## RESULTS

### *General efficacy for ascites in all groups*

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

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

### *Changes of azotemia*

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

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

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

### *Levels of endotoxin, NO, and ET*

Endotoxin content decreased in the comprehensive

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

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

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

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

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

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

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

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

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

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

### *Comparison of blood flow in portal and splenic veins*

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

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

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

### *Adverse reactions and follow-up*

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

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

## DISCUSSION

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

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

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

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

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

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

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

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

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

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

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

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*RAPID COMMUNICATION*

# An algorithm for family screening for coeliac disease

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## Abstract

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

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

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

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



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

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## INTRODUCTION

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

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

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

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

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

**Table 1 Sensitivity, specificity and reproducibility of screening coeliac serology**

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

## MATERIALS AND METHODS

### Study subjects

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

### Controls

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

### ELISA for anti-tissue transglutaminase antibodies

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

### Indirect immunofluorescence for anti-endomysial antibody

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

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

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

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

### Total IgA quantification

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

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

### HLA-DQ typing

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

## RESULTS

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

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

**Table 3 Single affected families-relationships of individuals to probands**

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

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

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

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

### HLA-typing

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

**Table 4 Mutliply affected Families-Relationships of Individuals to probands**

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

### *Follow-up*

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

## DISCUSSION

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

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

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

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

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

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

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

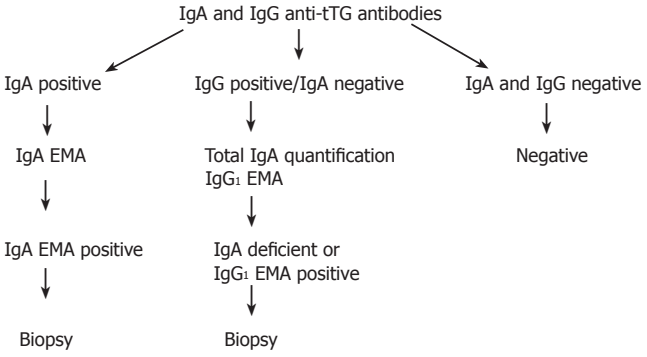


**Figure 1** Algorithm for familial screening of relatives of probands affected with coeliac disease.

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*RAPID COMMUNICATION*

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

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## Abstract

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

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

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

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



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

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## INTRODUCTION

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

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

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

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

## MATERIALS AND METHODS

### Patients and methods

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

### Statistics analysis

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

**Table 1 Main characteristics of the patients**

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

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

## RESULTS

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

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

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

**Table 2 Previous bleeding and prophylaxis given before the index bleeding**

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

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

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

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

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

## DISCUSSION

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

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

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

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

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

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

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

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

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

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

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## COMMENTS

### Background

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

### Research frontiers

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

### Innovations and breakthroughs

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

### Applications

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

### Peer review

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

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*RAPID COMMUNICATION*

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

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## Abstract

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

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

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

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



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

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## INTRODUCTION

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

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

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

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

## MATERIALS AND METHODS

### Patients and controls

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

### Determination of H pylori infection and CagA status

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

### Determination of anti-Hsp60 antibodies

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

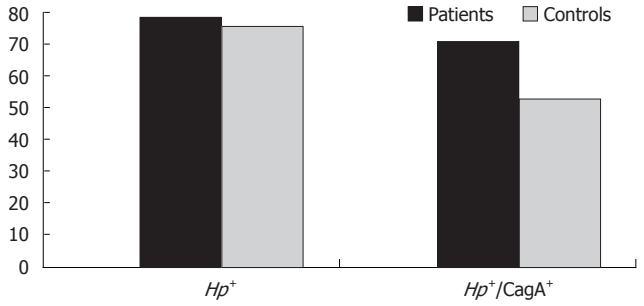


**Figure 1** Prevalence of overall *H pylori* and CagA+ *H pylori* infection in patients and controls.

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

### Determination of other pathogens

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

### Statistical analysis

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

## RESULTS

### Overall prevalence of H pylori infection

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

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

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

### Determination of CagA status in infected patients and controls

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

### Prevalence of infections by pathogens other than H pylori

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

### Determination of anti-Hsp60 antibodies in patients and controls

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

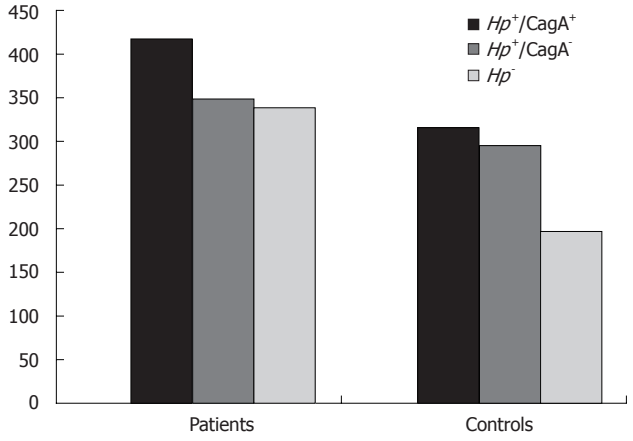


**Figure 2** Mean levels (in optical density) of anti-Hsp60 systemic antibodies in patients and controls.

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

## DISCUSSION

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

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

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

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

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*RAPID COMMUNICATION*

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

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## Abstract

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

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

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

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



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

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## INTRODUCTION

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

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

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

features.

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

## MATERIALS AND METHODS

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

### Patients

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

### Evaluation of glucose metabolism and insulin sensitivity

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

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

### Liver histology

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

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

### Statistical analysis

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

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

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

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

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

## RESULTS

### Patient characteristics

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

### Glucose metabolism

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

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

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

### Liver histology

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

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

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

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

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

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

BMI-SDS: body mass index standard deviation score; AST: aspartate aminotransferase; ALT: alanine aminotransferase; PLT: platelets count; PT: prothrombin time; HOMA-IR: homeostatic model assessment-insulin resistence; ISI: insulin sensitivity index.

## DISCUSSION

Over the last decades, the prevalence of overweight and obesity among children has increased dramatically, becoming an important public health problem. The negative consequences of paediatric overweight can occur during childhood or adulthood and may result in metabolic, physical, psychosocial and economic consequences. Among these, non alcoholic fatty liver disease has become a growing clinical problem and a major cause of liver-related morbidity.

The accurate means to distinguish between simple fatty liver and steatohepatitis is a liver biopsy as NASH is diagnosed when tissue histology shows fat along with inflammation and damage to liver cells. This distinction has a prognostic relevance because whereas NAFLD is a benign nonprogressive condition, NASH can progress to cirrhosis, liver failure and hepatocellular carcinoma[15]. However, the need for histological evaluation of NAFLD remains controversial as no efficacious treatment strategies have been yet designed. Persistent hypertransaminasemia of non-alcoholic steatohepatitis in obese children may resolve after weight reduction and high doses of vitamin E treatment, but the impact on the natural history needs to be validated by large-cohort controlled studies[16-19]. Thereby, the search for non invasive diagnostic tests of liver fibrotic deposition should be preferred to the expensive and invasive procedure of liver biopsy, whereas the latter should be limited to those cases with uncertain diagnosis[7].

Our previous validation study regarding non invasive diagnosis of liver fibrosis in adults with chronic hepatitis C to cirrhosis showed a significant inverse correlation between stage of fibrosis and platelet count with the highest platelets count in patients with fibrosis 0-2, lower in those with grade 3 and lowest in those with grade 4[20]. Also, an inverse correlation between spleen size and platelets count has been observed ($r$ = - 0.54, $P < 0.0001$) and thrombocytopenia presents in 71% and 23% of patients with or without splenomegaly respectively[21]. The accuracy of platelet count was not significant in children ($P$ = 0.5) as most of the children with NAFLD had stage 1 of fibrosis (52.2%), and less than 8% had a fibrotic score ⩾ 2. The amount of scar deposition that characterizes these stages of fibrosis is too low to favour hemodynamic changes with portal hypertension, altered production of thrombopoietin and enlarged spleen sequestration.

At multivariate analysis of baseline clinical and biochemical parameters in the present study, BMI was an independent predictor of fibrosis (OR = 5.85, 95% CI = 1.6-21); at the cut off value of 26.3 kg/m$^2$, the BMI evaluation showed a sensibility and specificity of 66% and 71%, respectively, in ascertaining the presence of fibrosis in children. Conceivably, the BMI evaluation may be useful in picking up those children at higher risk of disease progression. Future studies investigating the natural history and the long-term sequelae of our histological findings in children are warranted to corroborate our claim.

In conclusion, increased BMI appears to correlate with long term progression to fibrosis and cirrhosis. Reversal of obesity with a gradual weight reduction can improve laboratory abnormalities, histologic changes and liver size in children with NAFLD. BMI may be considered a good non invasive indicator of the underlying disease. Although little controversy exists about the role of liver biopsy as the best accurate method available to assess the stage of the disease, the decision to perform it should be weighed against the risk of the procedure and the impact of the information obtained. In particular, in paediatric population, the timing of biopsy should be individualized and postponed to non-invasive diagnostic tools.

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## COMMENTS

### Background
Interest is growing regarding the nonalcoholic steatohepatitis (NASH) in pediatric age and in the usefulness of liver biopsy for its detection.

### Research frontiers
Non invasive detection of liver fibrosis may help better define new therapeutical approaches in a larger number of pediatric NASHs.

### Innovations and breakthroughs
Still new diagnostic parameters for non invasive detection of liver fibrosis need to be searched and validated in paedriatic population. This work proves that BMI helps identify children with NASH who might have fibrotic deposition in the liver.

### Applications
BMI as one of the future criteria can be used for detection of pedriatic patients with NASH-fibrosis.

### Peer review
This study tested the accuracy of various clinical and biochemical parameters for the diagnosis of NASH in 69 children. The results show that only increased BMI is significantly associated with fibrosis, whereas other parameters have not been identified as predictive. This study is accurate and is of importance due to the absence of any non invasive method for the diagnosis of liver fibrosis in the paediatric population. While being interesting, this observation should be further confirmed.

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*RAPID COMMUNICATION*

# Insulin sensitizers in treatment of nonalcoholic fatty liver disease: Systematic review

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## Abstract

**AIM:** To summarize the evidence available for the clinical effectiveness of insulin sensitizers in the treatment of nonalcoholic fatty liver disease (NAFLD) systematically.

**METHODS:** Relevant articles were located using computer-assisted searches of Medline (1966-March 2006), EMBASE (1988-March 2006), CINAHL (1982-March 2003), Educational Resource Information Center (1966-March 2006), Library, Information Science & Technology Abstracts (1967-March 2006), Cochrane Database of Systematic Reviews, Database of Abstracts of Reviews of Effects (1994-2006), dissertations in ProQuest and FirstSearch databases. Manual searches were made in the abstracts from meetings of the American Gastroenterological Association (1999-2006), and the American Association for the Study of Liver Diseases (2003-2005). Studies were retrieved using the following selection criteria: (1) clinical trials using insulin sensitizers in subjects with NAFLD, (2) adult patients, (3) published as full manuscripts or abstracts, and (4) English, Spanish, German, and French languages only. Data were abstracted independently by two reviewers following standardized procedures. A face-to-face comparison of data was conducted to ensure the completeness and reliability of the abstraction process.

**RESULTS:** Nine studies were included, six using metformin and three using thiazolidinediones. Only two studies were placebo-controlled trials. The median sample size for all studies was 18 subjects. In the placebo-controlled trials, metformin improved insulin resistance markers and liver function tests, but not histological scores. In the single-arm trials, metformin and thiazolidinediones improved insulin resistance markers and liver function tests, and beneficial histological changes were reported. There is limited high-quality information available from which to draw categorical conclusions about the clinical use of insulin sensitizers in NAFLD.

**CONCLUSION:** Current information indicates that the use of insulin sensitizers in NAFLD improves insulin resistance and liver function. Histological changes must be corroborated in randomized controlled trials.



**Key words**: Diet; Metformin; Rosiglitazone; Pioglitazone; Fatty liver; Steatohepatitis; Evidence based medicine; Systematic review

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## INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) is an increasingly recognized condition that may progress to end-stage liver disease, ranging from simple steatosis to steatohepatitis, advanced fibrosis, and cirrhosis (in 1.6% of patients with NAFLD). The pathological picture resembles that of alcohol-induced liver injury, but it occurs in patients who do not abuse alcohol[1]. The true prevalence of NAFLD in the USA is unknown. Based on the percentage of people in the Third National Health and Nutrition Examination Survey (NHANES-Ⅲ) with unexplained elevated levels of serum aminotransferase, up to 7.3% of the USA population could be suffering from NAFLD[2]. When the diagnostic criteria are modified, the estimated prevalence of NAFLD reaches 24%[3]. According to

Byron *et al*[4], NAFLD is the third most common diagnosis in gastroenterological referrals, accounting for 11% of patients. NAFLD is expected to become one of the most important liver diseases in the near future as a result of the obesity epidemic[5].

NAFLD was first described more than 20 years ago[1] and many advances in our understanding of its pathophysiological mechanisms have been made. Diet and exercise constitute the central strategies in NAFLD treatment[6]. Considering the pathogenic mechanisms that may be involved, several pharmacological strategies for NAFLD have been tested that focus on correcting the risk factors for insulin resistance and decreasing hyperinsulinemia, as hepatoprotective effects, using diverse drugs: gemfibrozil, metformin, betaine, *N*-acetylcysteine, and vitamin E[7]. However, no consensus regarding an effective therapy for NAFLD has been reached[8].

Over the last five years, clinical trials evaluating the use of insulin sensitizers in the treatment of NAFLD, such as metformin and thiazolidinediones, have been conducted. Mixed results, heterogeneous therapeutic approaches, and the small numbers of subjects have limited their application as clinical guidelines. We performed a comprehensive systematic review to summarize the evidence available for the clinical effectiveness of insulin sensitizers in the treatment of NAFLD.

## MATERIALS AND METHODS

### Search strategy

Relevant articles were located using computer-assisted searches of Medline (1966-March 2006), EMBASE (1988-March 2006), Cumulative Index to Nursing & Allied Health Literature (CINAHL) (1982-March 2003), Educational Resource Information Center (ERIC) (1966-March 2006), Library, Information Science & Technology Abstracts (LISTA) (1967-March 2006), Cochrane Database of Systematic Reviews (CDSR), Cochrane Controlled Trials Register (CCTR), Database of Abstracts of Reviews of Effects (DARE) (1994-2006), dissertations in ProQuest and FirstSearch databases, and Literatura Latinoamericana y del Caribe en Ciencias de la Salud (LILACS). Manual searches were conducted in the abstracts from the Digestive Disease Week of the American Gastroenterological Association (1999-2006), the American Association for the Study of Liver Diseases Meetings (2003-2005), and the reference lists included in the retrieved articles. Searching terms included: nonalcoholic fatty liver disease, nonalcoholic steatohepatitis, hepatic steatosis, treatment, clinical trial, metformin, thiazolidinediones, rosiglitazone, troglitazone, pioglitazone, englitazone, PPAR-gamma, peroxisomal proliferator activated receptor.

### Study selection criteria

Two investigators (ChN, BT) independently reviewed the titles and abstracts of all the citations identified. Potentially relevant studies were retrieved based on the following selection criteria: (1) clinical trials using one or a combination of insulin sensitizers (metformin and thiazolidinediones: rosiglitazone, troglitazone, pioglitazone, englitazone) in subjects with NAFLD, (2) adult patients, (3) data published in full manuscript form or abstract form, and (4) English, Spanish, German, and French languages only.

### Eligibility and data abstraction

After retrieval, the articles were subject to evaluation to ensure their compliance with the inclusion and exclusion criteria considered for data abstraction. The exclusion criteria for data abstraction from the selected studies were: (1) use of a concomitant therapeutic approach (ursodeoxycholic acid, antioxidants, *etc*.) with insulin sensitizers, and (2) less than 10 participants at the beginning of the study. Inclusion criteria were: (1) controlled trials of insulin sensitizers versus placebo or diet, (2) single-arm studies of rosiglitazone, troglitazone, pioglitazone, or englitazone, and (3) NAFLD or nonalcoholic steatohepatitis (NASH) based on histological diagnosis or imaging studies (computed tomography, abdominal ultrasound, or magnetic resonance imaging) and/or aberrant liver function tests in the absence of alcohol consumption. Unblinded evaluation of the inclusion/exclusion criteria was conducted separately by all authors. Discrepancies in selection were resolved by consensus. Checks for repeated references were conducted based on the authors' names, publication dates, and reported population characteristics. Data abstraction was conducted independently by ChN and BT following the standardized procedures developed by the research team. The criteria included: publication and study characteristics, study population, diagnostic criteria, intervention description, and baseline and postintervention clinical characteristics. In cases where the information to be abstracted was not presented in the published reports, the authors attempted to contact the corresponding authors (Blaszyk, Azuma, and Duseja), and the responses (Duseja) were included in the abstraction formats. After all studies were abstracted, a face-to-face comparison of data retrieved by ChN and BT was conducted to ensure the completeness and reliability of the abstraction process. Minor discrepancies were recorded and resolved by referring to the original paper.

## RESULTS

A total of 94 studies were retrieved from the broad search terms used (Figure 1). After elimination of editorials, reviews, and repeated reports, 10 studies[9-18] remained candidates for evaluation. One study was excluded because of concomitant use of vitamin E[18] (Table 1). Heterogeneity of treatments, methodologies, and reporting quality in the studies precluded any attempt to estimate summary measures, forcing a narrative presentation of our findings. All studies were classified into one of three groups based on the characteristics of the pharmacological interventions: metformin *vs* diet trials, metformin single-arm trials, and thiazolidinedione single-arm trials.

Most (77%) studies were designed as single-arm trials, one was designed as a randomized controlled trial, and one as a nonrandomized controlled trial. Sample sizes ranged

**Table 1 Details of trials eligible for this systematic review**

| Ref. | Design | Participants | Intervention | Outcomes | | |
|---|---|---|---|---|---|---|
| | | | | IR | Biochemical | Histological |
| [9] | Nonrandomized open-label controlled | Italy. 20 consecutive subjects. No diabetic or severely obese subjects. | Treatment: metformin 500 mg/d for 4 mo Control: diet | + | + | NE |
| [10] | Randomized open-label controlled | Turkey. 36 nondiabetic subjects. | Treatment: metformin 1.7 g/d for 6 mo Control: diet (1600–1800 cal/d) | + | + | – |
| [11] | Nonrandomized open-label single-arm trial | USA. 15 subjects. One diabetic. | Metformin 20 mg/kg per day for 48 wk | + at 3 mo + at end of study | + at 3 mo –(+) at end of study | +[2] |
| [12] | Nonrandomized open-label single-arm trial | USA. 10 subjects. | Metformin 2 g/d for 48 wk | - | - | - |
| [13] | Randomized controlled trial[1] | Italy. 17 subjects. | Metformin 2 g/d for 48 wk | + | + | + |
| [14] | Nonrandomized open-label single-arm trial | India. 22 subjects. Three diabetics. | Metformin 1.5 g/d for 6 mo | + | + | NE |
| [15] | Nonrandomized open-label single-arm trial | USA. 25 subjects. | Rosiglitazone 8 mg/d for 48 wk plus diet and physical activity | + | + | + |
| [16] | Nonrandomized open-label single-arm trial | USA. 18 nondiabetic subjects. | Pioglitazone 30 mg/d for 48 wk | + | + | + |
| [17] | Nonrandomized open-label single-arm trial | Japan. 12 subjects. | Pioglitazone 30 mg/d for 12 wk | + | + | NE |

IR: Insulin resistance; NE: Not evaluated. [1]In this study, metformin was compared with vitamin E. We have included only the metformin-treated group, with biopsies at baseline and at the end of the study; [2]Hypothesis test was not provided.

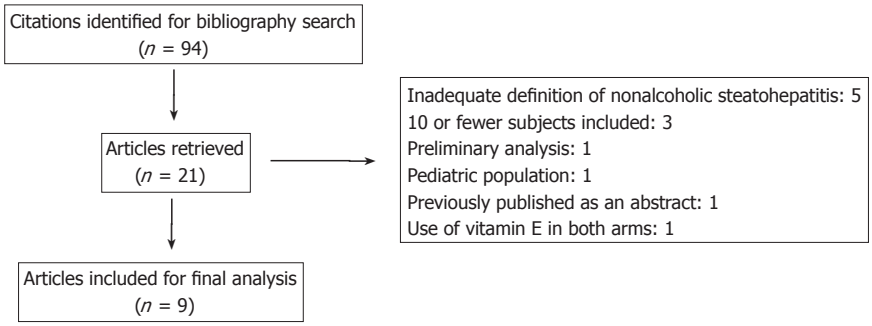


**Figure 1** Literature search and selection.

from 10 to 36 subjects, with a median of 18 subjects. All studies used a histological diagnosis of NASH as the inclusion criterion, and posttreatment biopsies were available in only six studies.

### *Description of the studies*

**Metformin versus diet trials**: Two studies compared the efficacy of metformin versus diet in the treatment of NAFLD. Marchesini *et al*[9] studied 20 consecutive patients (no diabetic or severely obese subjects were included) with liver function tests, and tests for insulin and insulin resistance (by euglycemia and a hyperinsulinemic glucose clamp). Liver biopsies were conducted in 14 subjects who received metformin (500 mg tid) and six were treated with diet alone for four months. The only significant difference between the two groups was in their alanine aminotransferase values. Histological improvement was not evaluated. The diet group did not differ from the drug group in weight reduction, which could reflect the effect of metformin. The most common adverse effect was gastrointestinal. Although subjects undergoing active treatment showed increased levels of lactic acid (by 30% in actively treated patients), just one patient was above the normal range of 2 mmol/L (2.2 mmol/L).

Uygun *et al*[10] studied 36 patients with NAFLD. The treatment group received metformin (850 mg bid) plus dietary treatment. The control group received only a restricted diet (1600-1800 calories per day). Compared with the controls, the treatment group showed improvements in: alanine aminotransferase (37.1 *vs* 17.4 U/L, respectively, $P = 0.003$), aspartate aminotransferase (22.1 *vs* 6.8 U/L, respectively, $P = 0.0001$), body mass

**Table 2 Characteristics of single-arm metformin studies**

| Publication characteristics | | | Study design | | | | Intervention | | | Diagnosis | Demographics | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| First author | Year | Publication type | Study design | Randomization | Blinding | Sample size | Drug | Doses reported | Treatment duration | NASH | Male (%) | Age (yr) | Obese (%) | Diabetics (%) |
| Nair[11] | 2004 | Article | Open label | N | N | 15 | Metformin | 20 mg/kg/d | 48 wk | Histology | 53 | 51 | NR | 6 |
| Blaszyk[12] | 2005 | Abstract | Open label | N | N | 70 | Metformin | 2 g/d | 48 wk | Histology | 70 | NR | NR | NR |
| Bugianesi[13] | 2005 | Article | RCT[1] | Y | N | 17 | Metformin | 2 g/d | 48 wk | Histology | 83 | 44 | 22–38 | 0 |
| Duseja[14] | 2006 | Article | Open label | N | N | 17 | Metformin | 1500 mg/d | 24 wk | Histology | 68 | 36 | NR | 13 |

| Author | AST (U/L) | | | ALT (U/L) | | | Insulin resistance | | | | Biopsies | | NASH score | | | Adverse effects | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Initial | Final | *P* | Initial | Final | *P* | Method | Initial | Final | *P* | Initial (*n*) | Final (*n*) | Initial | Final | *P* | Diarrhea | Lactate increase | Dropout rate | Loss to follow-up |
| Nair | 47 | 41 | NS | 62 | 68 | NS | QUICKI | 0.306 | 0.315 | < 0.05 | 15 | 10 | 1.4 | 1 | NR | 15 | 6 | 1 | 0 |
| Blaszyk | NR | NR | NR | 109 | 82 | NR | HOMA | 11.4 | 5 | NR | 10 | 10 | 2.4 | 2.1 | NS | NR | NR | 0 | 0 |
| Bugianesi | 44 | 25 | < 0.05 | 96 | 36 | < 0.05 | HOMA | 5.8 | 2.8 | 0.0006 | 17 | 17 | 6.5 | 4.4 | < 0.001 | NR | 1 | 0 | 0 |
| Duseja | NR | NR | NR | 124.6 | 74.4 | NR | KITT | 1.42 | 2.03 | NS | 22 | 0 | 12 | NA | NA | 0 | 0 | 0 | 0 |

[1]In this study, metformin was compared with vitamin E. We have included only the metformin-treated group, with biopsies at baseline and at the end of the study. RCT: Randomized controlled trial; NR: Not reported, NS: Not significant; NA: Not applied; QUICKI: Quantitative insulin sensitivity check index; HOMA: Homeostatic model assessment; KITT: K index of insulin tolerance test.

**Table 3 Characteristics of single-arm thiazolidinediones studies**

| Publication characteristics | | | Study design | | | | Intervention | | | Diagnosis | Demographics | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| First author | Year | Publication type | Study design | Randomization | Blinding | Sample size | Drug | Doses reported | Treatment duration | NASH | Male (%) | Age (yr) | Obese (%) | Diabetics (%) |
| Azuma[17] | 2002 | Abstract | Pilot study | N | N | 12[1] | Pioglitazone | 15 mg/d | 12 wk | Histology | 66 | 40 | NR | 0 |
| Neuschwander-Tetri[15] | 2003 | Article | Open label | N | N | 30 | Rosiglitazone | 8 mg/d | 48 wk | Histology | 46 | 45 | 13 | 6 |
| Promrat[16] | 2004 | Article | Pilot study | N | N | 18 | Pioglitazone | 30 mg/d | 48 wk | Histology | 39 | 45 | 61 | 11 |

| Author | AST (U/L) | | | ALT (U/L) | | | Insulin resistance | | | | Biopsies | | NASH score | | | Adverse effects | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Initial | Final | *P* | Initial | Final | *P* | Method | Initial | Final | *P* | Initial (*n*) | Final (*n*) | Initial | Final | *P* | Weight gain | Anemia | Dropout rates | Loss to follow-up |
| Azuma | NR | NR | NA | 110 | 39 | < 0.05 | Insulin | 54 | 30 | < 0.05 | 12 | 0 | NR | NR | NA | NR | NR | 5 | 0 |
| Neuschwander-Tetri | 60 | 34 | < 0.05 | 89 | 41 | < 0.05 | HOMA | 7.5 | 4 | < 0.05 | 26 | 22 | NR | NR | NA | 20 | [2] | 5 | 0 |
| Promrat | 61 | 34 | < 0.05 | 99 | 40 | < 0.05 | HOMA | 4.3 | 2.6 | < 0.05 | 18 | 18 | NR | NR | < 0.05 | 13 | 0 | 1 | 0 |

[1]Only seven patients in the treatment group. Information about the control group is not available; [2]This study reported anemia as an adverse effect, but numerical data are not available. NR: not reported; NA: not applied; HOMA: homeostatic model assessment.

index (2.4 *vs* 1.9 kg/m$^2$, respectively, $P = 0.01$), and index of insulin resistance (1.15% *vs* 0.02%, respectively, $P = 0.001$). In fact, a comparison of the treatment group at baseline and at six months showed improvements in alanine aminotransferase (83.5 ± 24.6 *vs* 46.4 ± 23.3 U/L, respectively, $P = 0.0001$) and aspartate aminotransferase (57.9 ± 17.3 *vs* 35.8 ± 10.5 U/L, respectively, $P = 0.0001$). However, this was also seen in the control group: alanine aminotransferase (72.8 ± 31.2 *vs* 55.4 ± 16.3 U/L, respectively, $P = 0.001$) and aspartate aminotransferase (48.1 ± 26.3 *vs* 41.3 ± 13.5 U/L, respectively, $P = 0.06$). No differences were observed in the liver biopsies of subjects after treatment. No patient discontinued metformin because of a lack of tolerance for the treatment. No patient reported symptoms of hypoglycemia. Four patients complained of gas and bloating and two patients complained of mild to moderate abdominal pain in the first month. However, these complaints did not require cessation of the drug.

**Metformin single-arm trials**: Four single-arm trials evaluated the use of metformin in NAFLD (Table 2)[11-14]. The mean age of the study participants ranged from 36 to 51 years in all but one study[14]. Males were predominant (ranging from 53% to 83%). The doses used in the different series ranged from 20 mg/kg per day (approximately 1.4 g per day in a subject of 70 kg) to 2 g/d. Treatment duration varied from six months[14] to 48 wk. Insulin resistance was assessed by the QUICKI, HOMA, or KITT methods.

All trials reported an improvement in the indices of insulin resistance, three studies[12-14] reported a reduction in liver function test values, and one study reported a nonsignificant increase in these values[11].

In terms of histological improvement, only one report[13] showed statistical differences in inflammation, steatosis, fibrosis, and global evaluations of NASH after treatment. The most common adverse effects were associated with poor gastrointestinal tolerance. One patient had an increase in serum lactate levels that required the patient to withdraw from the study.

**Thiazolidinediones single-arm trials**: The use of thiazolidinediones (pioglitazone, rosiglitazone, and

troglitazone) was evaluated in three studies (Table 3)[15-17]. The mean age in each study was 40-46 years. In one study, men were in the majority[17], and in two studies, diabetic subjects were included[15,16]. Of these studies, two used pioglitazone and one used rosiglitazone, at varying doses (pioglitazone 15-30 mg/d and rosiglitazone 4 mg, bid). The durations of the studies ranged from 12 wk[17] (pioglitazone 15 mg/d) to 48 wk. Two studies assessed insulin resistance with HOMA-IR[15,16] and the other with serum insulin levels[17]. Posttreatment hepatic biopsies were reported in two studies[15,16].

All studies showed significant improvement in insulin resistance. Alanine aminotransferase and aspartate aminotransferase showed significant decreases in all studies. Posttreatment biopsies showed statistically significant improvements with respect to baseline biopsies[15,16]. The adverse effects reported were weight gain, serum lactate increases, bad dreams, and heavy legs. The pooled dropout rate was high, at 11 of 60 subjects. No cases of liver failure were reported.

## DISCUSSION

This systematic review analyzes the clinical use of insulin sensitizers in the treatment of NAFLD. Although it has been more than 20 years since the first description of NAFLD[1], and much progress has been made in understanding its epidemiology and pathophysiology, few advances have been made in its treatment.

In this review, only two clinical studies compared pharmacological treatments with diet treatments. The methodological limitations are clear: the small numbers of subjects, nonrandomization and the lack of blinded measures, and the limited use of histological outcomes.

The fact that limited high-quality information available is interesting, especially because (1) NAFLD is a very common disease, with incidences between 3% in the low-risk population[19] and 93% in high-risk subjects[20,21], (2) subjects have some degree of histologically evident chronic liver damage, and at least 30% have fibrosis at diagnosis[22], (3) it is an important cause of chronic liver failure and adversely affects survival rates, with 7-10-year liver-related mortality rates of 12% to 25%[23], and (4) it is an important factor in cardiovascular-related mortality; in a 10-year prospective study of subjects with NASH or hepatitis C viral infection, the mortality rates were 5.2% *vs* 0.6%, respectively ($P < 0.03$)[24].

Analyzing the usefulness of insulin sensitizers by comparing metformin with thiazolidinediones in single-arm trials suggests that thiazolidinediones are the better option. However, when diet-controlled studies are considered, this conclusion is less clear because, contrary to the single-arm trials, these studies indicate that the use of metformin clearly benefits liver enzymes. Unfortunately, no data from a well-designed head-to-head comparative clinical trial are available to answer this question. In all the studies analyzed, a heterogeneity of drugs and doses was observed, which made it more difficult to evaluate the efficacy of insulin sensitizers in clinical practice.

The evidence presented in this systematic review indicates that the treatment of NAFLD with insulin sensitizers has been, until now, a nebulous field. However, new well-designed trials have been in progress during the preparation of this paper. Four trials using metformin and three studies on thiazolidinediones are in the recruitment phase[25]. Information derived from these studies should help in the clinical management of this disease.

Despite this (and future) information, many issues are not answered: (1) cost-analysis comparing diet and exercise with pharmacological treatment, (2) safety of insulin sensitizers in large samples, and perhaps one of the most important questions that (3) insulin sensitizers only treat one face of the metabolic syndrome and pharmacological approaches to treat all components of the metabolic syndrome sounds too simplistic[26]. This indicates that more creative prevention policy is mandatory.

In conclusion, current information indicates that the use of insulin sensitizers in the treatment of NAFLD improves insulin resistance and liver function. Single-arm studies have shown positive histological changes. However, placebo-controlled trials do not support this histological response. Future information derived from well-designed running trials will be useful in defining the clinical implications of insulin sensitizers in the treatment of NAFLD.

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*RAPID COMMUNICATION*

# Diagnosis and treatment of gallbladder perforation

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## Abstract

**AIM:** To present our clinical experience with gallbladder perforation cases.

**METHODS:** Records of 332 patients who received medical and/or surgical treatment with the diagnosis of acute cholecystitis in our clinic between 1997 and 2006 were reviewed retrospectively. Sixteen (4.8%) of those patients had gallbladder perforation. The parameters including age, gender, time from the onset of symptoms to the time of surgery, diagnostic procedures, surgical treatment, morbidity, and mortality were evaluated.

**RESULTS:** Seven patients had type Ⅰ gallbladder perforation, 7 type Ⅱ gallbladder perforation, and 2 type Ⅲ gallbladder perforation according to Niemeier's classification. The patients underwent surgery after administration of intravenous electrolyte solutions, and were treated with analgesics and antibiotics within the first 36 h (mean 9 h) after admission. Two patients died of sepsis and multiple organ failure in the early postoperative period. Subhepatic abscess, pelvic abscess, pneumonia, pancreatitis, and acute renal failure were found in 6 patients.

**CONCLUSION:** Early diagnosis and emergency surgical treatment of gallbladder perforation are of crucial importance. Upper abdominal computerized tomography for acute cholecystitis patients may contribute to the preoperative diagnosis of gallbladder perforation.



**Key words**: Acute cholecystitis; Gallbladder perforation; Early diagnosis; Computed tomography; Emergency surgery

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## INTRODUCTION

Gallbladder perforation (GBP) is a rare but life threatening complication of acute cholecystitis. Sometimes GBP may not be different from uncomplicated acute cholecystitis with high morbidity and mortality rates because of delay in diagnosis[1-3]. Thus GBP still continues to be an important problem for the surgeons. Most cases can only be diagnosed during surgery[1,4]. Male acute cholecystitis cases with high fever, high white blood cell (WBC) count, and associated systemic diseases should be meticulously investigated[1,5,6].

Niemeier[7] in 1934, classified free gallbladder perforation and generalized biliary peritonitis as acute or type Ⅰ GBP, pericholecystic abscess and localized peritonitis as subacute or type Ⅱ GBP, and cholecystoenteric fistula as chronic or type Ⅲ GBP. This classification is still in use. We aimed to present our clinical experience with GBP in this study.

## MATERIALS AND METHODS

### *Subjects*

Records of 332 patients who received medical and/or surgical treatment with the diagnosis acute cholecystitis in our clinic between January 1997 and February 2006 were reviewed retrospectively. Sixteen (4.8%) of those patients were found to have gallbladder perforation. Perforations due to trauma, iatrogenic causes, and gallbladder (GB) carcinoma were excluded.

### *Methods*

The original classification of Niemeier[7] was used to identify the patients. The parameters including age, gender, time from the onset of symptoms to the time of surgery, diagnostic procedures, surgical treatment, postoperative morbidity and mortality were evaluated. Direct abdominal X-ray series, abdominal ultrasound scanning (US), abdominal contrast-enhanced computerized tomography (CT), routine blood cell count, and blood chemistry tests were performed. Peritoneal spaces were lavaged thoroughly with isotonic saline and drains were placed for postoperative drainage in all of the patients.

## RESULTS

There were 10 male and 6 female patients. Their mean age was 69 (range, 54-85) years. Their complaints were abdominal pain, poor general condition, high fever, nausea, and vomiting on admission. Cholelithiasis was unknown prior to perforation in 5 patients. The patients

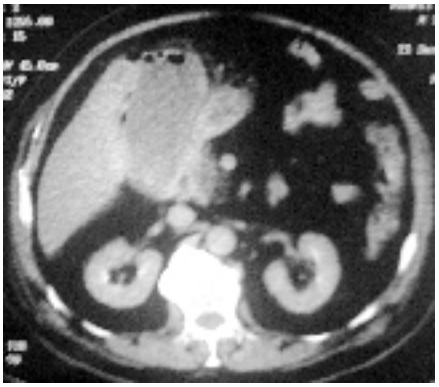

**Figure 1** Contrast-enhanced CT image at the level of gallbladder.

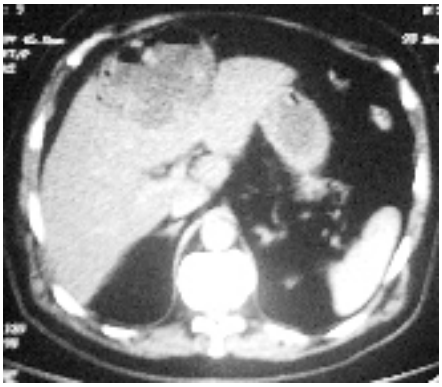

**Figure 2** Contrast-enhanced CT image of hepatic abscess adjacent to the superior part of gallbladder.

had several associated diseases, of which atherosclerotic heart disease (AHD) was the most common. One patient with type I gallbladder perforation was on long term steroid treatment for systemic lupus erythematosus. White blood cell (WBC) count was high in 14 patients, and 10 patients had high fever. Patients with type I gallbladder perforation had signs of peritoneal irritation such as extensive abdominal tenderness, guarding and rebound tenderness. Patients with type Ⅱ gallbladder perforation had local tenderness, guarding, positive Murphy's sign and 4 of them had palpable right subcostal mass while one patient had jaundice with a total bilirubin level of 28 mg/dL. Two patients with type Ⅲ gallbladder perforation had epigastric tenderness and one of them also had abdominal distension. Four patients had systemic inflammatory response syndrome, of them two patients with type I gallbladder perforation and two with type Ⅱ and type Ⅲ gallbladder perforations. The number of patients and their clinical features in each type of gallbladder perforation are shown in Table 1.

Abdominal X-ray series and abdominal US were performed for all of the patients and abdominal CT scanning for 14 patients. Only one patient with type Ⅲ gallbladder perforation had air-fluid levels on direct abdominal radiograms. Abdominal US showed gall stones in all of the patients with type I and type Ⅱ gallbladder perforations, extensive intraperitoneal free fluid in 7 patients with type I gallbladder perforation, and a small amount of pericholecystic free fluid in 6 patients with type Ⅱ gallbladder perforation. Abdominal US did not show GB wall defect in any of the patients. CT revealed GB wall thickening in all of the patients, gall stones in 10 patients, extensive intraperitoneal free fluid in 5 patients, a small amount of pericholecystic free fluid in 7 patients, and GB perforation sites in 5 patients. Abdominal CT and US detected liver abscesses in 2 patients, dilated extra and intrahepatic bile ducts in 1 patient. Abdominal CT showed dilated intestinal loops suggesting mechanical obstruction in one patient with type Ⅲ gallbladder perforation. Abdominal CT showed dilated stomach suggesting gastric outlet syndrome in another patient. The stomach was dilated with a normal mucosa, but the gall stone could not be seen during upper gastrointestinal endoscopy of this patient.

**Table 1 Clinical features of the patients, *n* (%)**

| Feature | Type Ⅰ | Type Ⅱ | Type Ⅲ |
|---|---|---|---|
| *n* (%) | 7 (43.7) | 7 (43.7) | 2 (12.6) |
| Mean age (yr) | 67.5 | 68.4 | 76.5 |
| Gender (Male/Female) | 5/2 | 5/2 | 0/2 |
| Fever (more than 38℃), *n* (%) | 5 (71.4) | 5 (71.4) | - |
| White blood cell count (mean) | 23966 | 17314 | 13200 |
| Mean duration of symptoms (d) | 5 | 8.6 | 15 |
| Systemic disorders[1], *n* (%) | | | |
| Atherosclerotic heart disease | 4 (14.3) | 4 (57.1) | 1 (50) |
| Diabetes mellitus | 2 (28.6) | 1 (14.3) | 1 (50) |
| Hypertansion | 1 (14.3) | 1 (14.3) | 1 (50) |
| Systemic lupus erythematosus | 1 (14.3) | - | - |
| Chronic obstructive pulmonary disease | 1 (14.3) | 2 (28.6) | 1 (50) |
| Systemic inflammatory response syndrome | 2 (28.6) | 1 (14.3) | 1 (50) |
| Preoperative diagnoses, *n* (%) | | | |
| Gallbladder perforation | 4 (14.3) | 1 (14.3) | - |
| Acute cholecystitis | 2 (28.6) | 4 (14.3) | - |
| Cholangitis | - | 1 (14.3) | - |
| Peptic ulcus perforation | 1 (14.3) | - | - |
| Perforated appendicitis | - | 1 (14.3) | - |
| Mechanical bowel obstruction | - | - | 1 (50) |
| Gastric outlet syndrome | - | - | 1 (50) |
| Sites of perforation, *n* (%) | | | |
| Fundus | 6 (85.7) | 3 (42.9) | - |
| Corpus | 1 (14.3) | 2 (28.6) | - |
| Infundibulum | - | 1 (14.3) | 2 (100) |
| Cystic duct | - | 1 (14.3) | - |

[1]Some patients had more than one sytemic disorder.

Enlarged gallbladder, irregularity of the gallbladder walls at the fundic region, air density lateral to the fundus and corpus, and increased density of the mesentery anterior to the fundus suggesting free fluid on CT image of a patient with type Ⅱ gallbladder perforation are shown in Figure 1. The abdominal CT image of a patient with hepatic abscess adjacent to the superior part of GB is shown in Figure 2.

The patients underwent surgery after administration of intravenous crystalloid solutions, and were treated with analgesics and antibiotic (third generation cephalo-

sporins) within the first 36 h (mean 9 h) after admission. The patients who had associated diseases such as diabetes, chronic obstructive pulmonary disease (COPD), and AHD underwent surgery after specific medical treatment was started. Antibiotic treatment was changed when required, according to the results of microbiological assessment of the infected bile specimens obtained from the gall bladder. The most common preoperative diagnoses were perforated cholecystitis in patients with type I gallbladder perforations, and acute cholecystitis in patients with type Ⅱ gallbladder perforations. Laparoscopic cholecystectomy was performed in 6 patients, two of them had type I gallbladder perforation, and four type Ⅱ gallbladder perforation. Conversion was required in 2 of them due to unclear anatomy. Laparotomy was performed in the remaining 10 patients. Perforations were recognized intraoperatively in 11 patients. The perforated site of the GB was the fundus in 9 patients, the corpus in 3 patients, the infundibulum in 3 patients, and the cystic duct in 1 patient. Two patients with type Ⅱ gallbladder perforation also had hepatic abscesses, which were covered by the omentum. Hepatic abscesses were also drained in these patients. All of the patients received a cholecystectomy. Common bile duct exploration was performed, gall stones were extracted, and a T-tube was placed into the common bile duct in addition to cholecystectomy in 1 patient with obstructive jaundice. One of the 2 patients had a cholecystoduodenal fistula (type Ⅲ gallbladder perforations) underwent surgery for gastrointestinal tract obstruction due to bile stones in the jejunum, which were removed through an enterotomy. Gall stones were also found in the duodenum of the other patient with type Ⅲ gallbladder perforation and removed through a duodenotomy.

The median hospital stay was 15 d (4-26 d). Two patients (12.5%) died of sepsis and multiple organ failure in the early postoperative period. One was a female patient at the age of 79 with type Ⅲ gallbladder perforation and COPD. The other was a 84 year-old male patient with delayed type Ⅱ gallbladder perforation and pericholecystic abscess associated with AHD and diabetes. Subhepatic abscess, pelvic abscess, pneumonia, pancreatitis and acute renal failure were found in 6 patients (Table 2). Subhepatic and pelvic abscesses were drained percutaneously under ultrasound guidance. The other diseases were treated conservatively.

**Table 2 Surgical procedures and morbidities of the patients *n* (%)**

| | Type Ⅰ | Type Ⅱ | Type Ⅲ |
|---|---|---|---|
| Surgery | | | |
| Laparoscopic cholecystectomy | 2 (12.5) | 4 (25) | - |
| Laparotomy and cholecystectomy | 5 (31.2) | 3 (18.7) | 2 (12.5) |
| Morbidity[1] | | | |
| Subhepatic abscess | | 1 (11.1) | 1 (11.1) |
| Pelvic abscess | | 1 (11.1) | 1 (11.1) |
| Pneumonia | 1 (11.1) | 1 (11.1) | 1 (11.1) |
| Pancreatitis | | 1 (11.1) | |
| Acute renal failure | 1 (11.1) | | |

[1]Some patients had more than one morbidity.

## DISCUSSION

Inflammation may progress and cause ischemia and necrosis, thus resulting GBP in 2% to 11% of acute cholecystitis patients[6,8,9]. GBP also develops following acalculous cholecystitis, although rare[10,11]. GB fundus, the most distal part with regard to blood supply, is the most common site of perforation[8,12]. The incidence of GBP was 4.5% and the most frequent site of perforation was the fundus (60%) in our study. Six of the 7 type I gallbladder perforations and 3 of 7 type Ⅱ gallbladder perforations were at the fundus. When GB is perforated at the fundus, it is less possibly covered by the omentum, thus the bile drains into the peritoneal space. If the perforation site is not at the fundus, it is easily sealed by the omentum or the intestines and the condition remains limited in the right upper quadrant with formation of a plastrone and pericholecystic fluid. This observation suggests that if the perforation site is at the fundus, it is more likely to end up with a type I perforation. The relation between the site and the type of GBP has not been defined. Although statistical analysis was not possible because of the insufficient number of patients in this series, this observation may be supported by larger series.

Acute uncomplicated cholecystitis is more common among females with a female to male ratio of 2:1[13]. However, GBP is more frequent in male gender[1,6]. In our study, male patients made up of 60% and the two patients with type Ⅲ gallbladder perforation were females. GBP is usually seen over 60 years of age[4,14]. Roslyn *et al*[1] reported that type I and Ⅱ GBP tend to occur in younger patients, especially more or less at the age of 50 years, whereas type Ⅲ gallbladder perforations are more common in the elderly. The patients with type I gallbladder perforation were relatively younger than those with type Ⅱ and Ⅲ gallbladder perforations in our study.

Type I gallbladder perforations are usually seen in patients with AHD, diabetes, malignancy, cirrhosis, and immunosupressive diseases, or during immunosupressive treatment, without a history of chronic cholecystitis. On the other hand, type Ⅲ gallbladder perforations most often occur in patients with a previous long time history of gall stones[1,9,12]. Severe AHD has been reported in 21% of patients with type I and Ⅱ gallbladder perforations, and diabetes, in 25% of patients with type I gallbladder perforation[8,14]. Such high rates are related to vascular disorders caused by these systemic diseases. It has been reported that type Ⅱ GBP occurs more frequently[1,3,9,12]. The incidence of type I and Ⅱ gallbladder perforations was equal and the most frequent associated systemic disease was AHD in the present study. Type Ⅲ gallbladder perforations usually occur in chronic cholecystitis patients with obstructive gastrointestinal symptoms[9,12,15]. Since the symptoms of type Ⅰ-Ⅱ GBP and uncomplicated cholecystitis are similar, differential diagnosis may be difficult based on physical examination, laboratory tests, and radiological methods and the diagnosis may not be established

preoperatively[6,12]. Delay in diagnosis is the major cause of its high morbidity and mortality[1,4,9,16]. Tanaka *et al*[17] reported that only one patient had a concrete diagnosis preoperatively in their series of 9 patients. The majority of GBP patients include those who undergo early surgery with the diagnosis of acute cholecystitis. The main complaint of the patients is abdominal pain accompanied with nausea and vomiting during the last 7 d[12,18]. The duration of symptoms was shorter in patients with type Ⅰ gallbladder perforation than in patients with type Ⅱ and Ⅲ gallbladder perforation in our study. High fever and elevated WBC count are not the diagnostic indications for gallbladder perforation. Parker *et al*[19] reported that high fever and high WBC count could be observed in 56% and 59% of patients with acute cholecystitis, respectively. The majority of type Ⅰ and Ⅱ gallbladder perforation cases had fever whereas type Ⅲ gallbladder perforation cases did not in our study. The patients with type Ⅰ and Ⅱ gallbladder perforation had elevated WBC count, but those with type Ⅲ gallbladder perforation had only a mild increase in WBC count.

US findings in acute cholecystitis, such as the GB wall thickening, GB distension, pericholecystic free fluid, and positive sonographic Murphy sign, may also be present in gallbladder perforation cases[2,9,20]. Sood *et al*[2] noted that the sonographic hole sign, in which the defect in GB wall is visualized, is the only reliable sign of gallbladder perforation. They reported that GB wall defect could be shown with a high resolution ultrasound scanner device in 70% of patients[2]. However, Kim *et al*[21] reported that the site of defect could not be visualized on US in any patients, which is similar to our study. On the other hand, CT can show more accurate signs of free intraperitoneal fluid, pericholecystic fluid, and abscess[1,2,22]. CT can also show GB wall thickness and the defect on the wall due to perforation[2,20]. In our study, all of the 5 patients who had the diagnosis of gallbladder perforation preoperatively were diagnosed by CT. Since the patients were admitted for acute abdominal pain, standard pelvic CT rather than upper abdominal CT was applied. Kim *et al*[21] reported that the defect could not be visualized on CT in 54% of patients. Doppler ultrasound, magnetic resonance imaging and radionuclide methods have been used in the diagnosis of gallbladder perforation[23-25]. The majority of our patients with type Ⅱ gallbladder perforation were initially treated conservatively and then underwent surgery as no improvement was observed during the first 3 d.

Cholecystectomy, drainage of abscess if present, and abdominal lavage are usually sufficient to treat gallbladder perforation[1,4]. Cholecystectomy may be difficult in type Ⅲ gallbladder perforations. If a cholesystectomy is performed, additional surgical procedures such as repair of the fistula may be required[15,16]. Cholecystectomy can be performed after the infection is relived by US guided percutaneus drainage in type Ⅱ gallbladder perforations[22]. Laparoscopic cholecystectomy can be performed for acute, gangrenous, and/or perforated cholecystitis as well as uncomplicated cholecystitis, but a conversion may be necessary in case of difficulties like an unclear anatomy[9,16]. In our study, laparoscopic procedure was initiated in 6 patients but conversion was required in two.

Since the difficulties in diagnosis cause delay in treatment, higher morbidity and mortality rates are often encountered[1,2,9]. Glenn and Moore[26] have reported that the mortality rate of gallbladder perforation patients is 42%, while other studies reported that the mortality rates are decreased to 12%-16% owing to the developments in anesthesiology and intensive care conditions[1,3]. The morbidity and mortality rates were 37.5% and 12.5%, respectively in the present study.

In conclusion, early diagnosis of gallbladder perforation and immediate surgical intervention are of crucial importance. Although standard abdominal CT has an important role in diagnosing gallbladder perforation, upper abdominal CT for acute cholecystitis in which pericholecystic fluid is found by US may increase the rate of preoperative diagnosis of gallbladder perforation.

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*RAPID COMMUNICATION*

# A head to head comparison of oral *vs* intravenous omeprazole for patients with bleeding peptic ulcers with a clean base, flat spots and adherent clots

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## Abstract

**AIM:** To compare the effect of intravenous and oral omeprazole in patients with bleeding peptic ulcers without high-risk stigmata.

**METHODS:** This randomized study included 211 patients [112 receiving *iv* omeprazole protocol (Group 1), 99 receiving *po* omeprazole 40 mg every 12 h (Group 2)] with a mean age of 52.7. In 144 patients the ulcers showed a clean base, and in 46 the ulcers showed flat spots and in 21 old adherent clots. The endpoints were re-bleeding, surgery, hospital stay, blood transfusion and death. After discharge, re-bleeding and death were re-evaluated within 30 d.

**RESULTS:** The study groups were similar with respect to baseline characteristics. Re-bleeding was recorded in 5 patients of Group 1 and in 4 patients of Group 2 ($P$ = 0.879). Three patients in Group 1 and 2 in Group 2 underwent surgery ($P$ = 0.773). The mean length of hospital stay was 4.6 ± 1.6 d in Group 1 *vs* 4.5 ± 2.6 d in Group 2 ($P$ = 0.710); the mean amounts of blood transfusion were 1.9 ± 1.1 units in Group 1 *vs* 2.1 ± 1.7 units in Group 2 ($P$ = 0.350). Four patients, two in each group died ($P$ = 0.981). After discharge, a new bleeding occurred in 2 patients of Group 1 and in 1 patient of Group 2, and one patient from Group 1 died.

**CONCLUSION:** We demonstrate that the effect of oral omeprazole is as effective as intravenous therapy in terms of re-bleeding, surgery, transfusion requirements, hospitalization and mortality in patients with bleeding ulcers with low risk stigmata. These patients can be treated effectively with oral omeprazole.



**Key words:** Oral omeprazole; Peptic ulcer; Bleeding

## INTRODUCTION

Acute upper gastrointestinal (UGI) bleeding remains the most common reason for emergency hospital admission managed by gastroenterologists. It is reported that it has an annual incidence ranging from approximately 50 to 150 per 100 000 of the population[1], and it is still rising steadily in the aspirin/nonsteroidal antiinflammatory drugs (NSAIDs) era. The most common cause of UGI bleeding in adult patients is peptic ulcer disease, which accounts for about 50% of the cases[2]. Patients with peptic ulcer bleeding account for an overall mortality rate that has remained around 5%-10% for the past five decades, despite improved therapy options and the availability of intensive care units[3].

Previous consensus guidelines and several studies have demonstrated that the risk for re-bleeding or continued bleeding from an ulcer is strongly associated with the stigmata seen at endoscopic examinations[4-7]. These hemorrhagic stigmata consist of a clean ulcer base, flat spots, adherent clots, nonbleeding visible vessels and active bleeding (oozing and spurting). Major stigmata of recent hemorrhage include spurting, oozing vessels, nonbleeding visible vessels or fresh adherent clots, while an old adherent clot was considered as minor stigmata[8]. There are also several studies that classified the high-risk stigmata as spurting, oozing, or nonbleeding visible vessels, excluding all adherent clots[9,10]. Currently, endoscopic hemostatic therapy is strongly recommended in patients with arterial spurting, oozing ulcers and nonbleeding visible vessels[11,12]. The optimum management of adherent clots has long been controversial. Although there are studies with opposite conclusions in this field[13,14], a cited meta-analysis showed that endoscopic therapy is of significant benefit in patients with active bleeding or a visible vessel but not in patients with adherent clots[15].

The overuse of parenteral proton pump inhibitors (PPI) in UGI bleeding is a common practice all over the world.

A recent meta-analysis pointed out that both intravenous (*iv*) and oral (*po*) PPIs are effective in UGI bleeding. However, the mortality is increased with *iv* PPI in the same report[16]. Moreover, it is reported that most patients who present with ulcer bleeding have low-risk stigmata and do not require *iv* PPI treatment but can be appropriately and adequately treated with oral PPIs in clinical practice[17]. In case intravenous treatment is particularly expensive, oral treatment would be appropriate.

Most previous studies on omeprazole have been performed with *iv* administration and not with *po* form. It is known that most physicians do not prefer *iv* PPI in patients with low-risk ulcers. In spite of this view, the issue needs a better confirmation. In the literature there are an abundant number of studies that compare oral PPI *vs* placebo, with[18] or without[19] endoscopic therapy, *iv* PPI *vs* placebo[20] or oral PPI *vs* endoscopic injection[11]. At the same time, to the best of our knowledge, there is no study in the literature that has been designed to allow head-to-head comparison of oral *vs* intravenous PPI treatment in UGI bleeding. We therefore designed this study to make a comparison of oral and intravenous omeprazole in patients with bleeding peptic ulcers without high-risk stigmata, in terms of re-bleeding, surgery, hospital stay, blood transfusion and mortality. We believe that it is important to add stronger study-supported evidence to the literature in this population.

## MATERIALS AND METHODS

### *Patients, definitions and study design*

Patients were enrolled in the study if they had any symptoms of upper gastrointestinal bleeding, such as hematemesis, melena or the presence of blood in a patient' s nasogastric tube lavage. They were all older than 18 years. Informed consent to participate in the study was obtained from all patients and the study was performed in accordance with the principles stated in the Declaration of Helsinki. Exclusion criteria were as follows: (1) a history of chronic liver disease and portal hypertension, (2) gastroduodenal malignancy, (3) gastric surgery, (4) known adverse drug reactions to the trial drugs, (5) current use of antisecretory drugs, $H_2$-receptor antagonists or PPIs, (6) a history of endoscopic therapy for bleeding ulcer within the past four weeks, (7) pregnancy or lactation, (8) had endoscopic findings of active bleeding (spurting, oozing vessels or nonbleeding visible vessels), (9) refusal to provide written informed consent. Moreover, patients found to have malignant ulcers after initial enrolment were also excluded.

Gender, age, current smoking, alcohol consumption, comorbid medical illnesses, use of aspirin/NSAIDs (any dose within last week), prior epigastric pain, history of previous upper gastrointestinal bleeding, prior major surgery, concomitant use of anticoagulants, antithrombocytic agents other than aspirin, and steroids and previous eradication treatment for *H pylori,* were investigated. The spectra of the comorbid illnesses included chronic obstructive pulmonary disease, pneumonia, end-stage renal disease with hemodialysis, chronic renal insufficiency or acute renal failure, congestive heart failure, coronary artery disease and cerebrovascular accident. Besides, we recorded the duration of hospitalization, number of re-bleeding episodes, initial hemoglobin level, coagulation parameters, need for blood transfusion, the endoscopic data and addresses/phone numbers of all patients.

Endoscopic examinations were performed using a videoendoscope (Olympus GIF-V70, Tokyo, Japan) within the first 24 h of admission. At endoscopy all primary and secondary lesions were recorded. The coagulation factors (prothrombin time, partial thromboplastin time, platelet count) were checked and corrected prior to any endoscopic intervention, if needed. Patients with an underlying anatomic cardiac abnormality were considered at a high risk for endocarditis, and recommended antibiotic regimens were given. An ulcer was defined as a circumscribed mucosal break at least 5 mm in diameter and with a perceptible depth. The ulcer size was measured using biopsy forceps, of which the fully opened cup was 5 mm in diameter. Besides, stigmata of recent hemorrhage were recorded. A 'fresh' adherent clot was defined as the presence of an adherent clot over the ulcer that could not be dislodged by vigorous washing with a jet of water delivered through the channel of the endoscope[21]. An 'old' clot was defined, on the contrary, as a clot dislodged easily by washing. These lesions were also excluded from the study due to their needs for an endoscopic therapy. All patients with benign gastroduodenal ulcers showing a clean ulcer base, flat spots or old adherent clots at endoscopy were included in the study. During the emergency admission, oral anticoagulant therapy was stopped in users and coagulation was corrected when applicable. The criteria for blood transfusion were as follows: hemoglobin levels of lower than 9 g/dL in older than 65 years, hemoglobin levels of lower than 8 g/dL in younger patients, or if the patient had a new episode of hematemesis in both age groups. Besides, if a state of shock existed, blood was transfused independent of haemoglobin levels. All patients with upper gastrointestinal bleeding were examined for *H pylori* in biopsy specimens taken from the antrum by hematoxylin and eosin (HE) staining.

We performed a single-center randomized clinical trial, comparing the effect of high dose intravenous omeprazole (Group 1) and oral omeprazole (Group 2) on bleeding peptic ulcer. The study was conducted between January 2004 and August 2006 at Gastroenterology Clinic of Dicle University Research Hospital in Turkey. After a stabilization period, patients were randomly divided into two groups in the endoscopy laboratory. A person outside from the study staff placed the two drug formulations into sealed non-transparent envelops and coded them based on random table numbers. Only this person knew the codes. The research assistant, other medical personnel, the endoscopists, and patients were blind to this information. The study was conducted in a double-blind manner as all treatment assignments were revealed at the end of the study. The high dose intravenous group received a bolus injection of omeprazole (Losec®, AstraZeneca, Molndal, Sweden), 80 mg, given at admission, followed immediately by a continuous infusion of 8 mg/h for 72 h, then 40

mg orally daily for 6 wk. The other group received oral omeprazole (Omeprol®, Ilsan-Hexal (Sandoz), Gebze, Turkey) 80 mg a day (20 mg capsule, two in the morning and two in the evening) for 72 h, then 40 mg orally daily for 6 wk. It is well-known that PPI treatment is an essential option in bleeding peptic ulcers and we did not include a placebo group for each treatment due to ethical problems.

The primary endpoints of the study were recurrent bleeding (early re-bleeding), surgery requirement, and death rates before discharge. Re-bleeding was defined as new hematemesis, melaena, or hypotension (< 100 mm Hg systolic blood pressure) associated with a drop in haemoglobin and/or endoscopic evidence of fresh re-bleeding. Patients with recurrent bleeding underwent urgent second endoscopy for confirmation and the lesion was classified as in previous description. Surgical intervention was considered if the bleeding could not be controlled by endoscopic therapy. Shock was defined as a pulse rate > 100 beats/min, systolic blood pressure < 100 mmHg accompanied by cold sweats, pallor, and oliguria. Secondary endpoints were duration of hospital stay, blood transfusion requirement, and re-bleeding or death within 1 mo after index bleeding (late re-bleeding). All patients were also evaluated in terms of risk analysis by Rockall scoring system, which is based on five variables (age, presence of shock, comorbidity, endoscopic diagnosis, and endoscopic stigmata)[22].

### Follow-up

Each patient was visited in the ward daily by a clinical research assistant who recorded information about their condition, their management, and results. After the treatment procedures, we observed the patients for complications such as recurrent bleeding, perforation or death in the hospital. Blood pressure and pulse rate were monitored hourly during the first 24 h and every 4 h, hemoglobin levels every 4 h during the first day and daily thereafter until discharge. Those who had no evidence of recurrent bleeding were discharged as soon as possible. After a treatment protocol of 72 h, patients with histologically proven *H pylori* were prescribed a 2-wk course of full dose omeprazole, twice daily, amoxicillin 1 g twice daily and clarithromycin 500 mg twice daily, irrespective of the treatment protocol. In addition, we recommended them not to use aspirin/NSAIDs if not needed anymore or use them in combination with PPIs. After discharge, all of the patients were informed about our contact phone number and the patients or their relatives were asked to report to us if any re-bleeding or death occurred within 30 d.

### Statistical analysis

Data were entered into a personal computer and analysed using the Epi-INFO 2000 software package (version 2000, CDC, Atlanta). Continuous variables were presented as mean (standard deviation). The results of the two treatment groups were compared by $\chi^2$ test, Student's *t* test and Fisher's exact tests in the analysis as appropriate. To test the association between outcomes and clinical covariables, we estimated risk ratios and 95% CI. In all analyses, statistical significance was defined as $P < 0.05$.

## RESULTS

During the study period, a total of 278 patients with bleeding gastroduodenal ulcers were admitted to our clinic. Of these ulcers, 21 were actively bleeding, 17 had nonbleeding visible vessels and 17 had a fresh adherent clot. At the beginning, all these 55 patients, together with 5 patients with malignant ulcer presentation, 4 patients currently known to take antisecretory drugs, $H_2$-RAs or PPIs and 3 patients with gastric surgery were excluded from the study.

Thus, a total of 211 eligible patients were included in the study and all of them completed the treatment protocols. The mean age of the patients was 52.7 (range, 18-93 years). The total number of patients with duodenal ulcer was 160 (75.8%) and gastric ulcer 51 (24.2%). Of the ulcers, 144 (68.2%) had a clean base, 46 (21.8%) had flat spots and 21 (10.0%) had old adherent clots. There were 112 patients in Group 1 (taking *iv* omeprazole) and 99 patients in Group2 (taking *po* omeprazole). The study groups were similar with respect to gender, age, stigmata of ulcer hemorrhage, use of aspirin/NSAIDs, *H pylori* status and previous eradication treatment, co-existing illnesses, previous abdominal surgery, alcohol consumption, smoking habit, previous epigastric pain, previous UGI bleeding, hematemesis, coagulopathy, shock, hematocrit, ulcer site (gastric-duodenal) and size. The characteristics of patients in both groups are summarized in Table 1. Multiple ulcers were found to be more common in Group 2 compared to Group 1 (6 and 17, respectively, $P = 0.007$). Besides, gastric antral and corporal ulcers were more common in Group 2 compared to Group 1 (33 *vs* 18, respectively, $P = 0.007$), while the number of duodenal anterior and posterior ulcers was similar in both groups. *H pylori* infection was present in 61.2% patients with duodenal ulcer and 41.2% patients with gastric ulcer ($P = 0.012$). Aspirin/NSAID use was recorded in 82% of gastric ulcers, while in 62% of duodenal ulcers ($P = 0.007$).

### Clinical outcomes during hospital stay (inpatient basis)

Recovery without major complications was seen in 107 (95.5%) patients of Group 1 and in 95 (96.0%) patients of Group 2 ($P = 0.945$). Recurrent bleeding was recorded in 5 (4.5%) patients assigned to Group 1 and 4 (4.0%) patients assigned to Group 2 ($P = 0.879$, Fisher's exact test). Re-bleeding rates were similar between duodenal and gastric ulcers, and posterior duodenal and gastric corporal ulcers ($P = 0.511$ and 0.673, respectively. Fisher's exact test). Only one ulcer with a clean base (11.1%), while 3 ulcers with old clots (33.3%) and 5 with flat spots (55.5%) showed rebleeding.

Three patients (2.7%) in Group 1 and 2 (2.0%) in Group 2 underwent surgery to control re-bleeding after a failure in second endoscopic intervention ($P = 0.773$, Fisher's exact test). Surgery requirement was mostly seen in patients taking aspirin/NSAIDs in both groups (2, for each). Four patients (1.9%), two in each group died ($P = 0.981$, Fisher's exact test). Three were older than 65 years. The Rockall score higher than 8 was present in 18.1% of Group 1 and 19.1% of Group 2. The causes of death were pneumonia in 2, myocardial infarction in 1 and pneumonia

**Table 1 Baseline characteristics of the study groups**

| Characteristics | Group 1 (*iv*) (*n* = 112) | Group 2 (*po*) (*n* = 99) | Total (*n* = 211) | *P* |
|---|---|---|---|---|
| Male / Female (*n*) | 79/33 | 66/33 | 145/66 | 0.545 |
| Age (mean ± SD) | 52.7 ± 17.05 | 52.8 ± 19.61 | 52.7 ± 18.12 | 0.966[1] |
| Age < 65 yr [*n* (%)] | 80 (71.4) | 64 (64.6) | 144 (68.2) | 0.291 |
| Age ⩾ 65 yr [*n* (%)] | 32 (28.6) | 35 (35.4) | 67 (31.8) | |
| Ulcer site (*n*): | | | | |
| -Duodenal | 94 | 76 | 160 | 0.083 |
| -Gastric | 18 | 23 | 51 | |
| Endoscopic signs (*n*): | | | | |
| -Clean base | 82 | 62 | 144 | 0.244 |
| -Flat spot | 20 | 26 | 46 | |
| -Old adherent clot | 10 | 11 | 21 | |
| Ulcer size (cm) | 1.05 ± 0.4 | 1.06 ± 0.6 | 1.05 ± 0.5 | 0.934[1] |
| Ulcer count (*n*): | | | | |
| -Single | 106 | 82 | 188 | 0.007[2] |
| -Multiple | 6 | 17 | 23 | |
| Ulcer locations (*n*): | | | | |
| -Posterior duodenal | 61 | 52 | 113 | |
| -Anterior duodenal | 33 | 14 | 47 | 0.007 |
| -Gastric corporal | 9 | 16 | 25 | |
| -Gastric antral | 9 | 17 | 26 | |
| Aspirin/NSAIDs use [*n* (%)] | 68 (60.7) | 70 (70.7) | 138 (65.4) | 0.072 |
| *H pylori* positive [*n* (%)] | 63 (56.3) | 56 (56.6) | 119 (56.4) | 0.963 |
| Previous eradication [*n* (%)] | 10 (8.9) | 3 (3.0) | 13 (6.2) | 0.09[2] |
| Coexisting illness [*n* (%)] | 41 (36.6) | 35 (35.4) | 76 (36.1) | |
| -Cardiac (*n*) | 18 | 14 | 32 | 0.850 |
| -Pulmonary (*n*) | 16 | 15 | 31 | |
| -Cerebral (*n*) | 7 | 6 | 13 | |
| Previous surgery [*n* (%)] | 19 (17.0) | 22 (22.2) | 41 (19.4) | 0.335 |
| Alcohol [*n* (%)] | 5 (4.5) | 2 (2.0) | 7 (3.3) | 0.452[2] |
| Smoking [*n* (%)] | 47 (42.0) | 38 (38.4) | 85 (40.3) | 0.597 |
| Previous pain [*n* (%)] | 72 (64.3) | 67 (67.7) | 139 (65.9) | 0.604 |
| Previous bleeding [*n* (%)] | 17 (15.2) | 17 (17.2) | 34 (26.1) | 0.694 |
| Hematemesis [*n* (%)] | 77 (68.8) | 69 (69.7) | 146 (69.2) | 0.882 |
| Coagulopathy [*n* (%)] | 4 (3.5) | 3 (3.0) | 7 (3.3) | 0.917[2] |
| Hematocrit (%, Mean) | 24.2 ± 3.2 | 23.6 ± 3.4 | 23.9 ± 3.1 | 0.567[1] |
| Index hematocrit < 25% (*n*) | 47 | 45 | 92 | 0.610 |
| Index hematocrit ⩾ 25% (*n*) | 65 | 54 | 119 | |
| Shock (*n*) | 6 | 5 | 11 | 0.381[2] |
| Rockall score ⩽ 3 [*n* (%)] | 63 (56.3) | 52 (52.5) | 115 (54.5) | 0.737 |
| > 8 [*n* (%)] | 21 (18.7) | 19 (19.1) | 40 (18.9) | 0.865 |

[1]Student's *t* test; [2]Fisher's exact test; NSAIDs: non-steroidal anti-inflammatory drugs.

**Table 2 Clinical outcomes of the study population**

| Outcome | Group 1 (*iv*) (*n* = 112) | Group 2 (*po*) (*n* = 99) | Total (*n* = 211) | *P* |
|---|---|---|---|---|
| **Inpatient basis** | | | | |
| Recovery [*n* (%)] | 107 (95.5) | 95 (96.0) | 202 (95.7) | 0.945 |
| Re-bleeding [*n* (%)] | 5 (4.5) | 4 (4.0) | 9 (4.3) | 0.879[2] |
| Surgery requirement [*n* (%)] | 3 (2.7) | 2 (2.0) | 5 (2.4) | 0.773[2] |
| Hospital stay (days, mean) | | | | |
| Total | 4.6 ± 1.6 | 4.5 ± 2.6 | 4.5 ± 2.8 | 0.710[2] |
| ⩽ 5 d (*n*) | 52 | 55 | 107 | 0.093 |
| > 5 d (*n*) | 60 | 44 | 104 | |
| Blood transfusion (units) | | | | |
| Total (mean) | 1.9 ± 1.1 | 2.1 ± 1.7 | 2.0 ± 1.6 | 0.350[1] |
| ⩽ 3 units (*n*) | 85 | 74 | 159 | 0.847 |
| > 3 units (*n*) | 27 | 25 | 52 | 0.610 |
| Death [*n* (%)] | 2 (1.8) | 2 (2.0) | 4 (1.9) | 0.981[2] |
| **Outpatient basis** | | | | |
| Re-bleeding [*n* (%)] | 2 (1.8) | 1 (1.0) | 3 (1.4) | 0.766[2] |
| Death [*n* (%)] | 1 (0.8) | 0 (0.0) | 1 (0.4) | 0.887[2] |
| **Overall** | | | | |
| Re-bleeding [*n* (%)] | 7 (6.2) | 5 (5.0) | 12 (5.6%) | 0.745[2] |
| Death [*n* (%)] | 3 (2.6) | 2 (2.0) | 5 (2.3) | 0.980[2] |

[1]Student's *t* test; [2]Fisher's exact test.

**Table 3 Probable effects of variables on re-bleeding**

| Factor | Re-bleeding (*n*) | *P* | OR (95% CI) |
|---|---|---|---|
| Co-existing illness | 5 | 0.288 | 2.3 (0.6-8.8) |
| Hematemesis | 6 | 0.971 | 0.8 (0.2-3.6) |
| Smoking | 5 | 0.490 | 1.9 (0.4-7.3) |
| Aspirin/NSAIDs | 7 | 0.721 | 1.1 (0.3-8.7) |
| Age ⩾ 65 yr | 5 | 0.117 | 2.1 (0.7-10.8) |
| Ulcer size > 1 cm | 6 | 0.001 | 11 (2.6-46.3) |
| Ulcer locations: | | | |
| Posterior duodenal | 4 | 0.041 | 7 (1.5-18.2) |
| Gastric corporal | 3 | 0.049 | 5 (1.0-14.3) |
| Ulcer stigmata: | | | |
| Flat spots | 5 | 0.001 | 12 (4.5-57.3) |
| Old clots | 3 | 0.023 | 5 (1.2-13.5) |

plus adrenal insufficiency in 1. Total hospital stay was 3 d at minimum and 20 d at maximum. The mean duration of hospital stay was 4.6 ± 1.6 d in Group 1 and 4.5 ± 2.6 d in Group 2. Length of hospital stay did not differ significantly between two groups ($P = 0.710$, Student's *t* test). Hospital stay more than 5 d was also similar between the groups ($P = 0.093$). The median number of units of blood transfused was approximately 2 in each group ($P = 0.350$, Student's *t* test). Blood transfusion requirement was more than 3 units in 27 (24.1%) patients of Group 1 and 25 (25.3%) patients of Group 2 ($P = 0.610$). Details about clinical outcomes are summarized in Table 2.

Bleeding from posterior duodenal (44.4%) and gastric corporal (33.3%) sites was more common compared to other sites [$P = 0.041$, OR 7 (1.5-18.2) and $P = 0.049$, OR 5 (1.0-14.3), 95% CI], respectively]. Of ulcers with re-bleeding, a diameter greater than 1 cm had a higher risk [$P = 0.001$, OR 11.0 (2.6-46.3), 95% CI]. Ulcers with flat spots and old clots had also higher risks for re-bleeding. Co-existing illnesses, hematemesis, smoking habit, aspirin/NSAIDs use and age older than 65 years did not have any effect on re-bleeding rates. The risk estimates of cofactors and their powers are summarized in Table 3.

### *Clinical outcomes after discharge in 30 d (outpatient basis)*

We strictly informed patients or their relatives that it was very important to report to us any problems (new bleeding attack or death) immediately that occurred during the discharge period. None of the patients took aspirin or NSAIDs during the 30 d follow-up period. Four patients (or a relative) re-contacted us within 30 d. A new bleeding occurred in 2 patients of Group 1 and in 1 patient of

Group 2 after the index bleeding episode. The overall re-bleeding was seen in 12 (5.6%) patients [7 (6.2%) in Group 1 and 5 (5.0%) in Group 2]. Additionally, one patient died from Group 1 due to a new cerebrovascular event. Hence, the overall death was seen in 5 (2.3%) patients.

## DISCUSSION

In the present study, we demonstrated that oral omeprazole was as effective as intravenous omeprazole in controlling bleeding peptic ulcers without high-risk stigmata. Importantly, the study implies that treatment with the oral agent is indicated for the bleeding instead of the *iv* approach when in reality, PPI use in this situation is simply to heal the ulcer. Although most of bleeding episodes from peptic ulcers resolve spontaneously and are not detrimental, recurrence of bleeding adversely affects prognosis. The overall re-bleeding rate in the oral treatment group (5.0%) was similar to that in the intravenous treatment group (6.2%) within 30 d, and both groups were also similar with respect to the need for surgery, duration of hospitalization, total amounts of blood transfusion, and mortality. Although clean base ulcers form the largest portion of the study population, the calculated Rockall scores were higher than 8 in approximately one fifth of the patients. As well-known, a score of higher than 8 is associated with a high risk of death[22]. Comorbidity and age were the predominant contributors to these high scores in our population.

Although the Federal Drug Administration has not approved intravenous proton pump inhibitors for the treatment of UGI bleeding, these agents are being used widely all around the world. A meta-analysis pointed out that PPI therapy in UGI bleeding was effective only in patients with UGI bleeding caused by peptic ulcers and with high-risk stigmata for re-bleeding[16]. Moreover, it has been reported that patients with endoscopy results showing a low risk of re-bleeding should not be treated endoscopically as their prognosis is excellent when treated conservatively[1].

What about oral versus intravenous drug administration? Most of the previous studies on omeprazole have been conducted with intravenous omeprazole and not with the oral drug. The oral absorption of omeprazole is 50%; however, as gastric pH rises, as much as 75% may be absorbed. It is highly protein-bound in plasma and is rapidly metabolized in the liver, and the metabolites are excreted in urine. The onset of antisecretory effect occurs within 1 h, with peak effects occurring in 2 h, depending on the dose[23]. Demonstration of effectiveness of oral treatment would be particularly attractive as it would allow treatment to be initiated outside, prior to hospital admission. However, it was concluded that pharmacotherapy alone could not replace endoscopic hemostasis for patients with actively bleeding ulcers or ulcers with nonbleeding visible vessels[12]. On the other hand, it was reported that oral omeprazole therapy can be a valid alternative to endoscopic therapy, especially when injection therapy is not readily available[11]. Replacement of endoscopy in bleeding may be more possible in ulcers with low risk stigmata. Interestingly, one meta-analysis warned readers that those patients with UGI bleeding with significant comorbid diseases (such as diabetes mellitus, collagen vascular disease and hypercoagulable states) need careful monitoring, as intravenous PPI therapy in such patients may be harmful. Besides, all-cause deaths and non-ulcer deaths in trials using intravenous PPI were higher in the treatment group and not in trials using oral PPI[16].

The role of oral omeprazole in bleeding peptic ulcers was studied, especially in ulcers with high-risk stigmata by some authors and they found the rebleeding rates of omeprazole groups to be 7%, 10.9%, 15%, 22.9% and 26%, respectively[11,18,19,24,25]. Bour *et al*[25], Khuroo *et al*[19] and Jung *et al*[11] did not perform endoscopic therapy in omeprazole groups, while Javid *et al*[24] and Kaviani *et al*[18] did so. Jung *et al*[11] reported that oral omeprazole administration was comparable to endoscopic ethanol injection therapy for prevention of re-bleeding in patients with nonbleeding visible vessels or adherent clots. Moreover, Kaviani *et al*[18] showed that oral omeprazole reduced the re-bleeding rate, hospital stay and need for blood transfusion even in high-risk ulcers after endoscopic therapy. Khuroo *et al* reported a lower re-bleeding rate with oral omeprazole alone compared to placebo[19], which is comparable to the re-bleeding rates achieved by endoscopic therapy alone[13,14]. Bour *et al* also reported that oral omeprazole was comparable to endoscopic injection therapy[25], but they administered a smaller dose of omeprazole (40 mg every 24 h) than we did. This dosage may not be sufficient to maintain intragastric pH > 4. Detailed studies *in vitro* have shown that at a pH of < 6, the extrinsic and intrinsic coagulation cascades are impaired, and platelet aggregation is virtually abolished[26]. In our study, omeprazole at a dose of 40 mg every 12 h was administered because the gastric pH was reported to be > 6 during 85% of the first 24 h period at that dose[19].

Although it was declared four years ago by the British Society of Gastroenterology Endoscopy Committee[1] that patients who have active bleeding from the ulcer, a non-bleeding visible vessel, or have adherent clots should be recommended to receive endoscopic therapy (grade A), it was subjected to strong objections[27] and the disapproval was supported by a meta-analysis[15]. After a clot has been diagnosed, approaches to its management are quite different. Removal of blood clots is probably more hazardous in centers where clinicians are less experienced in handling peptic ulcer bleeding. However, targeted irrigation has been shown repeatedly to be safe and should be widely adopted in managing ulcers with adherent clots[28]. Randomized, controlled trials of endoscopic therapy versus non endoscopic therapy for ulcers with adherent clots have yielded conflicting results[13,29], and a meta-analysis does not support routine use of endoscopic therapy[15]. Our study groups had a low count (10 *vs* 11) of adherent clots and they had a lower risk for re-bleeding. The risk for re-bleeding with clots that remained adherent after washing has been reported to be only 8%[30]. We repeated the analysis excluding clots and found out that re-bleeding rates were also similar in both groups. On the other hand, the rebleeding rate of 10.8% (5/46) in the flat spot group is higher compared with other studies[4]. Interobserver variability of stigmata classification could be

a major limitation in this condition.

*H pylori* infection and chronic aspirin/NSAID use are the two major risk factors among patients hospitalized for ulcer bleeding[17]. Eradication of *H pylori* has been demonstrated in many randomized, controlled trials[31,32], to reduce the rate of ulcer recurrences and rebleeding in complicated ulcer diseases. In a recent study, duodenal ulcers were more likely to be associated with *H pylori* infection than gastric ulcers. In contrast, gastric ulcers were more likely associated with aspirin/NSAID use than were duodenal ulcers[17]. In patients with upper gastrointestinal bleeding, the sensitivity of the rapid urease test is relatively low in detecting *H pylori*. It was reported that this was best accomplished by histologic examination with a sensitivity above 90%[33], and therefore we did so. In our study, *H pylori* infection was present in 98 (61.2%) patients with duodenal ulcer, and in 21 (41.2%) patients with gastric ulcer ($P = 0.012$). Aspirin/NSAID use was seen in 82% of gastric ulcers, while in 62% of duodenal ulcers ($P = 0.007$). It was also reported that prior use of aspirin/NSAIDs increases the risk of re-bleeding in bleeding ulcer patients, and leads to a higher need for urgent surgery[34]. In parallel with this conclusion, surgery requirement was mostly seen in patients taking aspirin/NSAIDs in both groups (2, for each) in our study. On the other hand, endoscopic features of high-risk included ulcer size (> 1 or 2 cm)[34,35] and the site of bleeding (the posterior lesser gastric curvature and posterior duodenal wall)[36-38]. Bleeding from posterior duodenal (44.4%) and gastric corporal (33.3%) ulcers was more common compared to other sites in our study (Table 3). The mean age of our study population was 52 years, and more than one third had co-existing illnesses.

In conclusion, our results suggest that the effectiveness of oral omeprazole administration is comparable to intravenous therapy in terms of re-bleeding, need for emergency surgery, transfusion requirements, length of hospital stay and mortality in patients with bleeding peptic ulcers without high risk stigmata. In most of the countries, most patients with bleeding ulcers have low risk stigmata, and thus, can be treated with oral omeprazole. These patients do not explicitly require expensive omeprazole infusions.

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## COMMENTS

### *Background*

As yet, there is no study in the literature that had a head-to-head comparison of oral *vs* intravenous proton pump inhibitor treatment in bleeding peptic ulcers. We designed a study to compare the effect of *iv* and *po* omeprazole in patients with bleeding peptic ulcers without high-risk stigmata.

### *Research frontiers*

We demonstrate that the effect of oral omeprazole is as effective as intravenous therapy in terms of re-bleeding, surgery, transfusion requirements, hospitalization and mortality in patients with bleeding ulcers with low risk stigmata. These patients can be treated effectively with oral omeprazole.

### *Innovations and breakthroughs*

Our results suggest that the effectiveness of oral omeprazole administration is comparable to intravenous therapy in terms of re-bleeding, need for emergency surgery, transfusion requirements, length of hospital stay and mortality in patients with bleeding peptic ulcers without high risk stigmata. Generally speaking, most patients with ulcer bleeding have low-risk stigmata, and thus, can be treated with oral omeprazole. These patients do not explicitly require expensive omeprazole infusions.

### *Peer review*

It is a practical research to compare the effectiveness of oral omeprazole *vs iv* omeprazole in peptic bleeding cases without high risk stigmata. The results showed that oral treatment is as effective as the iv treatment but less expensive. The study was well designed with enough material as well as statistical analysis.

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*RAPID COMMUNICATION*

# Depression in patients with irritable bowel syndrome in Jos, Nigeria

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## Abstract

**AIM:** To study the brain-gut interaction and the effect of behavioral or psychiatric conditions on irritable bowel syndrome (IBS) in an African population.

**METHODS:** IBS was diagnosed using the Rome Ⅱ diagnostic criteria. The entry of each patient was confirmed following detailed explanations of the questions. Four hundred and eighteen patients were studied. Subjects satisfying the Rome Ⅱ criteria for IBS were physically examined and stool microscopy was done to identify the presence of "alarm factors". Depression was diagnosed using the symptom-check list adapted from the Research Diagnostic Criteria (DSM-Ⅳ) of the American Psychiatric Association.

**RESULTS:** Seventy-five (56.8%) of the 132 IBS patients were depressed whereas only 54 (20.1%) of the 268 non-IBS patients were depressed. There was a significant relationship between IBS and depression ($\chi^2$ = 54.29, Odds ratio = 5.21, 56.8 ± 8.4 *vs* 20.1 ± 5.2, *P* = 0.001). Even though constipation predominant IBS patients were more likely to be depressed, no significant relationship was found between the subtype of IBS and depression ($\chi^2$ = 0.02, OR = 0.95, *P* = 0.68).

**CONCLUSION:** IBS is significantly associated with major depression but not gender and bowel subtypes of the patients. Patients with IBS need to be evaluated for depression due to the highly significant relationship between the two conditions.



**Key words:** Irritable bowel syndrome; Depression; Nigeria; Africa

Ladep NG, Obindo TJ, Audu MD, Okeke EN, Malu AO. Depression in patients with irritable bowel syndrome in Jos, Nigeria. *World J Gastroenterol* 2006; 12(48): 7844-7847

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## INTRODUCTION

Irritable bowel syndrome (IBS) has been widely studied in the western world and pathophysiologic mechanisms have been available to explain the constellation of symptoms[1]. IBS is a chronic disorder of unknown etiology clinically consisting of altered bowel habits, abdominal pain and the absence of any detectable organic pathologic process[1]. Along with the putative pathophysiologic mechanisms of post-infectious inflammatory disorder, disordered intestinal motility and visceral hypersensitivity, there has been much discussion regarding the brain-gut interaction and the influence of behavioral or psychiatric conditions on these symptoms[2].

IBS is recognized widely as one of the most commonly encountered gastrointestinal disorders[1-4]. Locally, there is paucity of data concerning IBS in Africa. Of the studies available however, IBS was reported in 30 percent of an African population at the University College Hospital, Ibadan[5] and 8% in Kenya[6]. Peak prevalence of IBS was reported in the third decade of life in the study in Kenya. A more recent report from our centre demonstrated a prevalence of 26.1% among a healthy student population[7]. In addition, patients with IBS are at increased risk for other non-gastrointestinal functional disorders such as fibromyalgia[8-11] and interstitial cystitis[12]. IBS is known to co-exist with some psychiatric disorders such as depression, anxiety disorders, somatoform disorders[9,13] and sexual dysfunction (e.g., dyspareunia)[14]. The situation is however, not known in African population. This study aims at determining the prevalence of depression among African IBS patients.

## MATERIALS AND METHODS

### *Patients*

This is a descriptive cross-sectional study. The principal investigator administered the questionnaire after obtaining informed consent to randomly selected subjects attending the General Outpatient (GOP) Clinics of the three hospitals in Jos. These hospitals were: Jos University Teaching Hospital (JUTH), Evangel Hospital (EEH) and

Plateau State Specialist Hospital (PSSH). The GOP clinics of these hospitals are where primary care physicians attend to patients prior to referrals for specialist care. On selected days of the week, the investigators went to the various clinics until the proposed study sample size was achieved.

The number of patients visiting each of the GOP clinics in the previous year was used to divide the total sample to be studied proportionately. This was obtained from the records of the hospitals. Individual patients were randomly selected in each GOP clinic. Every fifth patient at EEH, every fourth patient at JUTH and alternate patient at PSSH were selected, subject to satisfying the inclusion criteria.

The study was approved by the Ethics Committee of the three hospitals prior to the commencement of the study. The study at EEH was conducted between July 2003 and September 2003, PSSH between October and November 2003, and JUTH between December 2003 and February 2004. Each patient's entry was confirmed following detailed explanations of the questions. Subjects satisfying the Rome Ⅱ criteria for IBS were physically examined to identify the presence of "alarm factors." Major depression was diagnosed using the symptom-check list adapted from the Research Diagnostic Criteria (DSM-Ⅳ) of the American Psychiatric Association[15].

### Sample size

The minimum sample size was determined based on the prevalence reported in the literature using the following formula:

$$N = \frac{(Zi\text{-}a)^2 (P) (1\text{-}P)}{d^2}$$

N = minimum sample size; P = best estimate of prevalence of IBS from the literature review expressed as a fraction of 100 (in this case, 30% = 0.3)[5]; Zi-a = a constant at 95% confidence interval for a two-tailed descriptive study (= 1.96); d = absolute precision, i.e. value required (in percentage) which in actual terms describes the maximum difference between the population rate and the sample rate that can be tolerated. Five percent (0.05) was adapted for this study. Thus:

$$N = \frac{1.96^2 (0.3) (0.7)}{(0.05)^2} = 323$$

However, 400 subjects were recruited for the study. Systematically selected adult patients aged 18 to 50 years attending the GOP clinics of the three hospitals were included and the pregnant women and subjects using laxatives for any reason, those with memory problems and the presence of alarm symptoms, including a positive stool test for helminthes were excluded from the study.

The 400 patients who attended the three selected hospitals and who satisfied the inclusion criteria were studied with the questionnaires by the principal investigator. Four hundred and eighteen patients were initially evaluated, but 18 were excluded for various reasons. Six were above 50 years of age, 4 had significant weight loss, 5 could not give a coherent history, 2 declined to participate in the study and 1 had recurrent bloody stools. Of the 400 subjects, 174 were from ECWA Evangel Hospital, 148 from Jos University Teaching Hospital, and 78 from Plateau State Specialist Hospital.

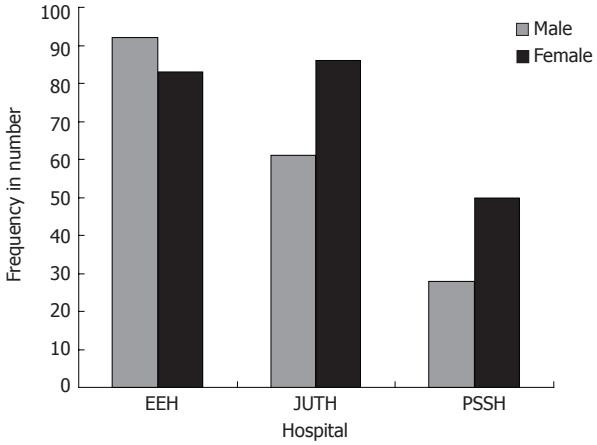


**Figure 1** Distribution of study population by gender in the three GOP clinics.

### Statistical analysis

Data were analysed using the Epi Info 2000 Statistical Program[16]. Means and standard deviations were used to describe continuous variables and proportions for categorical data. Two-tailed Student's *t* test and analysis of variance (ANOVA), a parametric test for inequality of population means, were used for comparison of group means while the significance of observed differences (proportions) was determined by Chi-square test. $P < 0.05$ was considered statistically significant.

## RESULTS

The mean age of the study population at EEH, JUTH and PSSH were 32.5 ± 9.3, 32.0 ± 10.0 and 31.1 ± 8.8 years, respectively and with an age range of 18-50 years at each hospital. There was no statistically significant difference in the mean age among the three study populations (f = 0.62, $P = 0.54$). Figure 1 shows the gender distribution of the study population in the three GOP clinics. Eighty-three (47.4%) females and 92 (52.6%) males were recruited at the EEH, while 86 (58.5%) females and 61 (41.5%) males were recruited at JUTH. Fifty (64.1%) females and 28 (35.9%) males were selected at PSSH.

### Age and gender distribution of study subjects

The mean age of this population was 32.0 ± 9.4 years with a range of 18-50 years. The mean age for females was 31.9 ± 9.5 years and males 32.1 ± 9.3 years, showing no statistically significant difference ($t = 0.2$, $P = 0.8$). Subjects in the third decade of life predominated over the other age groups, and the number of females was higher than their male counterparts.

### Irritable bowel syndrome and depression

Table 1 gives a summary of this relationship. Seventy-five (56.8%) of the 132 IBS patients were depressed whereas only 54 (20.1%) of the 268 non-IBS patients were depressed. There was a significant relationship between IBS and depression (Odds ratio = 5.21, $\chi^2 = 54.29$, $P = 0.001$). There was no significant relationship between the gender of the IBS patients and depression (Odds ratio = 0.95, $\chi^2 = 0.02$, $P = 0.89$) (Table 2). Even though

**Table 1 Relationship between IBS and depression**

| IBS | Depressed *n* (%) | Not depressed *n* (%) | Total (*n*) |
|---|---|---|---|
| Yes | 75 (56.8) | 57 (43.2) | 132 |
| No | 54 (20.1) | 214 (79.9) | 268 |
| Total | 129 (32.3) | 271 (67.7) | 400 |

OR = 5.21, 95% CI = 3.22-8.45, $\chi^2$ = 54.29, $P$ < 0.001.

**Table 2 Relationship between depression and gender among the IBS patients**

| Depression | Male *n* (%) | Female *n* (%) | Total (*n*) |
|---|---|---|---|
| Yes | 32 (42.7) | 43 (57.3) | 75 |
| No | 25 (43.9) | 32 (56.1) | 57 |
| Total | 57 (43.2) | 75 (56.8) | 132 |

OR = 0.95, 95% CI = 0.45-2.03, $\chi^2$ = 0.02, $P$ = 0.89.

**Table 3 Relationship between subtype of irritable bowel syndrome and depression**

| Subtype | Depression *n* (%) | No depression *n* (%) | Total (*n*) |
|---|---|---|---|
| Constipation | 36 (61.0) | 23 (39.0) | 59 |
| Diarrhoea | 31 (53.4) | 27 (46.6) | 58 |
| Alternator | 8 (53.3) | 7 (46.7) | 15 |
| Total | 75 (56.8) | 57 (43.2) | 132 |

$\chi^2$ = 0.77, df = 2, $P$ = 0.68.

constipation predominant IBS patients were more likely to be depressed, Table 3 shows that no significant relationship exists between the subtype of IBS and depression ($\chi^2$ = 0.77, $P$ = 0.68). No significant difference was observed between the gender of the population and the subtypes of IBS.

## DISCUSSION

Seventy-five (56.8%) IBS patients were depressed compared with 54 (20.1%) of the 268 non-IBS patients fulfilled the DSM Ⅳ criteria for depression. There was a highly significant relationship between IBS and depression ($\chi^2$ = 54.29, Odds ratio = 5.21, $P$ < 0.001). This compares well with western studies that reported 60% psychiatric co-morbidity among their IBS patients who have sought healthcare[17,18]. Abnormal psychologic features have been recorded in a large percentage of patients with IBS. In a study by McDonald and others[19] involving 35 patients with IBS and 32 patients with non-IBS disease, 20% of the non-IBS group had diagnosable psychiatric disorders when compared with 53% of the IBS group. This high prevalence of depression among our cohort is most likely due to the fact that it was a study among a patient population. Most western community-based studies and a recent study done by us among stable students showed that the rate of depression was comparable to that among the non-IBS group[7,17]. Indeed, co-morbidities such as psychological symptoms are recognized factors that affect healthcare seeking of IBS patients[20].

Most investigators have found that psychiatric features predate the onset of bowel symptoms or occur concurrently, suggesting that it is not the bowel symptoms of IBS that induce psychiatric diseases[4]. Many theories have implicated the role of depression in IBS[9,14]. In addition, serotonin and catecholeamines (neurotransmitters that play some role in depression), have been shown to be in excess in patients with IBS[21-23]. Emotional stress can trigger bowel hypermotility both in normal subjects and in patients with IBS[24]. These events are associated with development of both organic and functional abdominal illness[25]. Moreover, the existing evidence suggests that treating depression and anxiety can improve the patient's gastrointestinal distress.

There was no significant difference between the gender of the IBS patients and depression, implying that both men and women are equally prone to depression. Even though, a trend was observed in constipation-predominant IBS and depression, there was no statistically significant relationship ($P$ = 0.68). This finding agrees with that by Whitehead *et al*[26].

IBS is significantly associated with major depression, but not with gender and bowel subtypes of the patients. Patients with IBS need to be evaluated for depression due to the highly significant relationship that exists between the two conditions.

The Rome Ⅱ criteria has been widely validated in the western world and found to have a positive predictive value of 98%[27]. In our setting however, where a lot of helminthic and protozoa infestations exist, it is unclear whether the subjects fulfilling these criteria truly have IBS or not. A subset of the patients who had their stools evaluated for these infestations did not change the pre-examination diagnosis of IBS. This is a subject for further evaluations.

## ACKNOWLEDGMENTS

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## COMMENTS

### Background

Irritable bowel syndrome (IBS) has been widely studied in the western world and many pathophysiologic mechanisms have been provided to explain the constellation of symptoms. Along with the putative pathophysiologic mechanisms of post-infectious inflammatory disorder, disordered intestinal motility and visceral hypersensitivity, there has been much discussion regarding the brain-gut interaction and the influence of behavioral or psychiatric conditions on these symptoms.

### Innovations and breakthroughs

IBS is known to co-exist with some psychiatric disorders such as depression, anxiety disorders, somatoform disorders and sexual dysfunction (e.g., dyspareunia). The situation is however, not known in African populations. This study aimed at determining the prevalence of depression among African IBS patients.

### Applications

IBS is significantly associated with major depression. Patients with IBS need to be evaluated for depression due to the highly significant relationship between the two conditions. The Rome criteria has been widely validated in the Western world and found to have positive predictive value of 98%. It is necessary to validate this criteria in the African setting, where a lot of helminthic and protozoa infestations exist that may be confused with IBS.

### Terminology

IBS is a chronic disorder of unknown etiology clinically consisting of altered bowel habits, abdominal pain and the absence of any detectable organic pathologic process. Depression is a state of low mood, loss of interest and energy that may result in suicidal ideation or act.

### Peer review

This is a well-done and interesting study on co-expression of depression and IBS in an African population.

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*RAPID COMMUNICATION*

# Novel *MLH1* frameshift mutation in an extended hereditary nonpolyposis colorectal cancer family

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## Abstract

**AIM:** To present novel frameshift mutation c.31delC [p.L11X] in the *MLH1* gene identified in an extended Bulgarian hereditary non-polyposis colorectal cancer (HNPCC) family and to analyze the molecular and clinical findings within the pedigree concerning the proposal of adequate individual prophylactic strategy for all mutation carriers.

**METHODS:** The pedigree of the family consists of 42 members in four generations. Search for mutations in the *MLH1* and *hMSH2* genes was performed in the proband. After PCR amplification of all exons including flanking intronic regions, amplicons were directly sequenced.

**RESULTS:** The mutation was found in nine from the thirteen pedigree members who signed informed consent to participate in the study. In three adenocarcinomas, microsatellite instability and lack of the MLH1 protein expression were detected. The only one tubulovillous adenoma analyzed was microsatellite stable and the MLH1 protein showed an intact staining.

**CONCLUSION:** The newly described mutation c.31delC is HNPCC causative. Besides the typical clinical features of the syndrome, we found a specific pathologic manifestation such as moderate to high differentiated adenocarcinomas of the colon. One of the mutation carriers developed a benign giant cell soft tissue tumor. The primary tumor localizations were frequently extracolonic and detailed yearly gastrointestinal and gynecological examinations have been proposed to the mutation carriers. We emphasize the importance of including the HNPCC genetic counseling and testing as well in the following surveillance of all patients at risk in the services covered by the health insurance in Bulgaria.



**Key words:** Colon cancer; Hereditary non-polyposis colorectal cancer; *MLH1*; Microsatellite instability

Kadiyska TK, Kaneva RP, Nedin DG, Alexandrova AB, Gegova AT, Lalchev SG, Christova T, Mitev VI, Horst J, Bogdanova N, Kremensky IM. Novel *MLH1* frameshift mutation in an extended hereditary nonpolyposis colorectal cancer family. *World J Gastroenterol* 2006; 12(48): 7848-7851

http://www.wjgnet.com/1007-9327/12/7848.asp

## INTRODUCTION

Hereditary non-polyposis colorectal cancer (HNPCC) or Lynch syndrome is the most common type of hereditary colorectal cancer (CRC), which accounts for about 1% to 3% of all cases with CRC[1] and may be caused by germline mutations in DNA mismatch repair (MMR) genes. Mutations in the *MLH1*[2,3] and *hMSH2*[4,5] genes are responsible for the disease in the majority of HNPCC families. Some mutations have been found to be common in many population studies, whereas others are rare or unique[6] (http://www.insight-group.org/). When a predisposing mutation is found in the proband, the carrier status of the first degree relatives might be clarified after genetic counseling and signing of informed consent[7]. This process is hard and delicate. One of the most frequently observed problems is the anxiety from the result. For all mutation carriers, a prophylactic program is proposed[8]. In Bulgaria, the genetic counseling and testing in hereditary cancer syndromes and the following surveillance for the individuals at risk are not defined or covered by the health insurance. In the period of 1998-2006 the genetic counseling and testing have been financially supported by the National Science Fund

research grants. Our team analyzes individually the pedigrees with heterogenic localizations and particular clinical features, together with the official result from the DNA analysis. Mutation carriers receive information about the exact prophylactic exams to take and the recommended frequency.

In this paper we report a novel frameshift (c.31delC [p.L11X]) mutation in the *MLH1* gene due to a deletion of a cytosine at nucleotide position c.31 in the first exon of the gene in a proband of an extended Bulgarian HNPCC family, fulfilling the Amsterdam criteria. The aim of this study was to analyze the molecular and clinical findings within the pedigree concerning the proposal of adequate individual prophylactic strategy for all mutation carriers.

## MATERIALS AND METHODS

The pedigree of the family consists of 42 members in four generations (Figure 1). The proband, a 39 year old man (Ⅲ-7) was operated because of cancer of the cecum. In the family, 15 members were operated on 21 malignant tumors (15 colorectal and 6 extracolonic). The mean age of the first malignant localization was 55 years for the first generation, 63 years for the second generation, 38 years for the third generation and 25 years for the last generation.

A set of five polymorphic markers-BAT26, D2S123, D5S346, D18S35 and FGA, previously found as the most informative in our group of HNPCC patients, have been selected for analysis of microsatellite instability (MSI). Both normal and tumor tissue DNA samples were amplified for the five markers and electrophoresis was performed on an automated fluorescence sequencer (ALF Express, Pharmacia). MSI analysis was possible in four patients (Table 1).

Search for mutations in the *MLH1* and *hMSH2* genes was performed in the proband Ⅲ-7. After PCR amplification of all exons including flanking intronic regions, amplicons were sequenced in both directions using the ABI PRISM Dye terminator cycle sequencing reaction kit (Applied Biosystems Foster City, CA) and ABI-310 Genetic Analyzer. The additional pedigree members were tested only for the presence of the identified mutation in the *MLH1* gene.

In order to investigate the effect of this mutation on the MLH1 protein expression, we chose the immunohistochemistry (IHC) assay. This analysis was performed in four patients with available paraffin-embedded specimens (Table 1). The rabbit polyclonal antibody against the C-terminus of the MLH1 protein (Santa Cruz Biotechnology, Santa Cruz, CA) at 1:100 dilutions was used, following the manufacturer's protocol, with minor modifications. Two investigators assessed the slides for MLH1 staining independently. Informed consent for DNA analysis was obtained from individuals included in the study.

## RESULTS

We identified a novel frameshift mutation in the *MLH1* gene, due to a deletion of a cytosine (c.31delC [p.L11X]) leading to a stop codon 16 (TGA), 18 bases downstream (Figure 2). The mutation was found in nine out of the thirteen pedigree members who signed informed consent to participate in the study (Table 1). Individual Ⅱ-1 developed a colorectal cancer at the age of 80 years. Now he is 96 years old and none of his descendents developed cancer. The *MLH1* mutation c.31delC was excluded in this family member.

**Table 1 Diagnosis, microsatellite instability, c.31delC mutation and immunohistochemical analysis of ten affected and six healthy at risk relatives in the hereditary non-polyposis colorectal cancer (HNPCC) family**

| Family member | Diagnosis | Histological data | MSI Analysis | c.31delC mutation | MLH1 expression |
|---|---|---|---|---|---|
| Ⅱ-1 | Colon carcinoma | NT | NT | No | NT |
| Ⅱ-3 | Colon carcinoma | + | NT | NT | NT |
| Ⅲ-6 | Colon carcinoma | + | NT | NT | NT |
| Ⅲ-7 | Colon carcinoma | + | MSI | Yes | NS |
| Ⅲ-8 | Endometrial carcinoma | + | NT | Yes | NT |
| Ⅲ-12 | Healthy, at risk | NT | NT | Yes | NT |
| Ⅳ-13 | Healthy, at risk | NT | NT | Yes | NT |
| Ⅳ-14 | Colon carcinoma | + | MSI | Yes | NS |
| Ⅳ-15 | Tubulovillous Adenoma | + | MSS | Yes | PS |
| Ⅳ-16 | Healthy, at risk | NT | NT | No | NT |
| Ⅳ-17 | Healthy, at risk | NT | NT | Yes | NT |
| Ⅳ-18 | Colon carcinoma | + | NT | Yes | NT |
| Ⅳ-19 | Healthy, at risk | NT | NT | No | NT |
| Ⅳ-20 | Healthy, at risk | NT | NT | No | NT |
| Ⅳ-21 | Mixed germ cell tumor | + | NT | NT | NT |
| Ⅳ-22 | Colon carcinoma | + | MSI | Yes | NS |

NT: not tested; +: available; MSI: microsatellite instability of the tumor; MSS: microsatellite stability of the tumor; Yes: mutation was present; No: mutation was absent; PS: positive staining; NS: negative staining.

All three adenocarcinomas available from CRC patients for MSI analysis showed high instability. The IHC assay showed lack of expression of the MLH1 protein in these tumors. In contrast, microsatellite stability was characteristic of the tubulovillous adenoma and ICH of the tumor showed an intact nuclear staining.

From the remaining 23 members of this branch of the family, fourteen were operated on 20 different malignant tumors, four adenomas and one benign giant cell tumor of soft tissues. Synchronous tumors were found in three individuals, and metachronous in five. Eight colorectal cancers were right sided, three were left sided and no information on the exact tumor localization of the remaining three was available. Only one of the left sided colorectal tumors was the primary localization. The histomorphological study showed that all malignant colorectal tumors were adenocarcinomas, two lowly and nine moderately to highly differentiated. Mucinous production (predominantly extracellular type) was detected in all cases.

## DISCUSSION

In the present study we describe a novel *MLH1* mutation c.31delC in relation to the HNPCC phenotype and

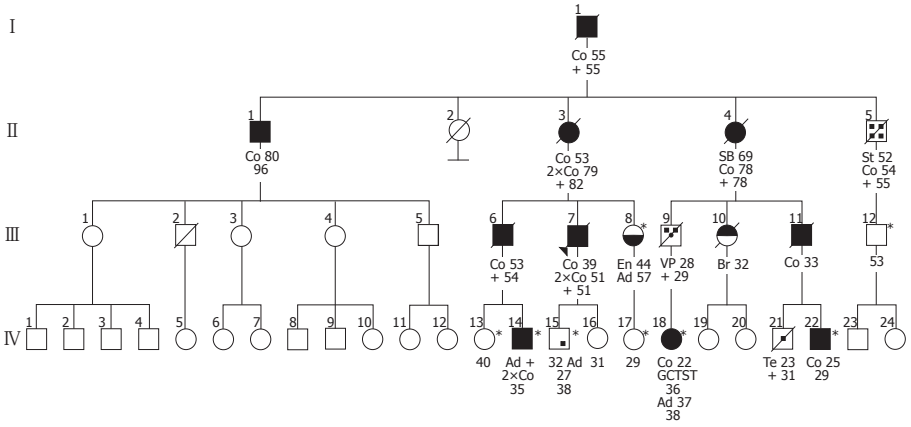


**Figure 1** Pedigree tree of the investigated family. Spouses, some of the unaffected and clinically undefined individuals are not presented in the figure. Co: colorectal cancer; VP: vater's Papilla cancer; SB: small bowel cancer; En: endometrial cancer; St: stomach cancer; Br: breast cancer; Te: testicular GCT; Ad: adenoma; GCTST: germ cell tumor of the soft tissue; *healthy mutation carriers.

MLH1 protein expression in colon tumors. Frameshift mutations are frequently found in both *MLH1* and *hMSH2* genes. Bisgaard *et al*[9] identified a mutation in the *MLH1* gene, c.9delC, resulting in a premature stop at the same location (codon 16) as in our study. In the family a skipped generation was present. However the authors conclude that stop codon and exon deletion mutations can be implemented for predictive testing without further analysis. In our study ICH and MSI analyses were used additionally to evaluate the role of this mutation in three adenocarcinomas and one adenoma.

Our genetic analysis showed that the CRC in Ⅱ-1 was not due to the mutation c.31delC. The late age of cancer development (80 years) in this patient, lack of metachronous carcinomas 15 years later and the lack of affected descendents support the sporadic origin of the malignant tumor in this case. Thanks to our study, this branch of the family was relieved of the excessive fear of inherited cancer. Patient Ⅳ-18 developed mixed germ cell tumor (GCT). He provided detailed clinical and genealogical information before his death but refused DNA analysis. The relation between c.31delC/*MLH1* and the risk of breast cancer could not be confirmed, since both daughters of Ⅲ-10 were not carriers of the mutation.

We found the typical features for HNPCC as early age of cancer development, dominant inheritance, high MSI and lack of detectable protein expression in the adenocarcinomas. The colorectal cancers were the most frequent lesions in the family, predominantly right sided, with extracellular mucinous production, presence of synchronous and metachronous tumors. All these observations support the data published by others[10-12]. Interesting findings were the anticipation in the last three generations and the moderate to high colorectal tumor differentiation. High differentiation has been described as specific for Chinese HNPCC families only[13].

The MLH1 protein expression in patient Ⅳ-15 was retained and the tumor showed microsatellite stability (MSS). Protein expression in a mutation carrier with adenoma was described by Stormorken *et al*[14]. The authors speculated that this event might be due to the sporadic origin of the adenoma or that the tumor did not reach the stage of protein loss. The early stage of the tumor diagnosis in our case might explain the presence of intact MLH1 protein, due to the proper functioning of the second, unaffected *MLH1* copy.

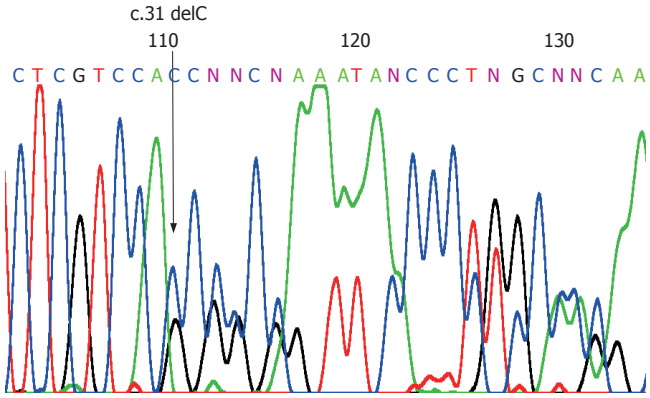


**Figure 2** Direct sequencing of the *MLH1* exon 1, demonstrating the mutation c.31delC. The arrow indicates the position of the C deletion.

The observed extracolonic malignant tumor localizations related to this mutation were in endometrium, stomach, Vater's papilla and small bowel. The last two are relatively rarely associated with HNPCC[15] and may be missed by the routine endoscopy.

Besides the tumors involved in the HNPCC spectrum, one of the family members (Ⅳ-23) developed a benign giant cell soft tissue tumor of the third finger, fourteen years after the primary colon localization. We have no data about the finding of such a metachronous tumor in other HNPCC families.

We conclude that the newly described mutation c.31delC is HNPCC causative. Besides the typical clinical features of the syndrome, we have found a specific pathologic manifestation as moderately to highly differentiated adenocarcinomas of the colon. The primary tumor localizations are frequently extracolonic and detailed yearly gastrointestinal and gynecological examinations have been proposed to the mutation carriers. We emphasize the importance of including the HNPCC genetic counseling and testing as well in the following surveillance of all patients at risk in the services covered by the health insurance in Bulgaria.

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*RAPID COMMUNICATION*

# Expression patterns and action analysis of genes associated with physiological responses during rat liver regeneration: Innate immune response

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## Abstract

**AIM:** To study the relationship between innate immune response and liver regeneration (LR) at transcriptional level.

**METHODS:** Genes associated with innate immunity response were obtained by collecting the data from databases and retrieving articles. Gene expression changes in rat regenerating liver were detected by rat genome 230 2.0 array.

**RESULTS:** A total of 85 genes were found to be associated with LR. The initially and totally expressed number of genes at the phases of initiation [0.5-4 h after partial hepatectomy (PH)], transition from G0 to G1 (4-6 h after PH), cell proliferation (6-66 h after PH), cell differentiation and structure-function reconstruction (66-168 h after PH) was 36, 9, 47, 4 and 36, 26, 78, 50, respectively, illustrating that the associated genes were mainly triggered at the initial phase of LR and worked at different phases. According to their expression similarity, these genes were classified into 5 types: 41 up-regulated, 4 predominantly up-regulated, 26 down-regulated, 6 predominantly down-regulated, and 8 approximately up/down-regulated genes, respectively. The expression of these genes was up-regulated 350 times and down-regulated 129 times respectively, demonstrating that the expression of most genes was enhanced while the expression of a small number of genes was decreased during LR. Their time relevance was classified into 14 groups, showing that the cellular physiological and biochemical activities during LR were staggered. According to the gene expression patterns, they were classified into 28 types, indicating that the cellular physiological and biochemical activities were diverse and complicated during LR.

**CONCLUSION:** Congenital cellular immunity is enhanced mainly in the forepart, prophase and anaphase of LR while congenital molecular immunity is increased dominantly in the forepart and anaphase of LR. A total of 85 genes associated with LR play an important role in innate immunity.



**Key words:** Partial hepatectomy; Rat genome 230 2.0 array; Innate immune response; Genes associated with liver regeneration

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## INTRODUCTION

Organisms can resist and remove endogenous and exogenous poisons *via* their innate immune cells and other factors. This process is known as innate immune response[1], a self-protection mechanism of living organisms which is absolutely indispensable to their survival[2]. Innate immune responses consist of three parts, namely barrier of self-tissue, innate cellular immunity and innate molecular immunity. Tissue barrier can excrete antibacterial and bactericidal matters to kill pathogens, innate cellular immunity can not only remove pathogens invading body *via* immune cells but also clear the broken, dead and abnormal cells, while innate molecular immunity can demolish and dissolve injurious substances *via* active molecules and cytokines[3]. Liver containing NK cells, T lymphocytes, macrophages, *etc*, is an important organ where innate immune response takes place[4]. After partial hepatectomy (PH), liver undergoes severe injury. How the remnant liver cells are protected by the innate immune system deserves intensive study[5].

In addition, PH[6] can activate the remaining hepatocytes to rapidly proliferate and compensate for the

loss of liver mass, which is known as liver regeneration (LR)[7,8]. Based on the cellular physiological activities, the regeneration proceeding is usually categorized into four stages: initiation (0.5-4 h after PH), transition from G0 to G1 (4-6 h after PH), cell proliferation (6-66 h after PH), cell differentiation and reorganization of the structure-function (66-168 h after PH)[8]. According to the time course, it can be classified into four phases: forepart (0.5-4 h after PH), prophase (6-12 h after PH), metaphase (16-66 h after PH), and anaphase (72-168 h after PH)[9], involving many physiological and biochemical events, such as cell activation, cell de-differentiation, cell proliferation and its regulation, cell re-differentiation, reorganization of structure-function[10], which are regulated by many factors including innate immune response. The action of genes associated with liver diseases caused by hepatitis virus infection and pathogen infection during LR, has been analyzed[11-13]. In the present study, rat genome 230 2.0 array containing 151 genes involved in innate immune response was used to detect the gene expression changes in regenerating liver after 2/3 hepatectomy as previously described[14-17] in order to investigate the relevance between LR and innate immune response at transcriptional level. The expression changes, patterns and action of these genes during LR were primarily analyzed. Our results indicate that 85 out of the 151 genes are associated with LR[18].

## MATERIALS AND METHODS

### *Regenerating liver preparation*

Healthy SD rats weighing 200-250 g were obtained from the Animal Center of Henan Normal University. The rats were divided into groups at random, 6 rats in each group (male: female = 1:1). PH was performed as previously described[6], the left and middle lobes of liver were removed. The rats were killed by cervical vertebra dislocation at 0.5, 1, 2, 4, 6, 8, 12, 16, 18, 24, 30, 36, 42, 48, 54, 60, 66, 72, 96, 120, 144 and 168 h after PH and the regenerating livers were observed at corresponding time points. The livers were rinsed three times in PBS at 4℃, then 100-200 mg liver tissue was taken from middle part of the right lobe. Six samples were collected from each group and mixed into 1-2 g (0.1-0.2 g × 6) liver tissue, then stored at -80℃. The sham-operation (SO) groups underwent the same PH without removal of the liver lobes. The animal protection laws of China were strictly followed.

### *RNA isolation and purification*

Total RNA was isolated from frozen livers according to the manual of Trizol kit (Invittrogen)[19] and then purified based on the guide of RNeasy mini kit (Qiagen)[20]. Agarose electrophoresis (180V, 0.5h) showed that total RNA sample exhibited a 2:1 ratio of 28S to 18S rRNA intensities. Total RNA concentration and purity were estimated by optical density measurements at 260/280 nm[21].

### *cDNA, cRNA synthesis and purification*

Total RNA (1-8 μg) was used as a template for cDNA synthesis. cDNA and cRNA synthesis was proceeded as previously described[16]. cRNA labeled with biotin was synthesized using 12 μL synthesized cDNA as a template, cDNA and cRNA were purified[16]. Measurement of concentration, purity and quality of cDNA and cRNA was performed as previously reported[21].

### *cRNA fragmentation and microarray detection*

Fifteen μL (1 μg/μL) cRNA incubated with 5 × fragmentation buffer at 94℃ for 35 min was digested into 35-200 bp fragments. The hybridization buffer was added to the prehybridized Rat Genome 230 2.0 microarry produced by Affymetrix, and then hybridization was carried out for 16 h at 45℃ on a rotary mixer at 60 rpm. The microarray was washed and stained by GeneChip fluidics station 450 (Affymetrix Inc., USA). The chips were scanned by GeneChip scan 3000 (Affymetrix Inc., USA), and the signal values of gene expression were observed[17].

### *Microarray data analysis*

The normalized signal values, signal detections (P, A, M) and experiment/control (Ri) were obtained by quantifying and normalizing the signal values using GCOS1.2[17].

### *Normalisation of microarray data*

To minimize the errors in microarray analysis, each analysis was performed three times by rat genome 230 2.0 microarray. Results with a maximal total ratio ($R^m$) and an average of three housekeeping genes β-actin, hexokinase and glyseraldehyde-3-phosphate dehydrogenase approaching 1.0 ($R^h$) were taken as a reference. Modified data were generated by applying a correction factor ($R^m/R^h$) multiplying the ratio of every gene in Rh at each time point. To remove spurious gene expression changes resulting from errors in the microarray analysis, the gene expression profiles at 0-4 h, 6-12 h and 12-24 h after PH were reorganized by normalization analysis program (NAP) software according to the cell cycle progression of regenerating hepatocytes. Data statistics and cluster analysis were done using the GeneMath, GeneSpring, Microsoft Excel softwares[17,22,23].

### *Identification of genes associated with liver regeneration*

First, the nomenclature of innate immune response was adopted from the GENEONTOLOGY database (www.geneontology.org) and input into NCBI (www.ncbi.nlm.nih.gov) and RGD (rgd.mcw.edu) to identify the rat, mouse and human genes associated with the biological process. According to the maps of biological pathways embodied by GENMAPP (www.genmapp.org), KEGG (www.genome.jp /kegg/pathway.html#amino) and BIOCARTA (www.biocarta.com/genes/index.asp), genes associated with innate immune response were collated. The results of this analysis were codified and compared with those obtained for humans and mice in order to identify human and mouse genes which are different from those of rats. Comparing these genes with the analysis output of rat genome 230 2.0 array, genes showing more than twofold change in expression level as meaningful expression changes[18], were referred to as rat homologous or rat specific genes associated with innate immune response

**Table 1 Expression of 85 innate immune response-associated genes during rat liver regeneration**

| Gene Abbr. | Associated to others | Fold difference | Gene Abbr. | Associated to others | Fold difference | Gene Abbr. | Associated to others | Fold difference | Gene Abbr. | Associated to others | Fold difference |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Innate immune cells | | | Colec12 | | 3.9 | Bdkrb2 | 3 | 0.4 | Cxcl10 | | 0.3, 9.2 |
| 1 Macrophage | | | Crp | | 0.5 | [1]Il1b | 1 | 0.4 | Cxcl12 | | 0.2 |
| Adora2a | | 0.5 | Hrh1 | 1 | 0.5, 9.9 | Il1f5 | 1 | 0.4, 2.8 | Darc | | 0.4, 8.5 |
| Anxa1 | | 4.3 | Hrh4 | 1 | 7.5 | Il1r1 | 1 | 0.5 | Others | | |
| Cebpb | | 3.1 | Mcpt6 | 1, 2 | 0.2 | Il1rn | 1 | 16.3 | Parp4 | | 0.5 |
| Clec7a | | 0.2 | Nr3c1 | | 4.7 | Il2 | | 0.3, 3.5 | Alox5 | | 0.2, 2.5 |
| Cybb | | 2.5 | Spp1 | | 0.5, 2.7 | Il5 | | 3.5 | Alox5ap | | 4.9 |
| Ereg | 2 | 0.4 | Nfatc4 | | 0.5 | Sarm1 | | 0.2, 4.3 | Apoe | | 0.1 |
| Ltb4r | | 0.5, 8.7 | Innate immune effectors | | | Sele | 3 | 12.9 | Casp12 | | 0.4,2.6 |
| Mif | | 3.2 | 4 Complement system | | | b Interferon and related factors | | | Dmbt1 | | 9.8 |
| Pap | | 68.6 | C1qa | | 0.3 | Ifnk | | 0.1, 5.7 | Hck | | 0.4 |
| Pla2g4a | | 2 | C1qr1 | 1, 2 | 5.5 | Ddx58 | | 11.8 | Map2k3 | | 0.4 |
| Ptgs2 | | 0.1, 2.1 | C2 | | 2.1 | Irf3 | | 2.6 | Prkca | | 4.6 |
| S100a8 | | 6.5 | [1]C3 | | 0.2 | Mx2 | | 9.4 | Ptafr | | 7.1 |
| S100a9 | | 4.9 | C3ar1 | | 0.3, 2.3 | c Iumor necrosis factor and related factors | | | Reg3a | | 0.1, 64.1 |
| Tgfb1 | 2, 3 | 4.0 | C4a | | 0.5 | Ager | | 0.4 | Reg3g | | 0.3, 7.5 |
| 2 NK cell | | | C4bpa | | 2.0 | Myd88 | | 2.1 | | | |
| Ncr3 | | 0.3 | C5ar1 | | 0.4, 2.6 | Tnf | 1 | 3.2 | | | |
| Ptger3 | | 0.2 | Cfh | | 2.5 | d Chemotactic numerator and telated factors | | | | | |
| Rela | | 0.5 | Cfi | | 6.4 | Ccl17 | | 0.1 | | | |
| Ripk2 | | 0.4 | Cr2 | 3 | 6.0 | Ccl19 | | 3.9 | | | |
| Tlr2 | | 10.6 | Crry | | 2.4 | Ccl2 | | 128.0 | | | |
| Tlr4 | | 0.5 | Masp1 | | 3.0 | Ccl20 | | 8.0 | | | |
| 3 Other cells | | | Mbl2 | | 0.2 | Ccl24 | | 4.0 | | | |
| Aoc3 | 1 | 6.1 | 5 Cell factors | | | [1]Ccl4 | | 0.2, 3.0 | | | |
| Atrn | 1 | 4.4 | a Interlenkin and related factors | | | Ccl7 | | 22.6 | | | |
| Clu | 1, 2 | 3.0 | Bcl3 | 1 | 0.4 | Ccr1 | | 0.4,.24.9 | | | |

[1]Reported genes associated with liver regeneration; associated to others: genes are involved in another kind of the responses besides one kind of innate immune responses; others: other genes associated with innate immune response, but cannot be clearly categorized.

under evaluation. Genes displaying reproducible results in three independent analyses with the chip and more than twofold change in expression level at least at one time point during LR with a significant difference ($P \leqslant 0.01 < 0.05$) or an extremely significant difference ($P \leqslant 0.01$) between PH and SO, were referred to as genes associated with liver regeneration.

## RESULTS

### *Expression changes of innate immune response-associated genes during liver regeneration*

According to the data from databases at NCBI, GENEMAP, KEGG and BIOCARTA, a total of 275 genes are involved in innate immune response, of which 151 are contained in the rat genome 230 2.0 array. In the resent study, 85 out of these 151 genes revealed meaningful expression changes at least at one time point after PH, showing a significant difference or an extremely significant difference in expression when PH was compared with SO and reproducible results checked by three detects with rat genome 230 2.0 array, suggesting that the genes were associated with LR. Their expression was up-regulated 2 to 128 times and down-regulated 2 to 10 times of the control (Table 1). The expression was up-regulated in 41, down-regulated in 26, and up/down-regulated in 18 genes, respectively, during LR. The expression of these 85 genes was up-regulated 350 times and down-regulated 129 times (Figure 1A). At the initiation stage of LR (0.5-4 h after PH), the expression was up-regulated in 28 and down-regulated in 8 genes. At the transition phase from G0 to G1 (4-6 h after PH), 21 was up-regulated in 21 and down-regulated in 5 genes. At cell proliferation period (6-66 h after PH), the expression was up-regulated in 42, down-regulated in 29, and up/down-regulated in 8 genes, respectively. At cell differentiation and reorganization of the structure-function stage (66-168 h after PH), the expression was up-regulated in 32, down-regulated in 14, and up/down-regulated in 4 genes, respectively (Figure 1B).

### *Initiation expression time of innate immune response-associated genes during liver regeneration*

At each time point of LR, the number of initially and totally up-regulated, down-regulated genes respectively was both 10 and 2 at 0.5 h; 7,6 and 15, 6 at 1 h; 7, 0 and 18, 1 at 2 h; 4, 0 and 20, 1 at 4 h; 1, 4 and 14, 5 at 6 h; 0, 0 and 16, 3 at 8 h ; 1, 0 and 19, 3 at 12 h ; 7, 4 and 19, 4 at 16 h ; 3, 9 and 19, 14 at 18 h ; 1, 0 and 17, 14 at 24 h ; 0, 3 and 12, 8 at 30 h ; 1, 3 and 19, 9 at 36 h ; 1, 1 and 15, 5 at 42 h ; 2, 1 and 26, 12 at 48 h; 0, 2 and 15, 11 at 54 h ; 0, 1 and 16, 7 at 60 h ; 0, 0 and 12, 4 at 66 h ; 0, 0 and 10, 4 at 72 h ; 1, 0 and 14, 7 at 96 h ; 3, 0 and 18, 6 at 120 h ; 0, 0 and 12, 5 at 144 h ; 0, 0 and 14, 6 at 166 h (Figure 2). Generally, gene expression changes occurred during the

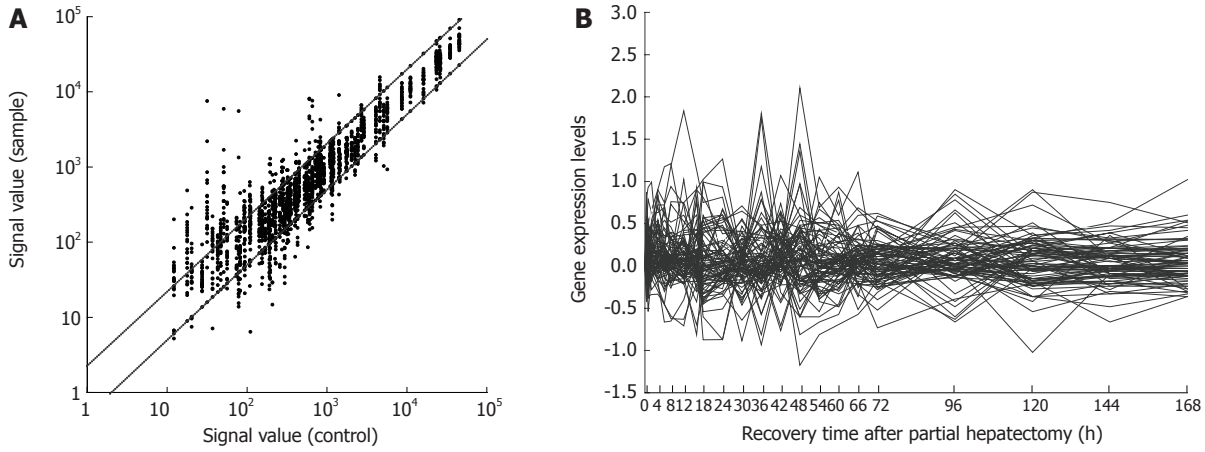


**Figure 1** Expression frequency (**A**) and changes (**B**) of 85 innate immune response-associated genes during rat liver regeneration. Data detected by rat genome 230 2.0 array were analyzed and graphed by Microsoft Excel. The dots above bias indicate that the expression of genes was increased more than two folds and up-regulated 350 times, the dots under bias indicate that the expression of genes was decreased more than two folds and down-regulated 129 times, the dots between biases indicate that the expression of genes has no alteration. The expression of 59 genes was increased 2-128 folds, while the expression of 44 genes was increased 2-10 folds.

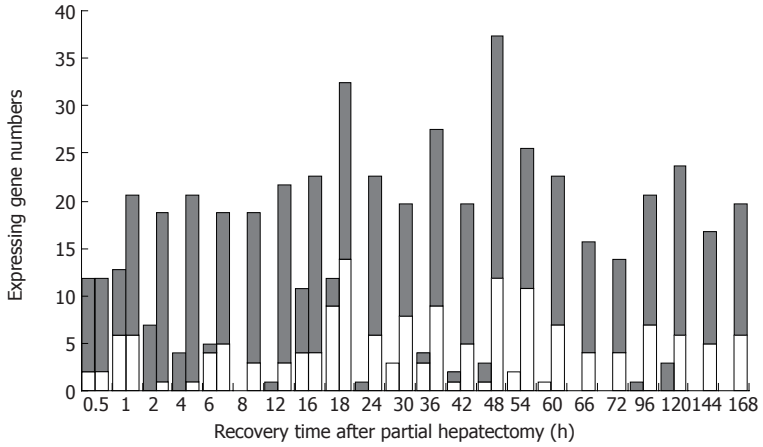


**Figure 2** Initial and total expression profiles of 85 innate immune response-associated genes at each time point of liver regeneration. Grey bars: up-regulated expression gene; white bars: down-regulated expression gene; blank bars: initially expressed genes in which up-regulated genes are predominant in the forepart, and down-regulated genes in the prophase and metaphase, whereas very few in the anaphase; dotted bars: the total number of expressed genes, in which the expression of some genes is up-regulated and the expression of others is down-regulated during LR.

whole LR. The expression of these genes was up-regulated 350 times and down-regulated 129 times. The expression of the genes was predominantly initially up-regulated in the forepart, and initially down-regulated in the prophase and metaphase, whereas the initial expression of very few genes was observed in the anaphase.

### *Expression similarity and time relevance of innate immune response-associated genes during liver regeneration*

Based on their similar expression, the 85 genes during LR could be divided into 41 up-regulated, 4 predominantly up-regulated, 26 down-regulated, 6 predominantly down-regulated, and 8 up/down-regulated genes, respectively (Figure 3). Based on their time relevance, they could also be classified into 14 groups (0.5 h, 1 and 66 h, 2 h, 4 and 8 h, 12 and 36 h, 16 and 96 h, 18 and 24 h, 30 and 42 h, 48 h, 54 and 60 h, 72 h, 120 h, 144 and 168 h), in which their expression was up- and down-regulated at 10 and 2 h, 27 and 10 h, 18 and 1 h, 20 and 1 h, 30 and 8 h, 38 and 12 h, 33 and 11 h, 36 and 20 h, 26 and 12 h, 31 and 18 h, 10 and 4 h, 18 and 6 h, 26 and 11 h (Figure 3). The up-regulated expression of genes was mainly associated with cellular immunity. The down-regulated expression of genes was significantly associated with molecular immunity.

### *Expression patterns of innate immune response-associated genes during liver regeneration*

According to their expression changes during LR, the patterns of the above 85 genes might be categorized into 28 types: 5 up-regulated genes at one time point, i.e. at 4, 48, 96, 120 h after PH (Figure 4A); 4 up-regulated genes at two time points, i.e. at 12 and 60 h, 42 and 120 h, 16 and 42 h (Figure 4B); 1 up-regulated gene at three time points (Figure 4B); 5 up-regulated genes at more time points (Figure 4B); 3 up-regulated genes at one phase, i.e. at 0.5-8 h, 4-8 h, 120-168 h (Figure 4C); 1 up-regulated gene at two phases, i.e. at 16-36 h, 42-48 h (Figure 4C); 1 up-regulated gene at three phases (Figure 4C); 1 up-regulated gene at more phases (Figure 4C); 1 up-regulated gene at one time point/one phase, i.e. at 120 and 2-72 h, 48 and 2-24 h, 18 and 48-60 h, 42 and 120-168 h (Figure 4D); 1 up-regulated gene at two time points/three phases (Figure 4E); 1 up-regulated gene at two time points/one phase (Figure 4E); 1 up-regulated at one time point/ three phases (Figure 4E); 1 up-regulated gene at two time points/three phases (Figure 4E); 3 up-regulated genes at two time points/two phases (Figure 4E); 1 up-regulated gene at three time points/ one phase (Figure 4F); 1 up-regulated gene at three time points/two phases (Figure 4F); 3 up-regulated genes at

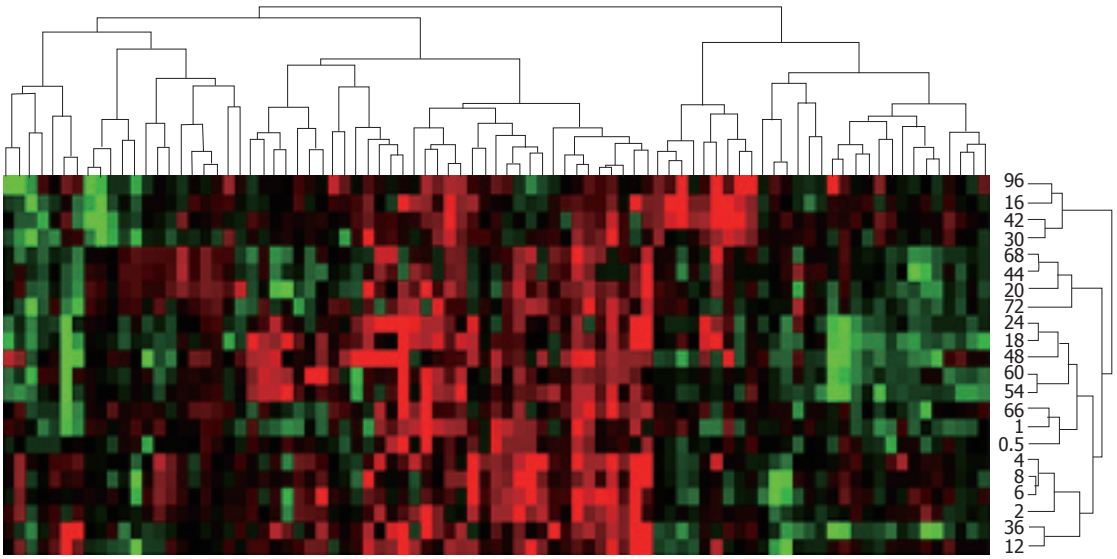


**Figure 3** Expression similarity and time relevance clusters of 85 innate immune response-associated genes during liver regeneration. Data detected by rat genome 230 2.0 array were analyzed by H-clustering. Red indicates up-regulated gene expression chiefly associated with cellular immunity; green indicates down-regulated gene expression mainly associated with molecular immunity; black indicates meaningless change in gene expression. The upper and right trees show the expression similarity and time series clusters, by which the above genes were classified into 5 and 14 groups respectively.

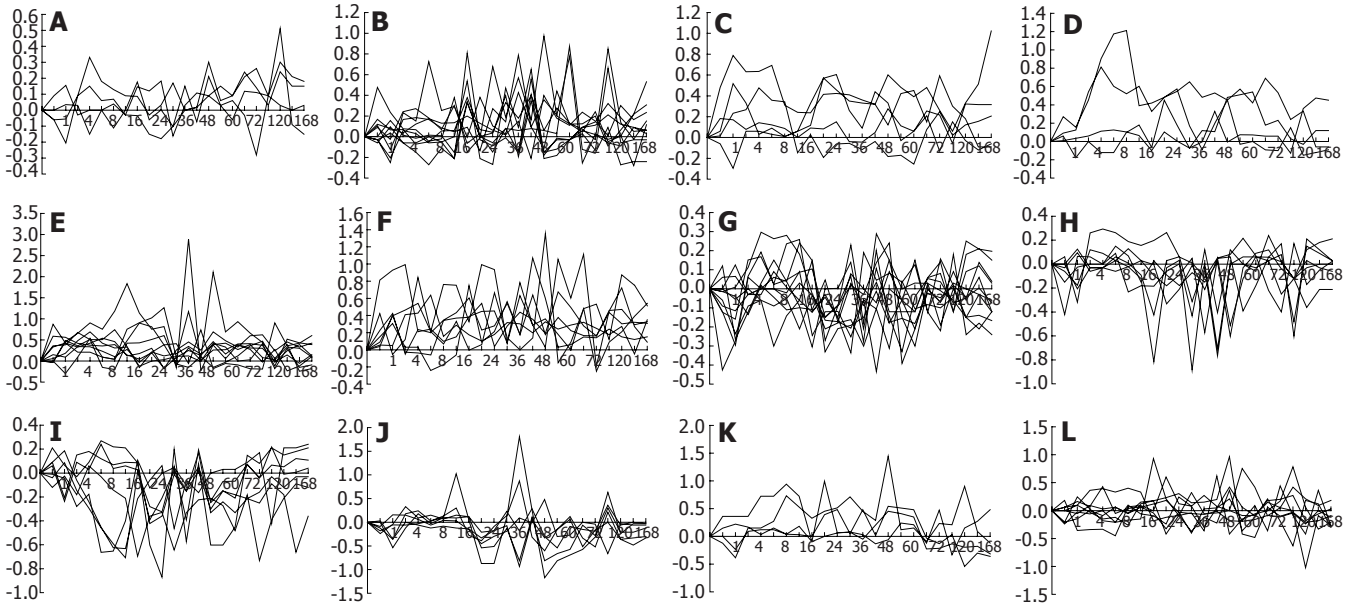


**Figure 4** Expression patterns of 85 innate immune response-associated genes during liver regeneration. Twenty-eight expression patterns were obtained by the analysis of data detected by rat genome 230 2.0 array with Microsoft Excel. **A**-**F**: 41 up-regulated genes; **G**-**I**: 26 down-regulated genes; **J**-**L**: 18 up/down-regulated genes. X-axis represents recovery time after PH (h); Y-axis shows logarithm ratio of the signal values of genes at each time point to control.

more phases (Figure 4F); 11 down-regulated genes at one time point, i.e. 0.5, 6, 16, 18, 30, 36, 42, 48, 54, 60 h (Figure 4G); 5 down-regulated genes at two time points, i.e. at 0.5 and 48, 1 and 72, 18 and 54, 30 and 42h (Figure 4H); 1 down-regulated gene at three time points (Figure 4H); 2 down-regulated genes at more time points (Figure 4H); 2 down-regulated genes at two time points/one phase (Figure 4I); 1 down-regulated gene at one phase, i.e. 6-12h (Figure 4I); 2 down-regulated genes at one time point/two phases (Figure 4I); 2 down-regulated genes at two time points/one phase (Figure 4I); 6 first down- and then up-regulated genes (Figure 4J); 4 first up- and then down-regulated genes (Figure 4K); 8 up/down-regulated genes (Figure 4L).

## DISCUSSION

Innate immune response, which is a self-protection mechanism formed during the long-term evolutionary process, includes tissue barrier, innate cellular immunity and innate molecular immunity, being closely linked to existence of high animal[2]. Of the proteins associated with innate cellular immunity, seven proteins including toll-like receptor 2 (TLR2) have a role in recognition of pathogens, interferon excretion of NK cells and activation of congenital immune system[24,25]; four proteins including attractin (ATRN) positively regulate antigen representation[26,27]; fifteen proteins including CCAAT/

enhancer binding protein beta (CEBPB) and S100 calcium binding protein A8 (S100A8) promote macrophage phagocytosis[28-32]; macrophage migration inhibitory factor (MIF), also called glycosylation-inhibiting factor, inhibits the function of macrophages[33]; glucocorticoid receptor (NR3C1) inhibits action of dendritic cells[34]. In the present study, the expression of the above genes was identical or similar at some time points, while different at other time points, indicating that they co-regulate cellular immune response. Among them, *cebpb* expression was up-regulated at 0.5-8 h after PH and reached its peak at 1 h, which was 3.1 folds of the control. *s100a8* expression was up-regulated at 2-72 h and 120 h after PH, and reached its peak at 4 h, which was 6.5 folds of the control. *nr3c1* and *tlr2* expression was up-regulated at multiple phases during LR and reached their peak at 168 h and 42 h respectively, which was 4.7 folds and 10.6 folds of the control. *atrn* expression was up-regulated mainly at the metaphase, showing that the highest expression at 42 h was 4.4 folds that of the control. These findings suggest that the genes are the key to innate cellular immunity in regenerating liver.

Of the proteins associated with innate molecular immunity, eleven proteins, such as chemokine C-C motif ligand 2, 4 (CCL2, CCL4), are associated with recognition of pathogens and immune enhancement[35-38]. Two proteins, such as annan-binding lectin serine peptidase 1 (MASP1), activate the complement system[39]. Five proteins including complement component 2 (C2) enhance inflammation[40,41]. Four proteins including interferon regulatory factor 3 (IRF3) interfere with the multiplication of virus[42]. Two proteins including complement component 1 q subcomponent receptor 1 (C1QR1) are responsible for removal of apoptotic cells[43,44]. Ten proteins including interleukin 1 family member 5 delta (IL1F5) have a role in elimination of the pathogens in inflammatory response[45-47]. Three proteins including tumor necrosis factor (TNF) have the function of sterilization by promoting NK cell proliferation[48]. In the present study, the expression changes of the genes encoding these proteins were identical or similar at some time points and different at other time points, suggesting that they can co-modulate innate molecular immunity. *ccl4* expression was up-regulated only at 48 h after PH, which was 3-fold of the control. However, Masson *et al*[38] reported that it is down-regulated at 3 and 12 h. *ccl2* expression was up-regulated at 0.5-1, 12-24, 36, 48-72 and 120 h, and reached its peak at 48 h, which was 128-folds of the control. *c1qr1* expression was up-regulated at multiple time points post PH and reached its peak at 8 h, which was 5.6 folds of the control. *masp1* expression was up-regulated at 42 and 120-168 h and reached its peak at 144 h, which was 3 folds that of the control. These findings indicate that the genes are important in molecular immunity during LR.

In conclusion, the expression changes of congenital immune response-associated genes after rat PH can be analyzed with high-throughput gene expression assay. The congenital immunity is enhanced during LR. Rat Genome 230 2.0 array was a useful tool analyzing the above response at transcriptional level. Nevertheless, DNA→ mRNA→ protein is influenced by various factors including protein interaction. Therefore, such techniques as Northern blotting, protein chip, RNA interference, protein-interaction *etc*, are needed to further test the above results.

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*RAPID COMMUNICATION*

# Overexpression of Ets-like protein 1 in human esophageal squamous cell carcinoma

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## Abstract

**AIM:** To study the expression pattern of Ets-like protein 1 (Elk-1) in human esophageal squamous cell carcinoma (ESCC) and to analyze its relationship with clinicopathologic parameters.

**METHODS:** The expression of Elk-1 in fresh esophageal cancer tissues and their corresponding normal mucosae was detected immunohistochemically (IHC) by means of tissue microarray (TMA). Its correlation with clinical characteristics was evaluated and analyzed by univariate analysis. All statistical analyses were performed by SPSS version 13.0.

**RESULTS:** Expression level of transcription factor Elk-1 increased in 78.5% (84/107) ESCC tissues compared with their matched normal esophageal epithelium. However, the expression of Elk-1 did not show any obvious correlation with degree of differentiation of esophageal carcinoma (in well-differentiated, moderately-differentiated and poorly-differentiated tumors, the increased expression was 7/8, 60/74, and 19/25, respectively, $P > 0.05$). Moreover, no obvious correlation was found with lymph node metastasis and depth of invasion.

**CONCLUSION:** Increased expression of transcription factor Elk-1 may play an important role in esophageal carcinogenesis.



**Key words:** Ets-like protein 1; Esophageal squamous cell carcinoma; Immunohistochemistry; Tissue microarray

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## INTRODUCTION

Esophageal cancer ranks among the 10 most frequent cancers in the world, with a predominant distribution in developing countries. It is one of the most common malignant tumors in China[1,2]. Our previous study showed that genetic susceptibility to esophageal cancer is one of the important causes for the high prevalence and familial aggregation of this disease in some areas of northern China[3]. Ets-like protein 1 (Elk-1) is a member of the ternary complex factor (TCF) subfamily of E twenty-six (ETS)-domain transcription factors[4,5]. The three ternary complex factors (TCFs) Elk-1, Net and Sap-1 form a subfamily of the ETS domain transcription factors. Their characteristic property is the ability to form a ternary nucleoprotein complex with the serum response factor (SRF) over the serum response element (SRE) of the c-*fos* promoter. The molecular mechanisms underlying the function and regulation of these factors have been extensively studied and the TCFs are a paradigm for the study of transcriptional regulation in response to extracellular signalling through the mitogen-activated protein (MAP) kinase pathway. As final effectors of multiple signalling pathways and components of protein complexes on immediate early promoters, they represent key elements in the complex and dynamic regulation of gene expression[6].

Tissue microarray (TMA) was first introduced in 1998[7]. It is a high throughput technique that can significantly accelerate the processing of a large number of tissue specimens with excellent quality, good reliability and the preservation of original tissue. TMA studies can demonstrate their accuracy and reliability compared to those of standard histological techniques and correlate with clinicopathologic information to determine disease progression and prediction of the clinical outcome[8]. It allows simultaneous analysis of many tumors using small diameter cores sampled from larger blocks of tissue, but may be limited by tumor heterogeneity[9]. In this study, we used TMA to investigate the transcription factor Elk-1 in esophageal squamous cell carcinoma (ESCC), including

method for assessing immunohistochemical scoring of microarrays. TMA blocks were constructed from 107 cases of ESCC with corresponding normal tissues, taking two cores from different areas of each tumor and two cores from adjacent esophageal epithelia. Immunohistochemical labelling was performed for Elk-1. The extent and intensity of scoring were determined for each core and the degree of agreement was determined for results from the assessment of two, three or four cores for each case. The results show that TMA is a reliable tool to demonstrate cellular and molecular alterations in ESCC.

In this study, we investigated the protein expression of transcription factor Elk-1 in ESCC. The expression levels of Elk-1 increased in ESCC tissues compared with their normal counterparts. Therefore, Elk-1 might be related to human ESCC and further study on Elk-1 may provide insight into the mechanisms of carcinogenesis of esophagus.

## MATERIALS AND METHODS

### Materials

Specimens of cancer tissues and matched adjacent normal mucosa were taken from 107 consecutive patients with squamous cell carcinoma of the thoracic esophagus who underwent esophagectomy with regional lymph nodes dissected from July 2005 to April 2006 at the Department of Thoracic Surgery, the First Affiliated Hospital of Anhui Medical University. None of the patients received radiotherapy or chemotherapy before surgery. The patients included 79 men and 28 women with a median age of 60 (range 40-79) years. Fourteen tumors were located in the upper thorax, 60 in the middle thorax and 33 in the lower thorax (Table 1). The removed specimens were stained with hematoxylin and eosin, examined histologically, and then the clinicopathologic stage was determined according to TNM classification.

**Table 1 Clinical and histopathological characteristics of patients**

| Characteristic | *n* (%) |
|---|---|
| Sex | |
| Male | 79 (73.8) |
| Female | 28 (26.2) |
| Location of tumor | |
| Upper thoracic | 14 (13.1) |
| Middle thoracic | 60 (56.1) |
| Lower thoracic | 33 (30.8) |
| Degree of differentiation | |
| Well-differentiated | 8 (7.5) |
| Moderately-differentiated | 74 (69.2) |
| Poorly-differentiated | 25 (23.3) |
| Depth of invasion | |
| T1 | 6 (5.6) |
| T2 | 39 (36.4) |
| T3 | 62 (58.0) |
| T4 | 0 (0) |
| Lymph node metastasis | |
| Positive | 35 (32.7) |
| Negative | 72 (67.2) |

### Construction of tissue microarray

The collected samples were fixed with formalin and embedded with paraffin, and then tissue microarray was performed. Two pathologists selected representative areas from each donor tumor block, and then punched cores 1.0 mm in diameter, from the donor blocks, and then transferred these tissue cores to a recipient block using a tissue microarrayer (Beecher Instrument, Silver Spring, Maryland, USA). The resultant tissue microarray was cut into sections and transferred to glass slides for processing of Elk-1 by immunohistochemistry.

### Immunohistochemical staining

Immunohistochemical analysis was done retrospectively. Resected esophageal specimens, including both tumor and normal mucosae, were fixed in a 40 g/L formaldehyde solution and embedded in paraffin. Rabbit polyclonal IgG Elk-1 antibody (Santa Cruz Biotech Co, USA) was used in this study (diluted 1:100). Formalin-fixed and paraffin embedded tissue blocks of esophageal tumors were cut into 4-μm thick sections. TMAs were deparaffinized in xylene, rehydrated in ethanol and treated with 30 mL/L $H_2O_2$ for 30 min to block the endogenous peroxidase activity. Antigen retrieval was achieved by microwaving in 0.01 mol/L citrate buffer (pH 6.0) at 96℃ for 15 min. After incubation with 10% normal goat serum to block non-specific binding, they were then incubated with anti-Elk-1 antibody at 1:100 dilution overnight at 4℃. After antibody was washed in PBS, TMAs were incubated with the secondary antibody and the third antibody (Streptavidin/HRP) according to the manufacturer's instructions. Finally DAB was used as a chromogen and hematoxylin as a counterstain. Negative control was designed by using PBS instead of primary antibody.

### Assessment of staining

The percentage of Elk-1-positive tumor cells was determined semiquantitatively by assessing the entire tumor section and scored as: a = 0, < 5% of epithelial cells in the respective lesions; b = 1, 5%-25% of epithelial cells in the respective lesions; c = 2, 26%-50% of epithelial cells in the respective lesions; d = 3, 51%-75% of epithelial cells in the respective lesions; e = 4, > 75% of epithelial cells in the respective lesions. The intensity was graded as: a = 0, negative; b = 1 +, weak; c = 2 +, moderate; d = 3 +, strong. A final score between 0 and 12 was achieved by multiplication of the extent of positivity and intensity[10,11]. Positive staining of more than 5% in cell cytoplasm was defined as positive staining, less than 50% in cell cytoplasm as preserved expression, more than 50% in cell cytoplasm as increased expression[12].

### Statistical analysis

Paired-samples T test, chi square test or Fisher's exact probability test was used to assess the association between immunohistochemical features and clinicopathologic characteristics. A $P$ value less than 0.05 was considered statistically significant. All the statistical analyses were performed using the SPSS 13.0 V for Windows.

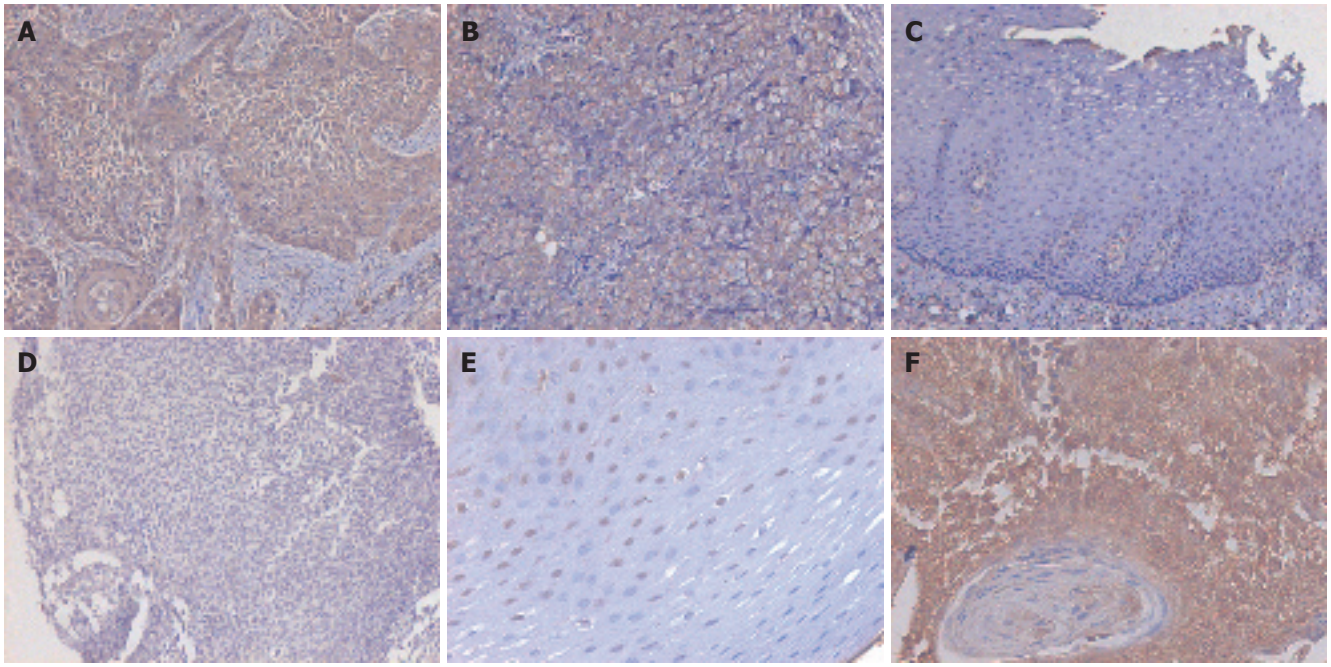


**Figure 1** Immunohistochemical analysis of Elk-1 in paired ESCC samples using anti-Elk-1 antibody (1:100) showing diffuse and strong staining in cytoplasm of esophageal cancer epithelial cells well-differentiated tumor (**A**), moderately-differentiated tumor (**B**), sporadic and weak staining in the cytoplasm of normal epithelial cells (**C**), negative control designed using PBS instead of primary antibody (**D**), strong staining in nuclei of normal epithelial cells (**E**) (**A-E** × 100), and in cytoplasm of well-differentiated esophageal cancer epithelial cells (**F**) (× 200).

## RESULTS

### *Expression of Elk-1 in esophageal squamous cell carcinoma*

Positive Elk-1 expression showed brown staining signals in ESCC cytoplasm and nuclei, reduced or negative expression of Elk-1 was found in normal squamous epithelium, with only a small number of expressions in cell membranes. The increased expression rate of Elk-1 in 107 esophageal cancer patients was 78.5% (84/107) compared to that in the matched normal tissue. A significant positive correlation was found in Elk-1 expression between esophageal carcinoma tissue and paired normal squamous epithelium ($P < 0.01$). The increased expression rate of Elk-1 was 80.4% (86/107) (Figure 1).

### *Relationship between Elk-1 expression and clinicopathologic variables in esophageal squamous cell carcinoma*

The expression of Elk-1 had no obvious correlation with the degree of differentiation of esophageal squamous cell carcinoma. The increased expression was found in 7/8 well-differentiated, 60/74 moderately-differentiated and 19/25 poorly-differentiated tumors, respectively, ($P > 0.05$). In addition, no significant correlation was found among Elk-1 expression, lymph node metastasis and depth of invasion (Table 2).

**Table 2 Relationship between clinicopathologic parameters and expression of Elk-1**

| Type | Case | Elk-1 | | *P* |
|---|---|---|---|---|
| | | Preserved | Increased | |
| Degree of differentiation | | | | |
| Well-differentiated | 8 | 1 | 7 | > 0.05 |
| Moderately-differentiated | 74 | 14 | 60 | |
| Poorly-differentiated | 25 | 6 | 19 | |
| Depth of invasion | | | | |
| Mucous layer (T1) | 6 | 1 | 5 | > 0.05 |
| Muscular layer (T2) | 39 | 6 | 33 | |
| Full-thickness (T3) | 62 | 14 | 48 | |
| Lymph node metastases | | | | |
| Positive | 35 | 5 | 30 | > 0.05 |
| Negative | 72 | 16 | 56 | |

## DISCUSSION

Regulations of cell growth are dependent on a number of gene families including proto-oncogene, growth factor, growth factor receptor and immediate early transcription factor gene. The first member of Ets gene family was discovered a decade ago by studying avian erythroblastosis virus, E twenty six (E26). Subsequently, a series of cellular Ets genes were isolated (Ets-1, Ets-2, Erg, Elk-1, Sap-1, PEA-3, PU.1, Fli-1 *etc.*)[13]. The Elk-1 gene is localized on human chromosomes Xp11.2-p11.1 and 14q32[14,15]. Elk-1 was first discovered in a fraction of HeLa cell nuclear extract that forms ternary complexes with SRF on the c-*fos* SRE. This novel component is called p62 due to its 62 kDa molecular mass[16]. Elk-1, Sap-1 and Sap-2/Net comprise the TCF subfamily of Ets-domain transcription factors. The TCF transcription factors play an important role in transducing extracellular signals into a nuclear response by acting as targets for the mitogen-activated protein kinase signaling pathways[4,5,17]. In addition to a N-terminal DNA-binding domain, Elk-1 contains a "B box" mediating its interaction with SRF, a "C domain" acting as a transcriptional activation domain, two repression domains,

and two domains that act as docking sites for multiple mitogen-activated protein kinases, including extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK) and p38[4,18,19]. The ERK cascade responds to growth factors and mitogens, whereas the JNK and p38 cascades are triggered by cytokines and stress.

The understanding of the molecular basis of tumor development has progressed dramatically in the last two decades. Since tumor is essentially a genetic disease, it is important to demonstrate what these oncogenes are and how they work in carcinogenesis. Identifying the genetic differences between normal and tumor cells or tissues will help discover the genes that directly cause tumor or are associated with tumorigenesis and provide novel markers for early detection and appropriate therapy.

Elk-1 is thought to impact neuronal differentiation[20], cell proliferation[4], tumorigenesis[21], and apoptosis[22]. Elk-1 plays a role in the neuronal expression of immediate-early genes like c-*fos* in the brain[23]. Elk-1 functions as a nuclear transcriptional activator via its association with SRF on serum response elements present in the promoters of many immediate-early genes, such as c-*fos*, *egr-1*, *egr-2*, *pip92*, and *nurr77*[17]. In addition to its regulation of growth-responsive genes, Elk-1 has been shown to play a role in regulating differentiation of smooth muscle, skeletal muscle, and neuronal cells[24-26]. Recently the SRF gene has also been identified as a target for Elk-1, thereby providing a positive-feedback loop where Elk-1 activation leads to enhanced expression of its partner protein, SRF[27]. Although the role of Elk-1 has been extensively studied, little information is available concerning its involvement in esophageal epithelia. In our study, we first investigated immunohistochemically the expression of Elk-1 protein in paired ESCC by TMA. The results of IHC revealed that the expression of Elk-1 was increased 78.5% (84/107) in tumor tissue compared to that in corresponding normal tissue. A significant positive correlation was found between esophageal carcinoma tissue and paired normal squamous epithelium ($P < 0.01$). Among the 107 histologically-examined esophageal squamous cell carcinomas, eight tumors were well-differentiated, 74 moderately-differentiated and 25 poorly-differentiated, suggesting that up-regulated Elk-1 expression has no difference in degree of tumor differentiation. No significant correlation was found among expression of Elk-1, degree of differentiation, lymph node metastasis and depth of invasion. Overexpression of two target genes of Elk-1 (c-*fos*, *egr*-1 mRNAs and their proteins) were found in dysplasia and esophageal squamous carcinomas, suggesting that these genes are involved in the development of esophageal carcinoma[28]. In addition to its nuclear location, Elk-1 is found throughout the cytoplasm of tumor and normal epithelial cells (Figure 1). This is in agreement with previous studies on nuclear transcription factor Elk-1 in neuronal cells[29,30].

In conclusion, Elk-1 may have alternative extranuclear functions in esophageal carcinogenesis. The mechanism of the involvement of Elk-1 in the development and progress of esophageal carcinoma remains to be further investigated.

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## COMMENTS

### Background

Esophageal squamous cell carcinoma (ESCC) has one of the highest malignant potentials of any tumor, and is characterized by poor survival and wide geographical variation in incidence. The molecular pathology underlying the development and progression of ESCC is poorly understood. In this study, the expression of Elk-1 was immunohistochemically examined in 107 ESCCs and its relationship with clinicopathologic parameters was analyzed.

### Research frontiers

The molecular mechanisms underlying the function and regulation of the three ternary complex factors (TCFs) Elk-1, Net and Sap-1 have been extensively studied and the TCFs are a paradigm for the study of transcriptional regulation in response to extracellular signalling through the mitogen-activated protein (MAP) kinase pathway. As final effectors of multiple signalling pathways and components of protein complexes play a role in immediate early promoters, they represent key elements in the complex and dynamic regulation of gene expression.

### Terminology

Tissue microarrays (TMAs) are means of combining tens to hundreds of specimens of tissues onto a single slide, using all types of *in-situ* analyses including immunohistochemistry (IHC), fluorescence *in situ* hybridization (FISH), and RNA *in situ* hybridization (RNA-ISH). Potential applications include the establishment of associations between molecular changes and clinical endpoints, testing of potential therapeutic targets using tissue samples from specific cancer patients, standardization of molecular detection of targets, and rapid translation of results from cell lines and animal models to human cancer.

### Peer review

The paper contributes to the mechanisms of carcinogenesis in the squamous epithelium of esophagus. The molecular study is focused on the Elk-1 transcription factor which is overexpressed in esophageal cancer. This fact is well demonstrated, showing that increased expression of transcription factor Elk-1 may play an important role in esophageal carcinogenesis.

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*RAPID COMMUNICATION*

# Colonic exclusion and combined therapy for refractory constipation

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## Abstract

**AIM:** To investigate the therapeutic effectiveness of colonic exclusion and combined therapy for refractory constipation.

**METHODS:** Thirty-two patients with refractory constipation were randomly divided into treatment group ($n$ = 14) and control group ($n$ = 18). Fourteen patients in treatment group underwent colonic exclusion and end-to-side colorectal anastomosis. Eighteen patients in control group received subtotal colectomy and end-to-end colorectal anastomosis. The therapeutic effects of the operations were assessed by comparing the surgical time, incision length, volume of blood losses, hospital stay, recovery rate and complication incidence. All patients received long-term follow-up.

**RESULTS:** All operations were successful and patients recovered fully after the operations. In comparison of treatment group and control group, the surgical time (h), incision length (cm), volume of blood losses (mL), hospital stay (d) were 87 ± 16 min *vs* 194 ± 23 min ($t$ = 9.85), 10.4 ± 0.5 cm *vs* 21.2 ± 1.8 cm ($t$ = 14.26), 79.5 ± 31.3 mL *vs* 286.3 ± 49.2 mL ($t$ = 17.24), and 11.8 ± 2.4 d *vs* 18.6 ± 2.6 d ($t$ = 6.91), respectively ($P$ < 0.001 for all). The recovery rate and complication incidence were 85.7% *vs* 88.9% ($P$ = 0.14 > 0.05), 21.4% *vs* 33.3% ($P$ = 0.73 > 0.05), respectively.

**CONCLUSION:** Colonic exclusion has better therapeutic efficacy on refractory constipation. It has many advantages such as shorter surgical time, smaller incision, fewer blood losses and shorter hospital stay.



**Key words:** Constipation; Colonic exclusion; Subtotal colectomy; Quality of life; Follow-up

## INTRODUCTION

Refractory constipation is a common clinical symptom. Because it is very obstinate and its etiological factors are unclear, it is difficult for medical workers to treat the patients. The problem of difficult defecation usually cannot be solved with drug treatment. According to our experience, surgical treatment is suggested for the patients who are unresponsive to cathartics or who need to ingest exceeding cathartics to evacuate their bowels. There are many conventional surgical methods, such as total colectomy, subtotal colectomy, hemicolectomy, *etc*. However, the operation time of these surgical methods is too long. Besides, these surgical methods with big trauma will give rise to many postoperative complications and negatively affect the quality of the patients' lives. Moreover, the very aged patients cannot bear these operations and their treatment is far from satisfactory[1]. Since 1998 we have adopted colonic exclusion with colorectal anastomosis and treated 14 patients of refractory constipation. All of them were satisfied with the therapeutic effects. The quality of patients' lives had been improved significantly after operations. Our clinical practice demonstrates that colonic exclusion is a safe and feasible operation. It has good therapeutic effects, shorter surgical time, and lower complication incidences.

## MATERIALS AND METHODS

### General data

Thirty-two patients were diagnosed as refractory constipation between January 1998 and April 2006. These patients received surgical intervention after ineffective medical treatment. They were divided into two groups randomly. There were two males and twelve females in treatment group ($n$ = 14). Their ages ranged from 31 to 77 years with a mean age of 45. Their courses of disease ranged from three to thirty years with 12 years on average. Among them, ten patients had rectocele, and eight patients had rectal prolapse. Five males and thirteen females entered control group ($n$ = 18). Their ages ranged from 28 to 75 years with a mean age of 51. Their courses of

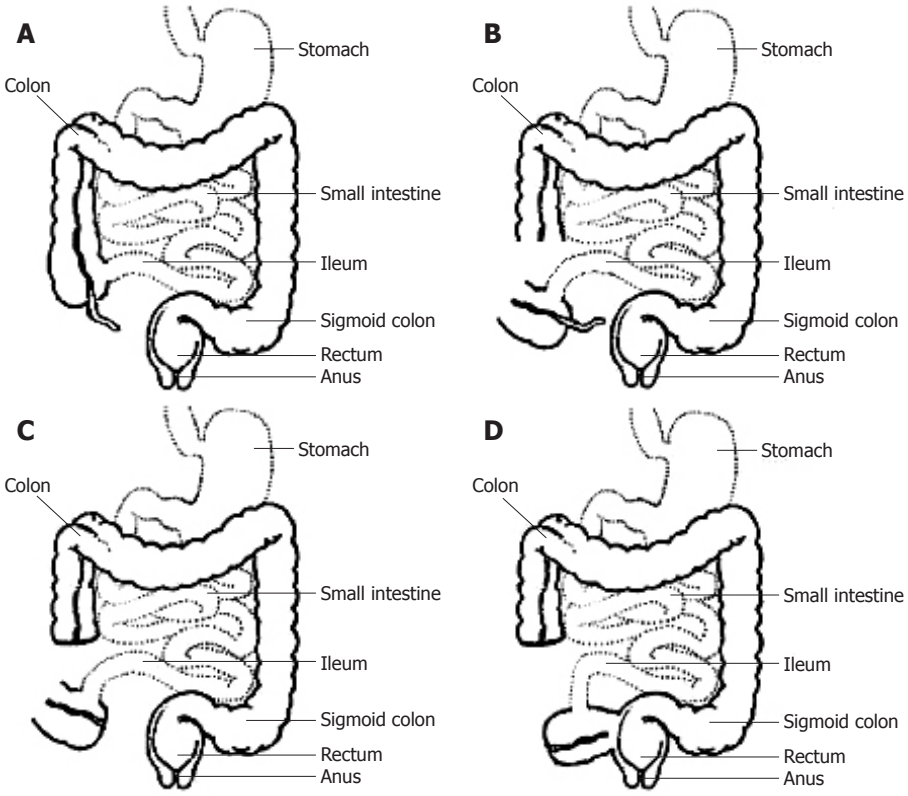


**Figure 1** Surgical procedures of colonic exclusion. **A**: Normal gastrointestinal structure in human body; **B**: Transect the ascending colon at the chosen level with good blood supply; **C**: Close the distal colon by U-shape sutures and sever the vermiform appendix; **D**: Lay the distal colon in abdominal cavity. The proximal ascending is made end-to-side anastomosis with rectum.

disease ranged from four to thirty-five years with 13 on average. Eleven patients had rectocele among them. There were no statistical differences in age and courses of disease between the two groups.

### *Clinical symptoms*

All patients had difficult defecation for three or more than three years. Some serious cases had difficult defecation beyond thirty years. They had been ingesting cathartics or other medicine for a long time. Patients usually complained of headache, general malaise, decreased appetite, abdominal pain, abdominal distension, straining at stooling, incomplete evacuation, or a need for digital manipulation to defecate. The frequency of defecation was about once four to eight days.

### *Diagnostic criteria*

Diagnostic criteria included (1) The time of difficult defecation exceeded one year; (2) The stool frequency was below three times per week for at least three months in one year. Patients had difficult defecation accompanied by abdominal pain and abdominal distension. Stools hardened gradually to form hard feces; (3) Digital rectal examination suggested that the patient had fecal impaction accompanied by anal stenosis, hemorrhoid, and rectal prolapse; (4) Gastrointestinal transit test showed that colonic transit became slower. Rectocele and long-winded sigmoid colon were confirmed with defecography; (5) Gastrointestinal organic diseases were excluded by electronic colonoscopy.

### *Surgical methods*

Patients in treatment group were in a lithotomy position after general anesthesia. We adopted median abdominal incision around the umbilicus. After entering the abdominal cavity, the peritoneal reflexion was severed, and the anterior wall of the rectum was dissected until the rectocele was exposed. We sewed three or four needles with 3-0 absorbable suture perpendicular to the longitudinal axis of the rectum, and subsequently sewed two needles along the longitudinal axis of the rectum. The needles should penetrate the serous membrane and muscular layer so as to restore the anterior wall of the rectum. Cautions must be taken not to penetrate the mucous membrane at the same time. Freeing of distal ileum, ileocecal junction and partial ascending colon allowed us to obtain enough slack proximal colon, which guaranteed a tension-free anastomosis. Colonic blood supply was carefully examined about 4 to 6 cm away from the ileocecal junction, and the ascending colon was transected at the chosen level with good blood supply. The distal colon was closed by U-shaped sutures to lay the indwelling colon in the abdominal cavity. Vermiform appendix was severed from the cecum by a conventional method. The proximal ascending colon and cecum were moved to the pelvic cavity in anticlockwise, and then end-to-side colorectal anastomosis was performed with stapler under peritoneal reflexion. Long-winded sigmoid colon was resected simultaneously. The rectum was raised and fixed with the lateral side of pelvic peritoneum in order to suspend the rectum. The basement of pelvic cavity was reestablished and the posterior peritoneal space was closed. Finally the abdominal cavity was closed after checking surgical instruments and gauzes. It was unnecessary to place drainage tubes in the abdominal cavity to prevent postoperative adhesion (Figure 1).

The patients in control group had median abdominal incision. After entering the abdominal cavity, peritoneal

**Table 1 Surgical therapeutic efficacy for 32 refractory constipation patients**

| Group | Cases (*n*) | Surgical time (min) | Incision length (cm) | Blood loss (mL) | Hospital stay (d) | Recovery (%) | Improvement (%) | Complication (%) |
|---|---|---|---|---|---|---|---|---|
| Treatment group | 14 | 87 ± 16 | 10.4 ± 0.5 | 79.5 ± 31.3 | 11.8 ± 2.4 | 12 (85.7) | 2 (14.3) | 3 (21.4) |
| Control group | 18 | 194 ± 23 | 21.2 ± 1.8 | 286.3 ± 49.2 | 18.6 ± 2.6 | 16 (88.9) | 2 (11.1) | 6 (33.3) |
| *t* | | 9.85 | 14.26 | 17.24 | 6.91 | *P* = 0.14 | *P* = 0.14 | *P* = 0.73 |
| *P* | | < 0.001 | < 0.001 | < 0.001 | < 0.001 | > 0.05 | > 0.05 | > 0.05 |

reflexion was severed. The anterior wall of the rectum was dissected until the rectocele was exposed. Then the rectocele was restored as above. The whole colon was freed. Vermiform appendix was severed. Subsequently the colon from the ascending colon, which was about 4 to 6 cm away from ileocecal valve, to sigmoid colon was resected subtotally. The proximal ascending colon and cecum were moved to the pelvic cavity in anticlockwise, and then the proximal ascending colon was end-to-end anastomosed with the rectum. The abdominal cavity was closed after closure of the posterior peritoneal space. We also did not place drainage tubes in the abdominal cavity.

### *Statistical analysis*

All data are expressed as mean ± SD. They were entered into SPSS 12.0 statistical package. Statistical comparison was done with group *t*-test and Fisher exact probabilities in 2 × 2 table. A *P* value less than 0.05 was considered statistically significant.

## RESULTS

### *Surgical results*

The surgical time (h), incision length (cm), volume of blood losses (ml), hospital stay (d) were 87 ± 16 min, 10.4 ± 0.5 cm, 79.5 ± 31.3 mL, 11.8 ± 2.4 d in treatment group, and 194 ± 23 min, 21.2 ± 1.8 cm, 286.3 ± 49.2 mL, 18.6 ± 2.6 d in control group, respectively ($P < 0.001$) (Table 1). The treatment group had several advantages such as shorter surgical time, smaller incision, less blood losses and shorter hospital stay. Compared with the control group, colonic exclusion could reduce local trauma for many patients.

### *Criteria for therapeutic effect*

(1) Recovery: Constipation and relevant symptoms disappeared. Auxiliary examinations showed that associated manifestations disappeared; (2) Improvement: Constipation and relevant symptoms were relieved. Auxiliary examinations showed that major relevant symptoms disappeared; (3) Inefficacy: Constipation and relevant symptoms were not obviously improved. Auxiliary examinations showed that constipation still existed.

### *Postoperative effects*

According to the above criteria, twelve patients recovered (12/14, 85.7%) and two patients improved (2/14, 14.3%) in treatment group. In control group sixteen patients recovered (16/18, 88.9%) and two patients improved (2/18, 11.1%).

### *Postoperative complications*

In treatment group, adhesive ileus occurred in one patient (relieved after expectant treatment later). Another patient developed grease liquefaction of incision. Acute pancreatitis occurred in one patient. Complication incidence was 21.4%. In control group, adhesive ileus occurred in three patients. The infection of incisional wound occurred in two patients. One patient developed adhesive stenosis of the ureter. Complication incidence was 33.3%. There were no death case, no postoperative stomal leak or anastomotic stricture in either groups.

### *Long-term follow-up*

All patients were followed for six to thirty-eight months. The follow-up rate was 100%. Most patients recovered very well after operations. Their constipation disappeared and the quality of their lives improved. One patient in treatment group still had abdominal distention. She was diagnosed as endometriosis later. And the indwelling colon was resected in another hospital. The other patients did not have any abdominal pain or abdominal distention. They evacuated their bowels one to three times every day. Their stools were almost forming. Three patients in control group still had slight abdominal distention. They evacuated their bowels one to four times every day. The shape of their stools was pasty.

## DISCUSSION

Over the recent years in China, along with the improvement of the economy, quickening up of the life pace, change of the structure of foods and drinks, as well as aging of the population, the incidence of refractory constipation rises year by year. It has become one of the common diseases that affect the quality of people's lives. Refractory constipation is usually classified into three types: slow transit constipation (STC), outlet obstructive constipation (OOC), mixed constipation (MC). Among them, mixed constipation is the most commonly seen in clinics.

### *Etiological factors*

The etiological factors of refractory constipation are very complicated and largely unclear; however, some of the etiological factors have been certain[2,3]. (1) Abnormalities in the enteric nervous system; (2) Abnormalities of extrinsic nerves; (3) Smooth muscle abnormalities; (4) Interstitial cell of Cajal dysfunction; (5) Structural abnormalities of the rectum and anus: such as rectal prolapse, rectocele, hemorrhoid; (6) Endocrine and metabolic conditions;

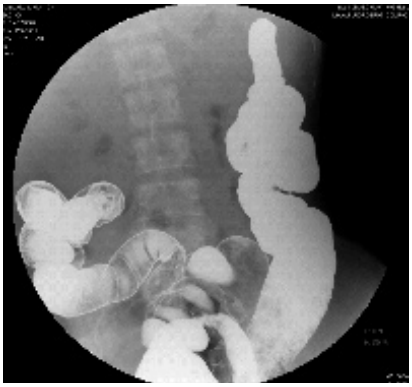

**Figure 2** The indwelling colon didn't inflate and anastomotic stoma transmitted normally.

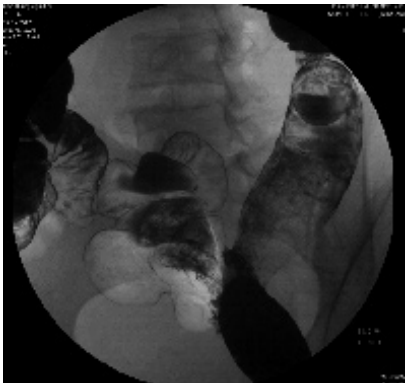

**Figure 3** After twenty-four hours barium in the indwelling colon was evacuated completely.

(7) Drugs; (8) Psychogenic conditions. Understanding of the etiological factors will help us make correct treatment plans for the disease. Effective combined therapy can lead to better therapeutic efficacy.

### *Surgical indications*

Refractory constipation is not a fatal disease. We suggest expectant treatment for the constipated patients with a short course of disease and light pathogenetic condition. Constipated patients who have serious clinical symptoms, poor quality of life and a strong desire for surgery, however, should be considered to take colonic exclusion, except for the aged patients. According to our clinical observation and experience over the years, there are the following surgical indications for colonic exclusion: (1) Patients have serious difficult defecation for at least three years. The stool frequency is below three times every week. (2) Drug treatment for at least half a year is confirmed ineffective or patients have to ingest exceeding cathartics for a long time. (3) Gastrointestinal transit test shows that colonic transit becomes slower, while the stomach and small bowel transit are normal. Accompanying outlet obstructive diseases are confirmed by preoperative defecography. (4) Constipation-predominant irritable bowel syndrome and constipations which are caused by drugs must be excluded. (5) The patient has no serious mental disorder and she (he) has a strong desire for operation. Surgeons should be cautious of surgical treatment for refractory constipation and handle surgical indications strictly. Patients should undergo preoperative psychological tests and clinical examinations of colorectal function. After that, individualized therapeutic strategies and surgical schema are made.

### *Variations of colonic propagated activity before and after colon exclusion*

The phenomenon of slow colonic transit more or less exists in patients with refractory constipation. We used gastrointestinal transit test to detect gastrointestinal function of 14 patients in treatment group before operation. Forty-eight hours later the photographs showed that baric markers were still stagnated in transverse colon or sigmoid colon in 12 patients. Seventy-two hours later the photographs displayed retention of baric markers in sigmoid colon or rectum in nine patients. Bassotti *et al*[4] studied colonic propulsive activity in constipated patients and found colonic dysfunction. Both colonic contraction amplitude and high-amplitude propagated contractions were significantly decreased, which might be the immediate cause of colonic inertia. We examined nine patients in treatment group with barium enema after colon exclusion a month later. The intestine did not inflate and the anastomotic stoma transmitted normally (Figure 2). Three patients had barium reflux into the indwelling colon, but it was evacuated completely 24 h later (Figure 3). Hereby it demonstrated that the indwelling colon still had itself movement. It did not lose its peristalsis under the condition of disuse. However, it is uncertain whether it has any practical clinical significance to lay the colon with neuromuscular diseases in the abdominal cavity. By now, there is no definite conclusion about the long-term influence of the indwelling colon on human bodies.

### *Characteristics and clinical application perspectives of colon exclusion*

Arbuthnot first adopted total colectomy with ileorectal anastomosis to treat slow transit constipation in 1908. Afterwards people gradually adopted total colectomy with caecorectal anastomosis. These surgical methods could relieve constipation symptoms in most patients with chronic idiopathic constipation; however, they were associated with a considerable morbidity and were less effective in resolving symptoms of abdominal pain and bloating[5]. Based on the result of gastrointestinal transit test, people adopted hemicolectomy to treat constipated patients. They only resected some segmental long-winded colon. Hemicolectomy could lead to fewer postoperative complications and a faster recovery[6], but the postoperative recurrence rate was very high[7]. This is because the excisional range of the pathological colon is not large enough. After operation the residual colon transmits slower, which will lead to recurrence of constipation. On the other hand, subtotal colectomy is a popular operation to treat refractory constipation. It seldom leads to serious diarrhea for the remaining of ileocecal valve. However, it still has some disadvantages, such as big trauma, long surgical time, delayed recovery, many complications. Besides, patients' postoperative quality of life is not so ideal[8]. We adopted colonic exclusion with colorectal anastomosis and neoplasty for symptomatic rectocele to treat constipated patients. The clinical results were satisfactory. Patients recovered excellently after operations. And there were no serious postoperative complications among them. Compared with the control group, it had

smaller incision, fewer blood losses, shorter operational time and shorter hospital stay.

In conclusion, colonic exclusion has such advantages as: (1) less trauma, fast recovery; (2) simplified operation, shorter surgical process; (3) preservation of some bowels with normal function; (4) higher clinical cure rate; (5) avoidance of serious postoperative complications. For most constipated patients, colonic exclusion is the best surgical method because it is convenient, economical, less trauma, and less painful. It is especially indicated for aged constipated patients whose surgical endurances are not so good. We assume that it has wide application value.

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## COMMENTS

### *Background*

The incidence of refractory constipation is very high in modern people, especially in aged people and middle-aged females. Some constipated patients have to be treated by surgery, while conventional surgical methods have big trauma. We recommend a new surgical method to treat refractory constipation. It is convenient, economical, less trauma, and less painful.

### *Research frontiers*

Surgical methods to treat refractory constipation are improving. Recently people pay more attention to microinvasive operation. Laparoscopic operations instead of conventional surgical methods are used to treat refractory constipation.

### *Innovations and breakthroughs*

Colonic exclusion is a technical innovation in treating refractory constipation. Compared with conventional surgical methods, it doesn't need to sever or resect too much colon and it retains some bowels with normal function. Rectocele is restored and the basement of pelvic cavity is reestablished. Therefore, it has less trauma and patients recover faster after operation.

### *Applications*

Colonic exclusion has better therapeutic efficacy for refractory constipation. It has many advantages such as smaller incision, fewer blood losses, less adhesive ileus, shorter surgical time and shorter hospital stay. It is especially indicated for aged constipated patients, whose surgical endurances are not so good. It has wide application prospect in clinical practice.

### *Terminology*

Colonic exclusion: It is a surgical method that needn't to resect the colon. The ascending colon is transected. Then the distal colon is closed and the proximal colon is end-to-side anastomosed with the rectum. An indwelling colon is laid in the abdominal cavity.

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*RAPID COMMUNICATION*

# CT diagnosis of 52 patients with lymphoma in abdominal lymph nodes

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## Abstract

**AIM:** To assess CT manifestations and its diagnostic value for lymphoma in the abdominal lymph nodes (LALN).

**METHODS:** CT findings in 52 cases of LALN proved by surgery or biopsy, including Hodgkin's disease (HD) in 16 cases and non-Hodgkin's lymphoma (NHL) in 36 cases, were retrospectively analyzed.

**RESULTS:** (1) CT manifestations based on distribution of the lesions of LALN: Solitary mass type was found in 10 cases, including solitary, round, uniform-density, enlarged lymph nodes in 3 cases; and multiple, enlarged lymph nodes fusing into singular lobular mass in 7 cases. Thirty-four cases of multiple-nodular type showed multiple, round, enlarged lymph nodes with uniform density and clear margins. Vessels-embedded signs, including mesenteric vessels, renal vessels, abdominal aorta or inferior vena cava, were seen in 6 cases, and duodenum-embedded signs were seen in 2 cases. Eight cases of diffuse type showed characteristic "cobblestone signs". (2) CT manifestations correlated with pathological type: CT manifestations of 12 cases of HD were different from those of 40 cases of NHL in distribution, size, quantity and fused lesion of enlarged lymph nodes. (3) Twenty-eight cases of 52 patients were accompanied with extra-nodal lymphoma in the abdomen, especially gastrointestinal lymphoma, which had characteristic CT findings. (4) In follow-up examinations, CT images showed uniform, heterogeneous or rim enhancement in 15 cases, and occasional calcifications accompanied with reduction of the lesion size and quantity in 12 cases, whereas the lesions disappeared in 3 cases after treatment.

**CONCLUSION:** CT images show many characteristic manifestations valuable for qualitative diagnosis of LALN, and it is also helpful for pathological classification of LALN and therapeutic evaluation in follow-up of patients.



**Key words:** Lymphoma; Lymph nodes; Computed tomography; Diagnosis; Classification

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## INTRODUCTION

Abdominal lymphoma is clinically not uncommon and lymphoma in the abdominal lymph nodes (LALN) is most frequently encountered[1]. Though it may be part of a systemic lymphoma, single onset of LALN is not rare. LALN can be divided into Hodgkin's disease (HD) and non-Hodgkin's lymphoma (NHL). HD is seldom seen in China[2-3] and mostly occurs in middle-aged and young people. The number of NHL patients is larger than that of HD with a diffuse distribution in age. In addition, AIDS patients are more susceptible to NHL[4].

CT is useful for diagnosis[1-2,5-14] and staging[15-21] of abdominal lymphomas. For evaluation of lymph node involvement, the sensitivity of contrast-enhanced CT is 88%, and specificity is 86%. For evaluation of organ involvement, the sensitivity of contrast-enhanced CT is 50%, and specificity is 90%[15]. To our knowledge, however, CT classification of LALN has not been reported in previous documents. We reviewed retrospectively CT findings of 52 patients with LALN confirmed by pathology and histology from 2000 to 2005 in our hospital, and analyzed its value in diagnosis, pathological classification and therapeutic assessment of LALN.

## MATERIALS AND METHODS

### *Subjects*

Of the 52 LALN patients, 29 were males and 23 females, with age ranging from 18 to 77 years (mean, 43.4 years), including Hodgkin's disease (HD, age range 18-59 years, mean 34.6 years) in 12 cases and non-Hodgkin's lymphoma (NHL, age range 20-77 years, mean 47.3 years) in 40 cases. The diagnosis was confirmed by surgery (20 cases) and lymph node biopsy (32 cases) including peripheral

lymph nodes (19 cases) and abdominal lymph nodes (13 cases). The duration of symptoms ranged from 1 to 18 mo. All the patients had evident improvement in clinical symptoms and signs, and reduction in size of the lesions or disappearance of the lesions after chemotherapy and radiotherapy.

### CT scanning

Twenty patients were performed with a Somatom HiQ CT machine (Siemens Medical Systems, Forchheim, Germany) and 32 patients were performed with a 4-detector row CT machine (Volume Zoom, Siemens Medical Systems, Forchheim, Germany). All patients were in routine fasting states. Some patients were given 1000-1500 mL of diluted iodinated contrast medium (10 g/L meglumine diatrizoate) orally 60-90 min before scanning. Scan scope ranged from the dome of diaphragm to symphysis pubica. All patients were examined with plain scanning at first and then examined using Ultravist (Schering, Berlin, Germany) 80 mL for enhanced scanning, with a section thickness of 10 mm and internal of 10 mm (Somatom HiQ CT machine) or 1.5 mm section thickness, a pitch of 1.25, 5 mm reconstruction interval (4-detector row CT machine).

## RESULTS

### CT manifestations based on distribution of the lesions of LALN

**Solitary mass type:** Ten cases of solitary mass type of LALN were located in the retroperitoneum, mesenterium and lesser omentum. Of them, 3 cases on CT plain scanning showed solitary, round, enlarged lymph nodes of uniform-density with diameters of 3.2-5.8 cm. The lesions had rim enhancement in one case and slight homogeneous enhancement in 2 cases on enhanced CT. The other 7 cases showed multiple enlarged lymph nodes fusing into lobular masses with diameters of 7.8-18 cm and clear margins, of which 5 cases showed uniform density and 2 cases showed heterogeneous density on plain CT. Enhanced CT showed slight uniform enhancement in 4 cases, and heterogeneous enhancement in 3 cases. Of the all cases, the mesenteric vessels, abdominal aorta and inferior vena cava were embedded (vessels-embedded signs) in 3 cases (Figure 1).

**Multiple nodular type:** Thirty-four cases were of multiple nodular type of LALN, which referred to regional distribution of lymph nodes. CT showed multiple, round, enlarged lymph nodes with clear margins and uniform density in the abdomen (Figure 2). Most enlarged lymph nodes were over 1.0 cm in diameter with exception of 2 cases of less than 1.0 cm, which located in the mesenterium. Among the 34 cases, 29 showed slight uniform enhancement, and 5 appeared slight uniform enhancement accompanied with rim enhancement after contrast administration. Nineteen cases revealed a fusion of lesions of different degrees and 6 cases showed embedding of vessels (vessels-embedded signs), including the mesenteric vessels (3 cases) (Figure 3), renal vessels (3 cases), abdominal aorta (2 cases) and inferior vena cava (1 case), and 2 cases showed embedding of the horizontal segment of the duodenum (duodenum-embedded signs).

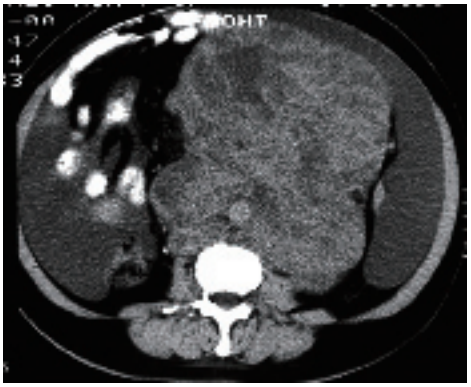

**Figure 1** Solitary mass type: Enhanced CT showing multiple enlarged lymph nodes fused into uneven density, huge lobular tumors with abdominal aorta and inferior vena cava encased and ascites.

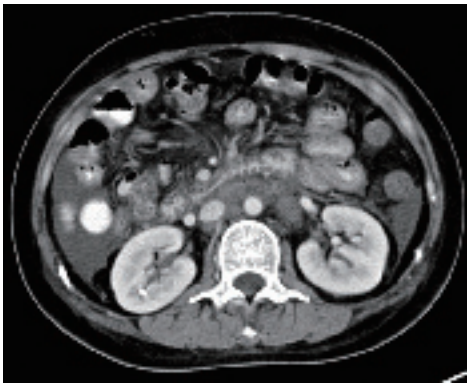

**Figure 2** Multiple-nodular type: Enhanced CT showing multiple, homogeneous density, enlarged lymph nodes in retroperitoneal region with ascites.

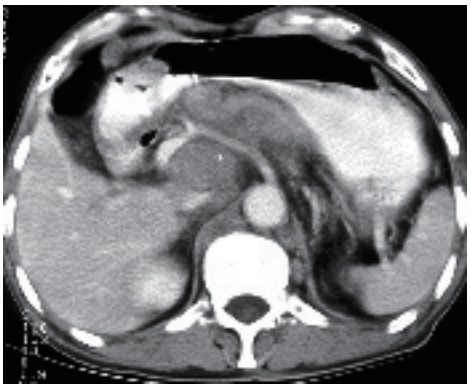

**Figure 3** Multiple-nodular type: CT of portal venous phase showing mesenteric multiple enlarged lymph nodes with superior mesenteric artery encased.

**Diffuse type:** The diffuse type of LALN involved the whole distribution areas of abdominal lymph nodes, mainly in the mesenteric and retroperitoneal regions in eight cases. CT revealed diffuse, round, non-confluent, enlarged lymph nodes with homogeneous density and clear margins, forming the characteristic "cobblestone signs" named by us. All the enlarged lymph nodes were slightly homogeneously enhanced after contrast administration (Figure 4).

### CT manifestations correlated with pathological types

**HD:** Twelve cases of HD included solitary mass type and diffuse type in one case each (8.3%, respectively), and multiple nodular type in 10 cases (83.4%). The enlarged lymph nodes were mainly distributed in the lesser omentum and retroperitoneal region adjacent to the abdominal aorta (Figures 2 and 5) and inferior vena cava. All the enlarged lymph nodes showed homogeneous

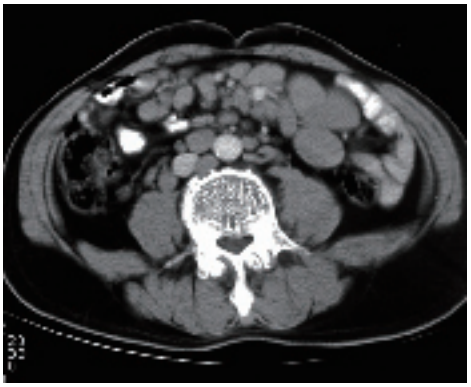

**Figure 4** Diff-use type: Enhanced CT showing diffuse, clear margins, homogeneous density enlar-ged lymph nodes in the mesenteric and retroperitoneal region.

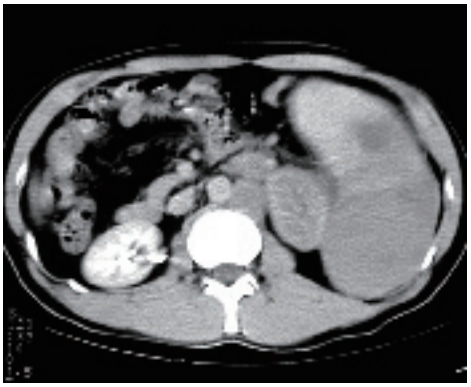

**Figure 5** Enhanced CT showing multiple, homogeneous density, enlarged lymph nodes in para-abdominal aorta and multiple, low-density lesions in the spleen (splenic lymphoma).

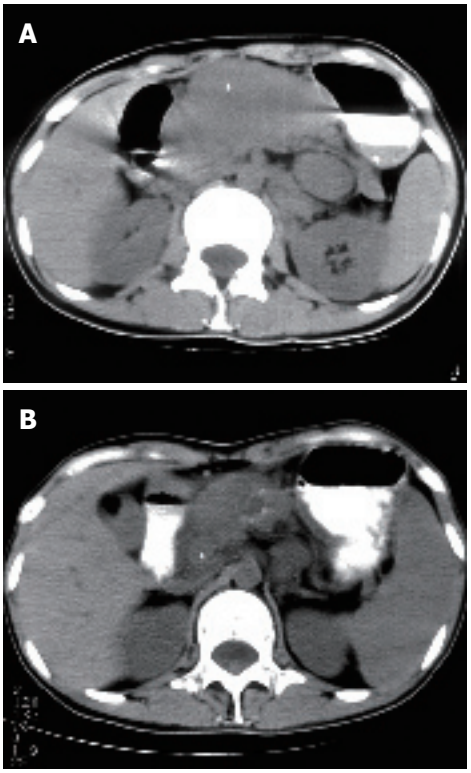


**Figure 6** **A**: Plain CT showing a uniform density, slightly lobular tumor (formed by multiple enlarged lymph nodes) in the lesser omentum; **B**: The tumor having a notable shrinkage in size and flecked calcifications found within the tumor after treatment on plain CT.

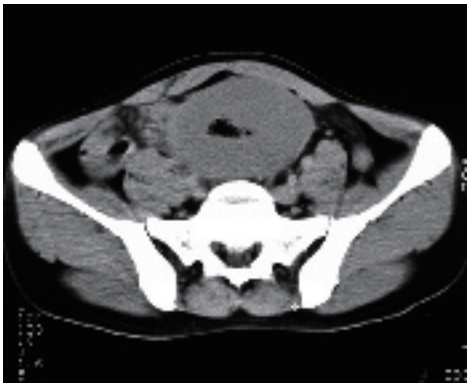

**Figure 7** Enhanced CT showing notable circular thickening of intestinal wall with homogeneous density.

enhancement except one rim enhancement after contrast administration. Among the 12 cases, only one case (8.3%) involved the mesenteric lymph nodes, which showed scattered distribution of lesions and a small number of lymph node involvement with less than 2.0 cm in size. Two cases (16.7%) had confluence of lesions and 7 cases (58.3%) showed involvement of other abdominal organs (Figure 5). **NHL:** Forty cases of NHL included solitary mass type (9 cases, 22.5%), multiple-nodular type (24 cases, 60%) and diffuse type (7 cases, 17.5%) (Figure 4). All the abdominal lymph nodes were involved, including 18 cases (45%) of mesenteric involvement. Among the 40 cases, 24 (60%) showed a fusion of the enlarged lymph nodes of different degrees, appearing as lobular masses (Figures 1 and 6); 4 exhibited embedding of vessels (Figures 1 and 3) and 2 had involvement of the intestine. Twenty-one cases (52.5%) were accompanied with involvement of other abdominal organs.

### *CT manifestations of LALN after chemo-radiotherapy*

In follow-up, CT was performed in 15 cases of LALN after chemo-radiotherapy, of which abdominal enlarged lymph nodes completely disappeared in 3 cases. The lesion size was reduced by different degrees in 12 cases, including 9 cases with homogeneous density and 3 cases with heterogeneous density, and one case with flecked calcification (Figure 6).

### *CT manifestations of the involvement of other abdominal organs with lymphoma*

Of the 52 cases of LALN, 28 involved other abdominal organs. Among the 28 cases, intestinal tract lymphomas (13 cases) showed notable circular thickening of the intestinal wall (25-38 mm, Figure 7) in 9 cases, including 2 cases with distinct dilation of intestinal cavity and one case with coexistence of narrowing and dilation of intestinal cavity, resulting in incomplete intestinal obstruction. The other 4 cases of intestinal lymphomas developed lobular masses with uneven margins in intestinal cavity. The CT manifestations of hepatic (7 cases) and splenic (6 cases) lymphomas were solitary or multiple nodular, low-density lesions (Figure 5). Ten cases of gastric lymphomas appeared notable circular thickening of gastric wall (22-33 mm). Renal lymphomas and adrenal gland lymphomas (2 cases each) were characterized by bilateral masses of both kidneys and adrenal glands. Involvement of the peritoneum and omentum (2 cases) resulted in a large amount of ascites (Figures 1 and 2) and omental cakes.

## DISCUSSION

### *Diagnostic value of CT for LALN*

CT plays an important role in the diagnosis and staging

of abdominal lymphomas. Based on the CT features of LALN, we classified LALN into solitary mass type, multiple-nodular type and diffuse type.

Solitary mass type of LALN included singular enlarged lymph nodes and fusion of multiple enlarged lymph nodes (most cases). CT featured a huge round mass or a lobular homogeneous density mass with uniform enhancement.

Multiple-nodular type of LALN, the most frequently seen, was characterized by enlarged lymph nodes with regional distribution. Parts of enlarged lymph nodes were fused together and formed a huge mass. CT showed uniform density lesions with mild homogenous enhancement. Sometimes coexistence of mild homogenous enhancement and rim enhancement was also observed, but fully rim enhancement of all lesions was rarely seen.

All the lesions of solitary mass or multiple-nodular type had clear margins. The vessels such as mesenteric vessels, renal vessels, abdominal aorta, and inferior vena cava could be imbedded by the fused multiple enlarged lymph nodes forming the unique "vascular-imbedded signs"[5,6], which were commonly seen in multiple-nodular type. The "duodenal-imbedded signs", named by us and found in multiple-nodular type, could be induced by LALN due to rich lymphoid tissues in the horizontal segment of the duodenum, which was different from intestinal lymphomas in CT manifestations. The former was marked by narrowing of the intestinal cavity surrounded by the fusion of multiple small nodular lesions with a streak appearance and the latter was manifested as circular thickening of intestinal wall and sometimes complicated with intestinal distension. Therefore, "intestinal-imbedded signs" have an important value for the diagnosis of LALN, although this was not reported in previous documents.

The CT features of diffuse type were diffuse but non-confluent enlarged lymph nodes in the mesenteric and retroperitoneal region with uniform density and cobblestone appearance, named as "cobblestone signs". It has an important value for the diagnosis of diffuse type of LALN because it has not been seen in other diseases. Moreover, the retroperitoneal lymph nodes below the level of lumbar 2-3 vertebrae were frequently involved (HD 94%, NHL 89%, respectively), which is of great importance in the differential diagnosis with tuberculous lymph nodes [1,6].

The above classification of CT manifestations into 3 types in LALN can not only help in the radiographic diagnosis of LALN but also guide the clinical management of LALN, e.g. the scope of radiotherapy.

LALN is frequently associated with abdominal extra-nodular lymphomas, and about 54% patients had gastrointestinal tract involvement[4]. This is of significance in the diagnosis of LALN, particularly lymphomas of the gastrointestinal tract, which have a high morbidity and show characteristic circular thickening of gastrointestinal wall with notable distension of intestinal cavity on CT. More than half of the patients in this series were associated with gastrointestinal lymphomas. In addition, the association with other solid organ lymphomas such as the liver and spleen is also helpful for the diagnosis. The peritoneum and omentum are seldom involved by LALN[7-11]. There were 2 cases in this series and CT showed a large amount of ascites and omental cakes.

CT findings of HD are different from those of NHL. According to the literature and this report[6,14], we summarize the differences as follows: The enlarged lymph nodes of HD are rarely seen in the mesentery, less than 5%[14], 8.3% in this paper, with a small number of small lesions distributed dispersedly. The confluence of enlarged lymph nodes of HD is seldom seen (16.7%), which mainly occurs in multiple-nodular type (60%). NHL has a wide distribution including all sites of abdominal lymph nodes, and the mesentery is frequently involved (45%). The fusion of enlarged lymph nodes is common (60%), showing vessel-imbedded signs, intestinal-imbedded signs and cobblestone signs. NHL is seen not only in the multiple-nodular type (60%), but also in nearly all of the diffuse type and solitary type (22.5%, 17.5%, respectively).

The CT manifestations of LALN before radio-chemotherapy are different from those of LALN after treatment[22], including reductions in extent of lesions and changes in internal nodal characteristics. The CT features of LALN after treatment are characterized by the reduction in the number of enlarged lymph nodes and the shrinkage of lesions. Though most enlarged lymph nodes remain uniform density on CT, an evident increase in heterogeneous or rim enhancement of lesions may occur due to intra-nodular necrosis after treatment and calcifications of lesions may occur in individual cases[5]. The results of this series revealed that 20% lesions disappeared after radio-chemotherapy and most of these occurred in multiple-nodular type with relative concentration of enlarged lymph nodes. Therefore, CT is valuable for the evaluation of therapeutic efficacy of radio-chemotherapy and helps guide the following treatment.

### *Differential diagnosis of LALN*

**Lymph node tuberculosis:** Lymph node tuberculosis may be easily confused with lymphomas and should be considered emphatically. Lesions of lymph node tuberculosis are relatively concentrated (non-hematogenous disseminated type) and the mesenteric lymph nodes are commonly involved. CT shows rim or multilocular enhancement of enlarged lymph nodes, and sometimes with intra-nodular calcifications. This disease is often associated with tuberculous peritonitis, which has characteristic signs of omental cakes, smudged mesentery signs and high-density ascites. LALN is relatively disperse and the mesenteric lymph nodes are not commonly involved in HD. CT shows homogenous enhancement of lymph node enlargement with fewer calcifications (only 0.84%) in untreated cases[12-13] and peritoneal and omental lymphomatosis is not often seen. In addition, rim enhancement of enlarged lymph nodes of LALN (including before and after radio-chemotherapy) on CT is different from that of lymph node tuberculosis. The lesions of LALN with clear margins and multilocular enhancement are not often found.

**Metastatic tumor:** The occurrence of abdominal lymph node metastasis usually follows the development of a

primary tumor. The enlarged lymph nodes are located near the primary lesion, showing heterogeneous or rim enhancement due to necrosis of lymph nodes, with frequent occurrence of ascites. Neither tuberculous lymph nodes nor metastatic lymphadenopathy has blood vessel-imbedded signs, intestinal-imbedded signs, or cobblestone signs.

In summary, LALN has three kinds of CT manifestations and each kind has some unique features valuable for qualitative diagnosis of LALN. CT is also helpful for the pathological classification of LALN and the therapeutic evaluation in follow-up.

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*CASE REPORT*

# Sporadic ganglioneuromatosis of esophagogastric junction in a patient with gastro-esophageal reflux disorder and intestinal metaplasia

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## Abstract

A 58-year-old female with a recurrent history of upper abdominal pain and intermittent dysphagia underwent endoscopic evaluation that demonstrated an irregular and nodular esophago-gastric (EG) junction and grade Ⅰ erosive esophagitis. Biopsies showed prominent intestinal metaplasia of Barrett's type without dysplasia, chronic inflammation and multiple aggregates of large cells within the mucosal lamina propria, some with spindle shaped nuclei. Immunohistochemistry stains for keratins AE-1/AE-3 were negative, while S-100 and NSE were positive. This, together with routine stains, was diagnostic for mucosal ganglioneuromatosis. The background of chronic inflammation with intestinal type metaplasia was consistent with long-term reflux esophagitis. No evidence of achalasia was seen. Biopsies of gastric antrum and fundus were unremarkable, without ganglioneural proliferation. Colonoscopy was unremarkable. No genetic syndromes were identified in the patient including familial adenomatous polyposis and multiple endocrine neoplasia type Ⅱb (MEN Ⅱb). Iansoprazole (Prevacid) was started by oral administration each day with partial relief of symptoms. Subsequent esophagogastroscopy repeated at 4 mo showed normal appearing EG junction. Esophageal manometry revealed a mild non-specific lower esophageal motility disorder. Mild motor dysfunction is seen with gastro-esophageal reflux disease (GERD) and we feel that the demonstration of localized ganglioneuromatosis was not likely related etiologically. In the absence of findings that might suggest neural hypertrophy, such as achalasia, the nodular mucosal irregularity seen with this instance of ganglioneuromatosis may, however, have exacerbated the patient's reflux.



**Key words:** Manometry; Motility; Lower esophago-gastric junction; Esophagus; Ganglioneuromatosis

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## INTRODUCTION

This paper presents a solitary ganglioneuroma of the esophago-gastric junction in a 58-year old woman with a chief complaint of recurrent bouts of abdominal pain and mild dysphagia over several years and a recent onset of diarrhea. There are several primary esophageal tumors, which show neuroid differentiation, most arising from innate innervations of the esophagus.

These may be solitary or disseminated with Schwann cell and ganglion cell components involving any portion of the gastrointestinal tract. Cases may demonstrate either an exophytic polypoid or endophytic configuration and tend to arise from the neural plexus in the bowel wall. Non-neoplastic neural proliferations involving the esophagus include achalasia with inflammation and localized neural proliferation, which closely mimics ganglioneuromatosis. Neoplastic neural tumors that can involve the esophagus include ganglioneuroma, gastrointestinal autonomic nerve tumor (GAN), schwannoma, neurofibroma, granular cell tumor; and gangliocytic paraganglioma. Ganglioneuroma is a benign tumo, and the solitary variant may be cured by excision. Lesions in syndromic ganglioneuromatosis may require more extensive surgery.

## CASE REPORT

### *Clinical presentation*

The patient is a 58-year old Caucasian woman who presented with a complaint of recurrent bouts of abdominal pain, mild dysphagia over several years and recent onset of diarrhea. Previous medical history was significant for hyperlipidemia, insulin dependent diabetes mellitus, coronary artery disease, pancreatitis and diagnosed meningioma. Previous surgical history included cholecystectomy, appendectomy, cervical spine

fusion and celiac axis stenting. Medicactions prior to endoscopic biopsy of esophagogastric EG junction included the following: Atorvastatin, pioglitazone HCl, diphenhydramine, Glipizide, Pyridoxine, isosobide and dihydrocodeinone.

### *Endoscopic and microscopic findings*

Endoscopy showed a somewhat nodular and irregular appearing EG junction with non-erosive mucosa (Figure 1). The antrum showed mild non-erosive gastritis. The pylorus and duodenum were unremarkable. Biopsies of the EG junction, antrum and duodenum were obtained. Hematoxylin and Eosin stained histologic sections from the EG junction showed aggregates of tangled fascicles of large cells mixed with Schwann like spindle cells expanding lamina propria (Figure 2). At low magnification this gave a pseudo granuloma appearance with distortion of the adjacent gland architecture. Higher magnification revealed a fibrillar cytoplasmic matrix with monomorphic bland nuclei without mitotic activity. Differential diagnosis for these features included ganglioneuroma, neurofibroma and schwannoma[1]. Immunohistochemical staining for S100 demonstrated a nerve sheath component. Focal positivity for Synaptophysin confirmed the presence of a ganglioneuronal tissue (Figure 3). The glandular epithelial component showed frequent goblet cells with no evidence of dysplasia. Chronic inflammatory cells were scattered in mucosal stroma. The biopsy diagnosis based on both HE and immunophenotypic characterization was of localized ganglioneuromatosis of EG junction within a background of mild chronic inflammation and prominent intestinal metaplasia without dysplasia.

### *Treatment and follow-up*

Clinical correlation excluded the presence of achalasia or MEN-ⅡB. Treatment following this diagnosis of localized ganglioneuroma associated with esophagitis included prevacid 30 mg daily. Neurontin had been considered if symptoms would have progressed; however, this was not the case. A follow-up observation with repeat endoscopic evaluation at 4 mo was performed and showed mild gastritis with mild chronic inflammation in an unremarkable EG junction without ulceration or nodularity. Early recognition of symptomatology that might suggest either lower esophageal sphincter (LES) dysfunction or esophageal dysmotility was reviewed with the patient. No other family members were symptomatic or presented a history that might suggest hereditary or familial factors. Colonoscopy showed no discrete lesions.

Except for occasional bouts of mild non-specific abdominal pain and mild dysphagia, the patient remained well for a year. There was no complaint, odynophagia or nausea. Esophageal manometry was performed for persistent dysphagia at one year following the initial biopsy. This demonstrated a distal esophageal amplitude of 106 mm mercury with a duration of 3.5 s, peristaltic contraction 90% with 10% simultaneous contractions. Detailed review of the manometric data suggested a mild non-specific lower esophageal motility disorder.

In the absence of changes that might suggest neural hypertrophy, occasionally seen with achalasia, the mild motor dysfunction in this patient may be related to GERD. The demonstration of localized ganglioneuromatosis was most likely fortuitous and not etiologically related to the gastroesophageal reflux. However, the patient's insulin dependant diabetes might have affected esophageal motility possibly associated with an increased incidence of reflux.

It is most likely that the generous biopsy sampling of the nodular EG may have removed a significant portion of the lesion; therefore, it was not overtly visible on the subsequent endoscopy.

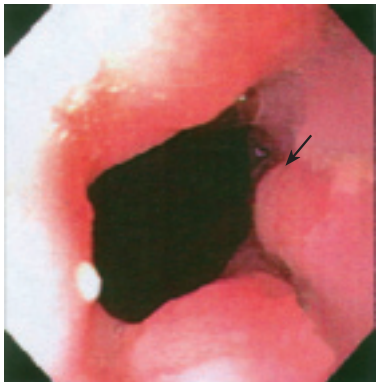

**Figure 1** Initial endoscopic image of localized nodularity in esophageal mucosal surface with irregular Z-line.

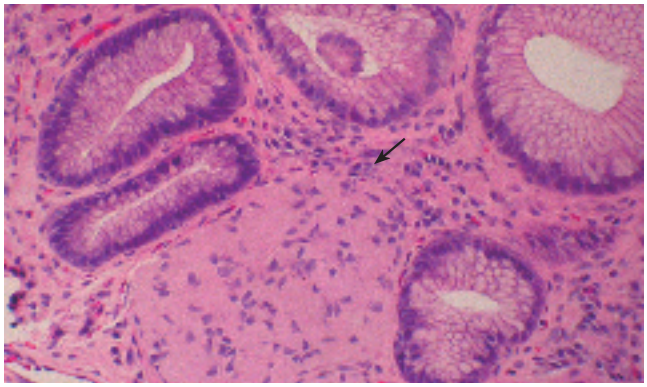

**Figure 2** Hematoxylin and eosin stained tissue section of biopsy from irregular EG-Junction showing neuroid proliferation within lamina propria (× 10).

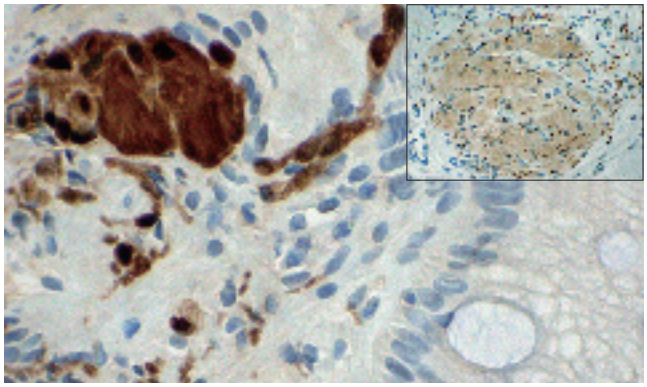

**Figure 3** Focal positivity for synaptophysin (insert × 10) confirms the presence of a neuronal (ganglio) component. S100 (× 20) confirms nerve sheath-neuromatous/ neuromatosis component.

## DISCUSSION

Primary esophageal tumors that show neuroid differentiation in addition to ganglioneuromata, include gastro-intestinal autonomic nerve tumor (GAN)[2,3], schwannoma[4,5] (some occasionally showing melanocytic differentiation)[6], and neurofibromas[7]. The innervation of the lower esophagus includes parasympathetic supply from the vagi and sympathetic innervation from the greater splanchnic and thoraci ganglia[8]. Histologically the neural components are seen within the muscle layer as myenteric plexus and in the submucosal neural plexus with branches entering the lamina propria[9].

Solitary or disseminated Schwann cell and ganglion cell proliferation anywhere in the gastrointestinal tract may appear as small intramucosal nodular lesions[10], exophytic polypoid lesions, or poorly demarcated transmural proliferations[11]. Ganglioneuroma, a fully differentiated tumor with no immature components[12], may occur as a solitary lesion (sporadic) or as multiple lesions called ganglioneuromatosis and may be associated with other diseases (syndromic).

Sporadic ganglioneuroma has been unknown to be associated with genetic syndromes and has been detected in patients of all ages with a mean age of 50 years. The majority of the solitary lesions are asymptomatic and, therefore, found incidentally, most frequently in the left colon[13].

Among the cases of multiple lesions, ganglioneuromatosis of an exophytic polypoid type (ganglioneuromatous polyposis) is characterized by interposition of neural proliferations with glandular components and is usually associated with familial adenomatous polyposis and multiple cutaneous lipomas. Ganglioneuromatosis of transmural proliferation type arising from the neural plexus in the bowel wall is frequently associated with other tumors, including MEN Ⅱb (medullary carcinoma thyroid, pheochromocytoma, oral-mucosal neuromas and skeletal deformities)[14], multiple ganglioneuromas and neurofibromas of the gastrointestinal tract, von Recklinghausen's disease, and neurogenic sarcoma[15]. Florid hyperplasia of submucosal or myenteric plexus is distinct for intramural ganglioneuromatosis and occurs with type Ⅰ neurofibromatosis[16]. Patients with syndromic ganglioneuromatosis present with symptoms, and the lesions are found much earlier in life, with a mean age approximately 35 years. There is no gender predominance in the incidence of this disease.

Non neoplastic neural proliferations involving the esophagus include achalasia, which is an esophageal motor disorder associated with a loss of myenteric ganglion cells with inflammation and secondary changes including neural proliferation, which closely mimics ganglioneuromatosis[17]. Neoplastic neural tumors that can involve the esophagus include ganglioneuroma, GAN, schwannoma, neurofibroma and other less prevalent forms like granular cell tumor with large eosinophilic cells[18]; gangliocytic paraganglioma showing predominantly spindle shaped cells with both ganglion and neuroendocrine features (more often seen in duodenum)[19]. Ganglioneuromatosis is visibly present throughout the gut showing predominantly spindle shaped neural proliferations with frequent ganglion type cells.

Ganglioneuroma is a benign tumor and the solitary variant may be cured by excision of the nodular lesion or complete polypectomy. Lesions in syndromic ganglioneuromatosis may require surgery, but the patient may die from the associated syndromes.

In summary, this case illustrates an instance of sporadic ganglioneuromatosis involving EG junction in a 58-year old female with a recurrent history of upper abdominal pain. Background of chronic inflammation and intestinal type metaplasia suggested that the symptoms were related to reflux esophagitis, possibly exacerbated by the nodular growth of the ganglioneuromatosis, which involved the gastro-esophageal junction. The attendant mild lower esophageal motility disorder (demonstrated by manometry) may reflect nonspecific reflux disorder. The patient was initially treated with Iansoprazole (Prevacid), which resulted in a partial relief of symptoms. This was followed by esomeprazole (Nexium) with additional relief of symptoms. The patient continued to experience mild intermittent dysphagia without odynophagia or nausea. Repeat EG endoscopy at 4 mo showed unremarkable GE junction. The importance of recognizing symptomatology indicative of lower esophageal sphincter dysfunction was reviewed with this patient with a discussion of follow-up studies over time[20]. It appears that the initial generous biopsy sampling removed the EG tumor nodularity, and this, together with the anti-acid treatment, decreased the intensity of the reflux esophagitis. Following the manometric study the patient was advised to swallow slowly with at least 30 s intervals, avoid beverages with extreme temperatures and was maintained on her current medications. We believe that although not etiologically associated, localized esophageal ganglioneuromatosis may exacerbate aspects of gastro-esophageal reflux disorder.

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*CASE REPORT*

# Osteoclast-like giant cell tumors of the pancreas and liver

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## Abstract

Osteoclast-like giant cell tumors (OGCT) are rare abdominal tumors, which mainly occur in the pancreas. The neoplasms are composed of two distinct cell populations and frequently show an inhomogenous appearance with cystic structures. However, due to the rarity of these tumors, only very limited clinical data are available. Imaging features and sonographic appearance have hardly been characterized. Here we report on two cases of osteoclast-like giant cell tumors, one located within the pancreas, the other within the liver, in which OGCTs are extremely rare. Both patients were investigated by contrast sonography, which demonstrated a complex, partly cystic and strongly vascularized tumor within the head of the pancreas in the first patient and a large, hypervascularized neoplasm with calcifications within the liver in the second patient. The liver OGCT responded well to a combination of carboplatin, etoposide and paclitaxel. With a combination of surgical resection, radiofrequency ablation and chemotherapy, the patient's survival is currently more than 15 mo, making him the longest survivor with an OGCT of the liver to date.



**Key words:** Osteoclast-like giant cell tumor; Liver cancer; Pancreatic cancer; Contrast sonography

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## INTRODUCTION

Osteoclast-like giant cell tumors (OGCTs) have been described in several extraskeletal sites. Within the alimentary tract, these rare neoplasms mainly occur within the pancreas. Since the first description by Rosai in 1968[1] and further classification by Alguacil-Garcia and Weiland in 1977[2], there have been at least 38 publications describing approximately 60 occurrences of OGCTs in the pancreas[1-15], whereas only 12 liver tumors have been reported[16-27].

## CASE REPORTS

### *Case 1*

A 54-year-old man regularly visited his urologist because of prostate hyperplasia. After a renal ultrasound was suspicious of a tumor within the left kidney, computed tomography (CT) of the abdomen was performed. CT only showed a parenchyma bridge within the left kidney but surprisingly demonstrated a 7 cm large inhomogenous solid liver tumor involving segments Ⅱ and Ⅲ. The patient was then referred to our clinic. He had no clinical symptoms, no history of liver diseases and denied alcohol and tobacco consumption. His further medical history was remarkable for peripheral arterial disease, hypertension, esophageal reflux, hiatal hernia and sleep apnea.

Physical examinations revealed an obese male (173 cm, 92 kg, BMI: 31) with normal vital signs. His abdomen was soft and nontender. Serum chemistries were within the normal range except for a gamma-glutamyl transferase level of 69 IU/L (normal range < 55 IU/L) and normocytic, normochromatous anemia [hematocrit 0.38% (0.4%-0.52%)]. Serum alpha-fetoprotein, carcinoembryonic cancer antigen and cancer antigen 19-9 were normal. Serology for hepatitis A, B, and C was negative.

B-mode sonography (HDI 5000, Philips) demonstrated a well-defined, inhomogenous, cauliflower-like tumor with multiple small calcifications, causing retraction of the liver contour. Within the center of the tumor, a focal nodular hyperplasia (FNH)-like stellar scar was present (Figure 1A). Contrast-enhanced sonography by use of an Acuson Sequoia 512 (Siemens, Germany) with Sonovue® (2 mL; Bracco, Italy) using coded pulse sequences (CPS) and a low mechanic index (MI) demonstrated an inhomogenous perfused tumor with a large feeding artery heading towards the center of the tumor, radially branching to the periphery. During the capillary and arterial perfusion phase, non-perfused tumor areas indicative of necrotic areas were observed, making a focal nodular hyperplasia highly unlikely (Figure 1B). Diagnosis of haemangioma or adenoma was excluded by the spoke-like architecture of tumor arteries with centrifugal filling (Figure 1B). The portal perfusion phase was characterized by a rapid decrease of perfusion

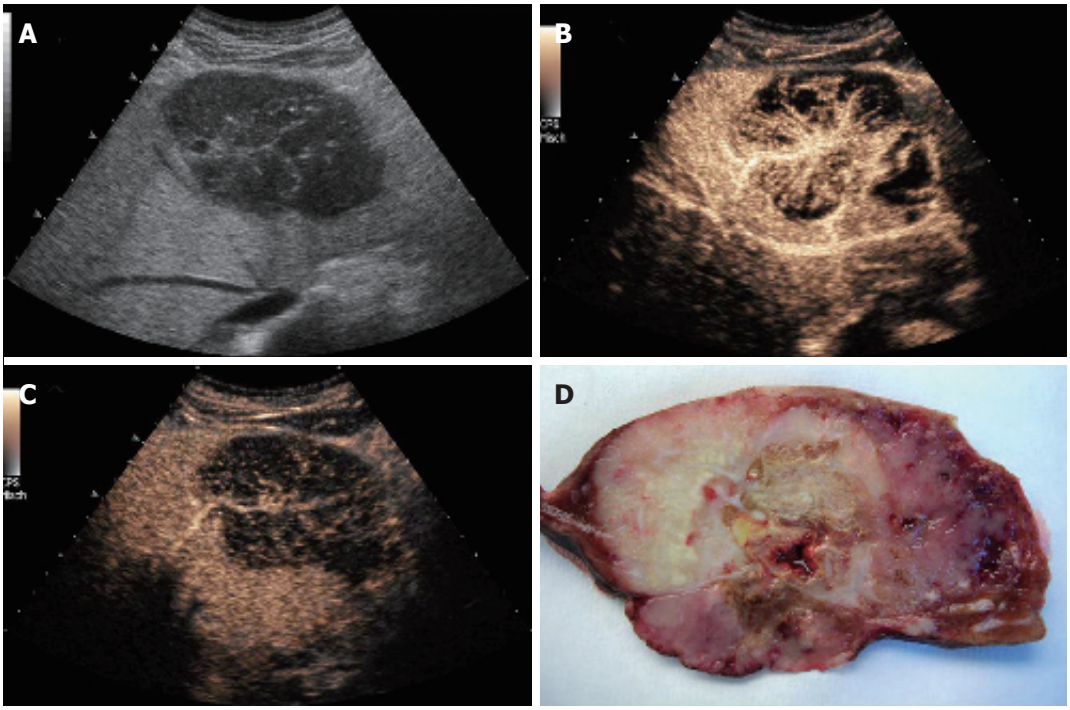


**Figure 1** Patient 1: Osteoclastic giant cell tumor of the liver: **A**: B-mode sonography (HDI 5000, Philips), contrast sonography during the **B**: arterial and **C**: portal phase (Siemens); **D**: resected tumor: cut surface shows distinct tumor areas with necrotic and hemorrhagic regions.

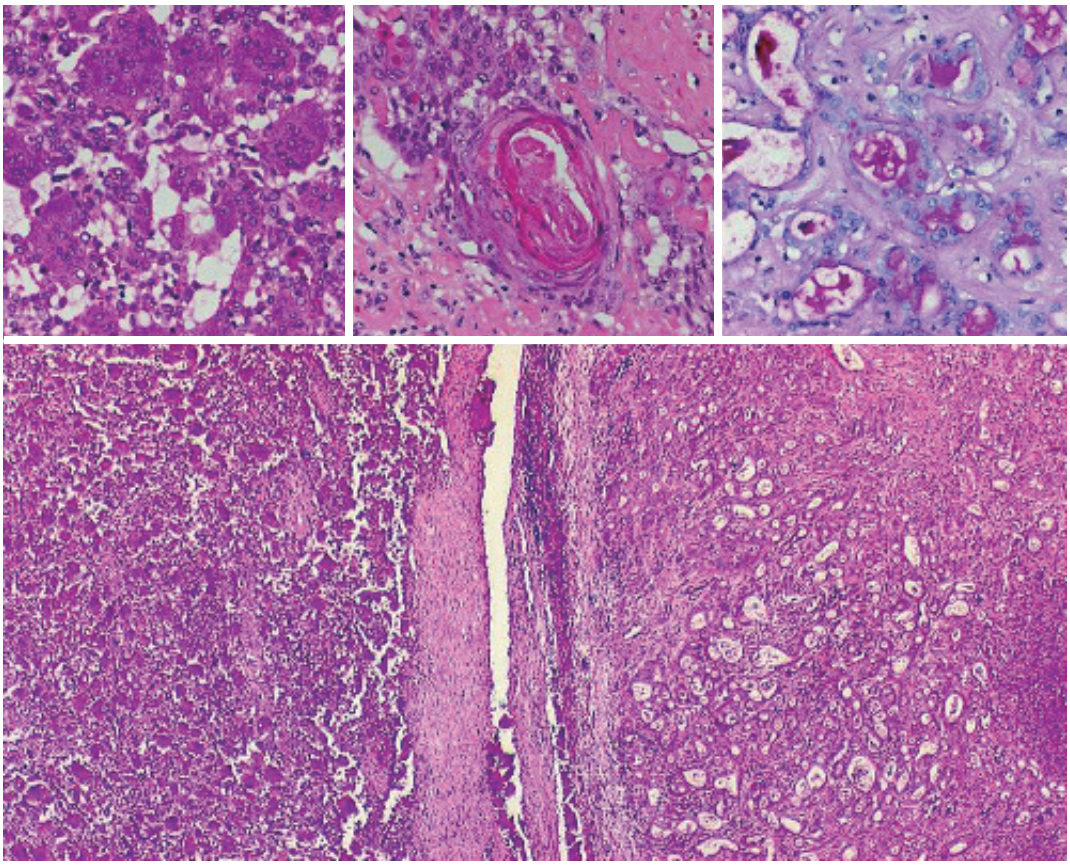

**Figure 2** Histology (HE, x 20-40) of OGCT of the liver with a mixed cell population of osteoclastic giant cells and pleomorphic mononuclear cells (left), adenocarcinomatous component with mucinous inclusions (PAS, upper right) and squamous cell differentiation (middle).

within the tumor (Figure 1C), indicating absence of portal vessels and thereby, together with the finding of a strong perfusion within the arterial phase, proving a malignant neoplasm.

Biopsy of the tumor was performed, demonstrating that the neoplasm was composed of pleomorphic mononuclear cells and scattered non-neoplastic osteoclast-like giant cells (OCGCs) with usually 10-20 uniformly small nuclei. Small mononuclear cells were differently characterized by cytological atypias and showed an increased number of mitoses with a proliferation rate of 70%. Focally there was an adenocarcinomatous component with mucinous inclusions and also areas of squamous cell differentiation (Figure 2). Immunofluorescence

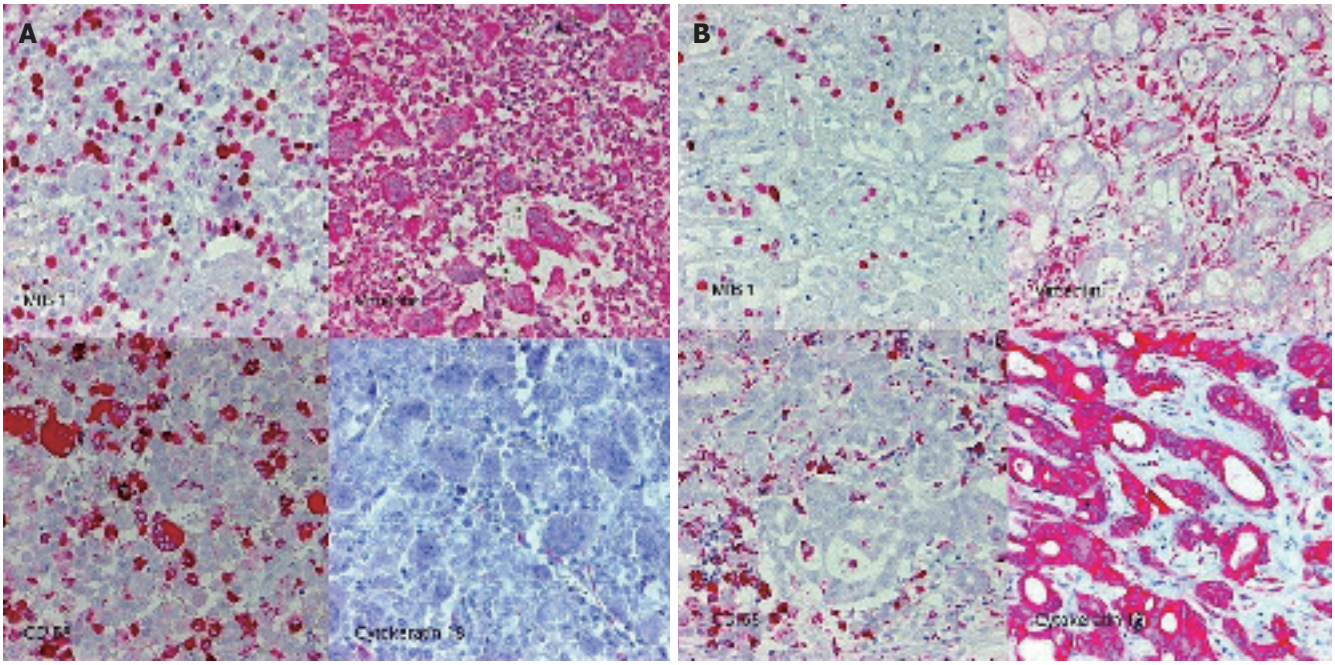


**Figure 3** Immunohistology of the OGCT of the liver. Panel **A**: mixed cell population of osteoclastic giant cells and pleomorphic mononuclear cells. CD68: histio-monocytic differentiation of osteoclastic giant cells, MIB 1: proliferation of mononuclear cells. Panel **B**: adenocarcinomatous component with mucinous inclusions: CK18+, CD68/ vimentin: -.

demonstrated expression of vimentin within both OCGCs and mononuclear cells, expression of macrophage marker CD 68 within OCGC and negative staining for CK18 (Figure 3). The adenocarcinomatous tumor components demonstrated expression of CK18 but negative staining for CD 68 and vimentin (Figure 3). In conclusion, the histopathological diagnosis revealed an osteoclast-like giant cell tumor of the liver.

As extensive further diagnostic procedures including gastroscopy, colonoscopy, endoscopic ultrasound and bone scintigraphy showed no evidence of another tumor or metastases, surgical resection of liver segments Ⅱ and Ⅲ with excision of regional lymph nodes was performed. Macroscopic examination of the resected specimen revealed an irregular shaped firm mass measuring 8.5 cm with hemorrhage and necrosis (Figure 1D). All surgical margins were free of tumors. However, two lymph nodes (retroduodenal and at the hepatic artery) showed metastases of an adenocarcinoma.

As OGCT's are typically aggressive tumors with a short life-expectancy, adjuvant chemotherapy consisting of carboplatin 250 mg/m$^2$ (d 1), etoposide (alternating doses of 50 and 100 mg, d 1-10) and paclitaxel (175 mg/m$^2$, d 1) was initiated. However, 6 wk after resection, as the first chemotherapy had just been completed, a single metastasis in the right lobe of the liver was diagnosed. The metastasis was successfully treated by radiofrequency ablation. Chemotherapy was proceeded for 6 mo. After restaging revealed no evidence of active tumor disease, chemotherapy was stopped. However, 3 mo after cessation of chemotherapy, tumor recurrence (liver and peritoneal metastases) was observed and chemotherapy (carboplatin, etoposide, paclitaxel) restarted. Restaging after 6 wk revealed partial remission with size reduction of metastases of 50%. Chemotherapy is currently proceeded. The patient's survival since diagnosis has been 15 mo.

### *Case 2*

A 49-year-old man suffered from pain in the upper abdomen since 4 mo ago, which was independent of food intake. During this time, his appetite and body weight were unchanged, however, his physical performance was reduced. Abdominal ultrasound revealed a 3 cm cystic mass in the region of the body and tail of the pancreas. The patient was then referred to our clinic for further diagnosis.

At admission he presented in fairly well condition with normal body weight (186 cm, 82 kg, BMI: 21). His abdomen was soft and nontender without evidence of ascites or signs of liver disease. Laboratory work-up showed no pathologic results except for moderate normocytic and normochromatous anemia (Hct 0.36% [0.4%-0.52%]). Neuron-specific enolase was slightly elevated (19.2 μg/L [< 15.2]), carcinoembryogenic antigen (CEA) and cancer antigen (CA) 19-9 were normal. He had stopped smoking 2 years ago and reported only moderate alcohol consumption. His past medical history was unremarkable. His family history revealed prostate carcinoma of his father.

B-mode sonography in our clinic (HDI 5000, Philips) indicated rapid enlargement of the pancreatic neoplasia, which presented as a well-defined complex lesion with several cysts and solid parts and had grown from 3 to 5 cm within 5 wk (Figure 4A). Contrast sonography by use of an Acuson Sequoia 512 with Sonovue® and CPS/low-MI technique demonstrated an intensively perfused tumor with strong vascularized margins and septs (Figure 4B), excluding a ductal pancreatic carcinoma. Strong vascularised pancreatic neoplasms have primarily to be differentiated

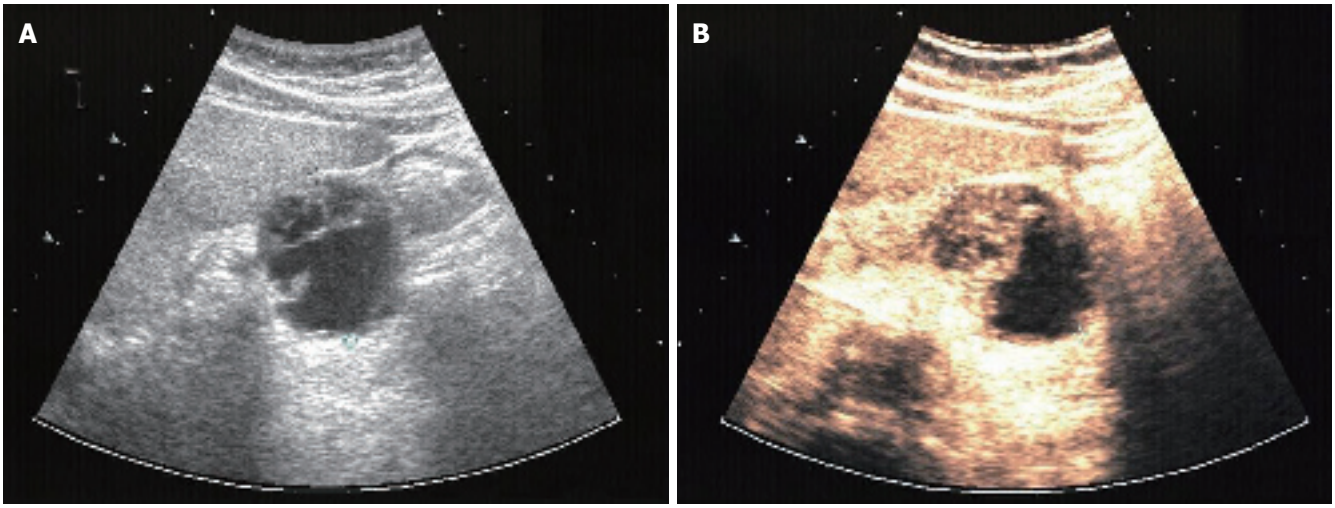


**Figure 4** Patient 2: osteoclastic giant cell tumor of the pancreas. **A**: B-mode (HDI 5000, Philips) and **B**: contrast sonography (Sonovue®, low MI/CPS, Acuson Sequoia 512).

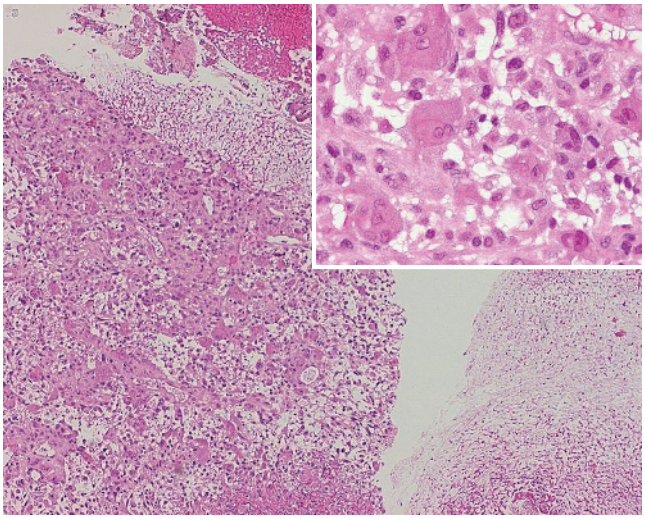

**Figure 5** Histology (HE, x 20-40) of OGCT of the pancreas with a mixed cell population of osteoclastic giant cells and pleomorphic mononuclear cells (left) and myxoid tumor areas (right).

from neuroendocrine tumors of the pancreas and from cystadenomas, which may also demonstrate strong vascularisation. However, malignant diseases become apparent after liver sonography. B-mode sonography demonstrated two complex nodular structures of 1 and 3 cm. Contrast sonography showed intensively perfused lesions with necrotic areas without portal perfusion, indicating metastases.

Biopsy revealed that the neoplasm was composed of pleomorphic mononuclear cells and scattered non-neoplastic osteoclast-like giant cells (OCGCs) with multiple uniformly small nuclei. OCGC's occasionally contained phagocytosed mononuclear cells. Immunohistochemically, the OCGC's were negative for cytokeratin but positive for vimentin and CD 68. Immunohistology of the pleomorphic cells demonstrated expression of vimentin, negative staining for cytokeratin and a proliferation rate of 30% (MIB-1). In conclusion, the histopathological diagnosis indicated osteoclast-like giant cell tumor of the pancreas (Figure 5).

As surgical resection was not possible, the patient received palliative chemotherapy with cisplatin 80 mg/m$^2$ (d 1), etoposide (100 mg/m$^2$, d 1, 3, 5) and ifosfamide (2 g/m$^2$, d 1-3) (PEI). After completion of three cycles of PEI, partial remission with significant size reduction of liver metastases was achieved. Thirteen mo after diagnosis chemotherapy is currently proceeded.

## DISCUSSION

Osteoclast-like giant cell tumors typically display an inhomogenous macroscopic appearance with cystic-liquid and necrotic areas and parenchymatous and calcified parts. Histologically, this mixed macroscopic structure corresponds to heterogeneous tissue structures of epithelial or undifferentiated tumor cells, which may contain foci of conventional adenocarcinoma, focal cartilagenous differentiation and bone formation[2,6,7,9]. The tumors are composed of two distinct cell types: a mononuclear cell population and in addition, osteoclastic tumor giant cells of uncertain lineage. The exceptional morphology and origin of the different cell types has been a matter of controversy since its first description in 1968. Recent evidence indicates that only the mononuclear cells constitute neoplastic tumor cells and that osteoclastic giant cells develop from secondary infiltrating cells.

Formation of osteoclast-like giant cells (OCGC) is speculated to result from fusion of bone-marrow derived mononuclear histiocytes/macrophages attracted to the tumor by chemotactic factors produced by the neoplastic cells[7,9,12,13]. Indeed, the CD-68/lysozyme reactivity of OCGCs suggests a histiocytic origin. Lack of mitoses and MIB-1 reactivity indicates a terminal stage of differentiation and non-neoplastic nature of the osteoclast-like giant cells, which are also K-ras negative[11]. Moderate staining of OCGCs with epithelial markers[20,25] is now explained by immunoreactivity of phagocytosed epithelial tumor cells[11,27]. The infiltrating mononuclear cells display

pleomorphism and neoplastic features and sometimes show features of epithelial tumors. However, other OGCTs lack epithelial differentiation[9,27]. Consequently, the term undifferentiated carcinoma with osteoclast-like giant cells has been proposed to more precisely describe these aggressive tumors.

Probably due to rapid tumor growth, osteoclast-like giant cell tumors of the pancreas only rarely present as small neoplasms. In more than 50 cases, in which tumor size was documented, only three neoplasias measured less than 3 cm. Typically OGCTs present as large tumors with cystic structures and necroses. The cystic component can become predominant, so that lesions may be misdiagnosed as pancreatic pseudocysts[3,4]. At time of diagnosis, more than 80% of tumors were already greater than 5 cm, 50% even greater than 10 cm. Most tumors arise in the head or body of the pancreas[1-15].

The differential diagnosis of pancreatic OGCTs includes cystic lesions like pancreatic cystadenomas, cystadenocarcinomas, serous and mucinous cystic tumors, pancreatic pseudocysts and also solid pancreatic tumors like ductal pancreatic carcinomas or neuroendocrine tumors. Solid tumors may be homogenous on computed tomographic imaging; however, they can also be very inhomogenous, as focal hemorrhage or necrosis is frequently found[9,12,15]. Vascularisation of these neoplasias has been described only in single cases. In one well-characterized patient, the tumor wall was slightly enhanced on contrast-enhanced CT[13]. Selective angiography demonstrated slight tumor staining[13].

In our patient with a pancreatic OGCT, contrast-enhanced sonography demonstrated a strong vascularisation within both the tumor and the liver metastases, making a pancreatic ductal carcinoma, which is typically only poorly vascularised, highly unlikely. A strong vascularised pancreatic neoplasm has to be differentiated from neuroendocrine tumors of the pancreas and from cystadenomas, which may also demonstrate strong vascularisation. In contrast, cystadenocarcinomas are generally poorly vascularised[28]. However, further studies are necessary to evaluate whether intense perfusion is a characteristic feature of OGCTs.

Osteoclast-like giant cell tumors of the liver are also generally large and inhomogenous neoplasias, ranging from 5 to 12 cm in size. Tumors typically feature necrotic or hemorrhagic regions and may also show cystic structures[16-27]. As published reports mainly focused on histopathology, radiological findings have only been documented in single cases. Magnetic resonance imaging in one case demonstrated a 10 cm large, fairly well circumscribed heterogenous solid mass with multiple fluid-like regions representing cystic components or necrosis on T1-weighted images[25]. Positron emission tomography scan in the same patient showed fluorine-18 fluorodeoxyglucose-uptake within the tumor[25]. In another patient, computed tomography described a 6 cm homogenous and hypervascularised tumor, which after resection presented as an inhomogenous tumor with hemorrhagic and necrotic areas[26].

In our patient, the liver tumor presented as an inhomogenous, cauliflower-like tumor with multiple small calcifications and a central stellar scar. However, contrast sonography revealed non-perfused tumor areas during the capillary and arterial perfusion phase, indicating necroses, thereby making a focal nodular hyperplasia highly unlikely[29,30]. Diagnosis of hemangioma or adenoma could be excluded by the spoke-like architecture of tumor arteries with centrifugal filling. The portal perfusion phase was characterized by a rapid decrease of perfusion within the tumor, indicating absence of portal vessels and thereby, together with the finding of a strong perfusion within the arterial phase, proving a malignant neoplasm[29,30].

A hepatocellular carcinoma was very unlikely, as the tumor demonstrated a very unusual vascular architecture and because there were no signs of cirrhosis. According to sonographic morphology and vascularisation pattern, the differential diagnosis included malignant primary liver tumors like peripheral cholangiocarcinoma (which, however, typically are poorly vascularized and do not show cysts or necroses), fibrolamellar carcinoma, epithelial hemangioendothelioma (which generally shows a multinodular structure) and a metastasis. However, a definitive sonographic diagnosis was impossible.

The average age of patients with osteoclast-like giant cell tumors of both pancreas and liver is around 60 years, ranging from 28 to 88 years. In pancreatic OGCTs, males and females appear to be affected in a fairly equal ratio[9,13]. The main symptoms are abdominal pain or discomfort and weight loss. Jaundice also frequently occurs if the tumor is located in the head. In cases of great cystic tumors, a palpable mass may be found. Invasion into adjacent structures is common. Nodal or intra-abdominal metastases are found in approximately 50% of patients at the time of diagnosis[1-15]. Overall, the prognosis of pancreatic OGCTs is unfavorable. As patients usually present with advanced diseases, complete resection can only rarely be performed. Leighton and Shiozawa reviewed 20 and 32 cases of pancreatic tumors, respectively, and determined the median survival rates to be less than 1 year. Interval to death or disease progression ranged from 4 mo to 5 years in these series[12,13]. In one patient, long-term survival of 15 years was documented; however, tumor recurrence within the pancreas after 10 years has also been reported[8]. Overall, the prognosis of pancreatic OGCTs is comparable to that of common ductal pancreatic carcinomas[12].

In contrast to pancreatic manifestations, OGCTs of the liver have been mostly described in male patients. Ten of 12 tumors (in one publication no data on patient's age and sex were reported)[27] occurred in men. The overall prognosis of OGCTs of the liver seems to be even worse than for pancreatic cases. Previous reports have demonstrated that these tumors are uniformly very aggressive and that survival ranges from 1 to 10 mo[16-26]. To date, there is only one report of chemotherapy and radiotherapy in the management of osteoclast-like giant cell tumors. Hood *et al* treated a patient with recurrent liver tumor with a combination of chemotherapy (5-fluorouracil and adriamycin), external beam radiation and radioimmunotherapy ($I^{131}$-labeled antiferritin immunoglobulin [IgG]), but could only achieve a partial response for several months[20]. In our case, the liver OGCT responded well to a combination

of carboplatin, etoposide and paclitaxel. With a combination of surgery, local ablative therapy and chemotherapy, our patient is currently the longest survivor reported. Due to epithelial features of the mononuclear neoplastic cells in some patients, agents like gemcitabine have been suggested as adjuvant or palliative therapy. As histology in our patients demonstrated undifferentiated tumors in both cases, we chose a polychemotherapy with cisplatin, etoposide and ifosfamide (PEI) and carboplatin, etoposide and paclitaxel, respectively. After three cycles of PEI, partial remission was observed in the patient with pancreatic OGCT. In the other patient with an OGCT of the liver, early tumor recurrence within the right lobe of the liver was observed 6 wk after surgical resection.

In conclusion, the histopathological features of these rare tumors have been precisely described in recent years, providing the basis for correct histological classification. OGCTs should also be included in the sonographic differential diagnosis of tumors of the pancreas and liver. B-mode sonography, as well as arterial perfusion of a liver OGCT resembles those of an FNH, reflecting its hepatic origin. However, demarcation of hemorrhagic necroses during the arterial and capillary phase together with a missing increase of signal intensity in comparison to the surrounding liver tissue during the portal and late phases allows exclusion of an FNH and diagnosis of a malignant neoplasm. For optimization of chemotherapy and other treatment strategies of osteoclast-like giant cell tumors, future studies should not only focus on histopathologic features, but also on diagnostic and therapeutic approaches.

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# Meetings

## MAJOR MEETINGS COMING UP

First Biennial Congress of the Asian-Pacific Hepato-Pancreato-Biliary Association
March, 2007
Fukuoka, Japan
http://www.congre.co.jp/1st-aphpba

American College of Gastroenterology Annual Scientific
20-25 October 2006
Las Vegas, NV

14th United European Gastroenterology Week, UEGW
21-25 October 2006
Berlin, Germany

APDW 2006: Asian Pacific Digestive Week 2006
26-29 November 2006
Lahug Cebu City, Philippines

## EVENTS AND MEETINGS IN THE UPCOMING 6 MONTHS

Falk Symposium 151: Emerging Issues in Inflammatory Bowel Diseases
24-25 March 2006
Sydney - NSW
Falk Foundation e.V.
symposia@falkfoundation.de

10th International Congress of Obesity
3-8 September 2006
Sydney
Event Planners Australia
enquiries@ico2006.com
www.ico2006.com

Easl 2006 - the 41st annual
26-30 April 2006
Vienna, Austria
Kenes International

Prague hepatology 2006
14-16 September 2006
Prague
Foundation of the Czech Society of Hepatology
veronika.revicka@congressprague.cz
www.czech-hepatology.cz/phm2006

12th International Symposium on Viral Hepatitis and Liver Disease
1-5 July 2006
Paris
MCI France
isvhld2006@mci-group.com
www.isvhld2006.com

Falk Symposium 152: Intestinal Disease Part I, Endoscopy 2006 - Update and Live Demonstration
4-5 May 2006
Berlin
Falk Foundation e.V.
symposia@falkfoundation.de

Falk Symposium 153: Intestinal Disease Part II, Immunoregulation in Inflammatory Bowel Disease - Current Understanding and Innovation
6-7 May 2006
Berlin
Falk Foundation e.V.
symposia@falkfoundation.de

ILTS 12th Annual International Congress
3-6 May 2006
Milan
ILTS
www.ilts.org

Internal Medicine: Gastroenterology
22 July 2006-1 August 2006
Amsterdam
Continuing Education Inc
jbarnhart@continuingeducation.net

6th Annual Gastroenterology And Hepatology
15-18 March 2006
Rio Grande
Office of Continuing Medical Education
cmenet@jhmi.edu
www.hopkinscme.net

World Congress on Gastrointestinal Cancer
28 June 2006-1 July 2006
Barcelona, Spain
c.chase@imedex.com

International Conference on Surgical Infections, ICSI2006
6-8 September 2006
Stockholm
European Society of Clinical Microbiology and Infectious Diseases
icsi2006@stocon.se
www.icsi2006.se/9/23312.asp

7th World Congress of the International Hepato-Pancreato-Biliary Association
3-7 September 2006
Edinburgh
Edinburgh Convention Bureau
convention@edinburgh.org
www.edinburgh.org/conference

Society of American Gastrointestinal Endoscopic Surgeons
26-29 April 2006
Dallas - TX
www.sages.org

Digestive Disease Week 2006
20-25 May 2006
Los Angeles
www.ddw.org

Annual Postgraduate Course
25-26 May 2006
Los Angeles, CA
American Society of Gastrointestinal Endoscopy
www.asge.org/education

American Society of Colon and Rectal Surgeons
3-7 June 2006
Seatlle - Washington
www.fascrs.org

## EVENTS AND MEETINGS IN 2006

10th World Congress of the International Society for Diseases of the Esophagus
22-25 February 2006
Adelaide
isde@sapmea.asn.au
www.isde.net

Falk Symposium 151: Emerging Issues in Inflammatory Bowel Diseases
24-25 March 2006
Sydney - NSW
Falk Foundation e.V.
symposia@falkfoundation.de

10th International Congress of Obesity
3-8 September 2006
Sydney
Event Planners Australia
enquiries@ico2006.com
www.ico2006.com

Easl 2006 - the 41st annual
26-30 April 2006
Vienna, Austria
Kenes International

VII Brazilian Digestive Disease Week
19-23 November 2006
www.gastro2006.com.br

International Gastrointestinal Fellows Initiative
22-24 February 2006
Banff, Alberta
Canadian Association of Gastroenterology
cagoffice@cag-acg.org
www.cag-acg.org

Canadian Digestive Disease Week
24-27 February 2006
Banff, Alberta
Digestive Disease Week Administration
cagoffice@cag-acg.org
www.cag-acg.org

Prague Hepatology 2006
14-16 September 2006
Prague
Foundation of the Czech Society of Hepatology
veronika.revicka@congressprague.cz
www.czech-hepatology.cz/phm2006

12th International Symposium on Viral Hepatitis and Liver Disease
1-5 July 2006
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Falk Seminar: XI Gastroenterology Seminar Week
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symposia@falkfoundation.de

European Multidisciplinary Colorectal Cancer Congress 2006
12-14 February 2006
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Congresscare
info@congresscare.com
www.colorectal2006.org

Falk Symposium 152: Intestinal Disease Part I, Endoscopy 2006 - Update and Live Demonstration
4-5 May 2006
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Falk Symposium 153: Intestinal Disease Part II, Immunoregulation in Inflammatory Bowel Disease - Current Understanding and Innovation
6-7 May 2006
Berlin
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14th United European Gastroenterology Week
21-25 October 2006
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United European Gastroenterology Federation
www.uegw2006.de

World Congress on Controversies in Obesity, Diabetes and Hypertension
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Internal Medicine: Gastroenterology
22 July 2006-1 August 2006
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Continuing Education Inc
jbarnhart@continuingeducation.net

6th Annual Gastroenterology And Hepatology
15-18 March 2006
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Hepatitis 2006
25 February 2006-5 March 2006
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hepatitis2006@mangosee.com
mangosee.com/mangosteen/hepatitis2006/hepatitis2006.htm

World Congress on Gastrointestinal Cancer
28 June 2006-1 July 2006
Barcelona, Spain
c.chase@imedex.com

International Conference on Surgical Infections, ICSI2006
6-8 September 2006
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European Society of Clinical Microbiology and Infectious Diseases
icsi2006@stocon.se
www.icsi2006.se/9/23312.asp

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25-26 May 2006
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3-7 June 2006
Seatlle - Washington
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Venetian Hotel, Las Vegas, Neveda
The American College of Gastroenterology

AASLD 57th Annual - The Liver Meeting™
27-31 October 2006
Boston, MA
AASLD

New York Society for Gastrointestinal Endoscopy
13-16 December 2006
New York
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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 U S A* 2006; In press

*Organization as author*

4 **Diabetes Prevention Program Research Group**. Hypertension, insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension* 2002; **40**: 679-686 [ PMID: 12411462 ]

*Both personal authors and an organization as author*

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

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

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*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 ]

**Books**

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

*Chapter in a book (list all authors)*

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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 Tumour Conference; 2001 Sep 13-15; Leeds, UK. New York: Springer, 2002: 30-56

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**Electronic journal** (list all authors)

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

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

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