Attenuation of experimental liver fibrosis by hepatocyte growth factor gene delivery mediated by adenovirus

Research Article

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**Key words**: Liver fibrosis; Hepatocyte growth factor; Transforming growth factor; Gene therapy; Adenovirus

**Abbreviations**: adenovirus, (Ad); albumin, (ALB); carbon tetrachloride, (CCL\textsubscript{4}); extracellular material, (ECM); glutamate pyruvate transaminase, (GPT); glutamic oxaloacetic transaminase, (GOT); hepatocyte growth factor, (HGF); hydroxyproline, (Hyp); plaque-forming units, (PFU); total bilirubin, (TBil); total protein, (TP); transforming growth factor-\textbeta\textsubscript{1}, (TGF-\textbeta\textsubscript{1})

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Received: 7 November 2008; Revised: 5 January 2009
Accepted: 8 January 2009; electronically published: January 2009

**Summary**

The hepatocyte growth factor (HGF), originally identified and cloned as a potent mitogen for hepatocytes, has an essential part in the development and regeneration of the liver. In this study, HGF expression in vitro and in vivo was determined using ELISA. Rats were injected subcutaneously with CCL\textsubscript{4} for eight weeks to induce liver fibrosis, and then divided randomly into groups for administration of various doses of adenovirus HGF (Ad-HGF) or vehicle. All rats were sacrificed 8 weeks after obtaining samples of serum and hepatic tissue. The results showed that glutamic oxaloacetic transaminase (GOT), glutamate pyruvate transaminase (GPT), total protein (TP), albumin (ALB), and total bilirubin (TBil) in serum were significantly recovered after gene therapy ($P<0.05$). Ad-HGF also attenuated the expression of TGF-\textbeta\textsubscript{1} and the deposition of collagen. We conclude that intravenous administration of Ad-HGF may be useful for the treatment of fibrotic liver by promoting liver function recovery and collagenolytic capacities.

I. Introduction

Liver fibrosis is a common result of chronic hepatic diseases caused by various types of injurious trauma and inflammatory agents, such as hepatitis virus, chemical toxicity, alcohol and autoallergy. Liver consists of parenchymal (hepatocytes) and non-parenchymal cells (Kupffer, stellate, and endothelial cells), and activation of Kupffer cells by membrane components from impaired hepatocytes and infiltrating inflammatory cells leads to the secretion of pro-fibrotic factors, such as reactive oxygen species. These pro-fibrotic factors activate hepatic stellate cells, leading to a change of myofibroblast phenotype and an increase in the production of extracellular matrix components, such as lammin and collagen, which results in liver fibrosis (Hayashi et al, 2008). There is no effective treatment for this disease, other than liver transplantation (Pinzani and Rombouts, 2004).

The pathogenesis of liver fibrosis is characterized by the excess production and deposition of collagen, which could frustrate the normal hepatic parenchyma (Xia et al, 2006). There are several reports that many growth factors participate in the progression of liver fibrosis, for instance, hepatocyte growth factor (HGF), transforming growth factor-\textbeta\textsubscript{1} (TGF-\textbeta\textsubscript{1}) and platelet-derived growth factor
(PDGF), which regulate various cellular functions and act antagonistically against each other (Borkham-Kamphorst et al., 2007; Doh et al., 2008; Ogura et al., 2001). Matsuda et al reported that expression of HGF mRNA in the liver decreases gradually following initial fibrosis induced by treatment with dimethylsulfoxide (DMS), whereas TGF-β1 mRNA is over-expressed persistently during the entire period. Consistent with this, the level of plasma HGF decreased during the onset of hepatic fibrosis following the initial elevation due to treatment with DMN, which is a consequence of apoptotic and fibrotic stress (Matsuda et al., 1995). Both chronic and acute liver injury can induce transient elevation of the level of HGF (Cui et al., 2008). Tsubouchi et al developed an enzyme-linked immunosorbent assay (ELISA) with high levels of specificity and sensitivity for HGF in human serum. They found that levels of HGF in the serum of patients with acute hepatitis, chronic hepatitis and cirrhosis were higher than those in normal subjects (Tsubouchi et al., 1991). These results suggest strongly that HGF may be a key factor in the pathogenesis of liver fibrosis.

HGF is a multifunctional polypeptide that elicits mitogenic, motogenic and morphogenic activities, and is known to be the most potent mitogen for parenchymal hepatocytes but not for non-epithelial liver stromal cells (Matsuda et al., 1995). HGF stimulates proliferation of epithelial and endothelial cells (Nakamura et al., 1986; Bussolino et al., 1992), and triggers the ’scatter’ effect by inducing cell dissociation and migration (Stoker et al., 1987). HGF increases the formation of branching tubules by epithelial cells grown in a collagen gel matrix (Montesano et al., 1991; Brinkmann et al., 1995; Medico et al., 1996), and prevents cell death induced by inhibition of adhesion (Frisch and Francis, 1994). The receptor for HGF is the heterodimeric tyrosine kinase encoded by the c-met proto-oncogene (Giordano et al., 1989; Bottaro et al., 1991; Naldini et al., 1995). HGF receptor signaling is mediated by phosphorylation of a multifunctional docking site located in the C-terminal region that interacts with multiple SH2-containing signal transducers (Ponzetto et al., 1994). The biological response is elicited by the concomitant activation of multiple intracellular effectors, including phosphatidylinositol 3-kinase and the Ras pathway (Graziani et al., 1993; Ponzetto et al., 1993).

In vivo, HGF produced resolution of fibrosis in the cirrhotic liver, inhibited hepatocyte apoptosis and stimulated hepatocyte mitosis, resulting in the survival of rats with an otherwise lethal illness (Ueki T et al., 1999). Reduction of TGF-β1 expression and collagen deposition is the most important pathway for the attenuation of liver fibrosis via HGF (Jiang et al., 2008). The curative effect of HGF has been elucidated by many studies. The half-life of exogenous HGF in blood circulation is only 3–5 min; therefore, the application of HGF is limited (Kawaida et al., 1994). Gene therapy is a potential strategy to overcome this problem, which could allow cells to secrete HGF persistently and maintain it in the serum (Xue et al., 2003).

Carbon tetrachloride (CCL4) is widely used to induce toxic liver injury and fibrosis in laboratory animals, and it was used to induce liver fibrosis in this study. It has been suggested that CCL4-induced hepatotoxicity involves the metabolism of CCL4 by cytochrome P450 to the trichloromethyl radical (CCL3•), leading to lipid peroxidation (Lee et al., 2007). This process is followed by the activation of Kupffer cells, which is accompanied by the production of proinflammatory mediators and profibrotic factors (Planagumá et al., 2005).

HGF has an important role in liver regeneration and amelioration of fibrosis, and we supposed that systemic administration of Ad-HGF might have therapeutic potential in the treatment of CCL4-induced liver fibrosis. To test this possibility, the anti-fibrotic role and therapeutic effect of Ad-HGF were determined in a rat model.

II. Materials and Methods

A. HGF expression in vitro and in vivo

Hepatocytes were isolated from adult male Wistar rats by collagenase perfusion as described (Cui et al., 2008). The isolated hepatocytes, showing more than 90% viability by trypan blue exclusion, were suspended in complete culture medium (Williams’ medium E supplemented with 10% fetal calf serum, 5 mM Hapes, 100 U/ml penicillin, 0.1 mg/ml streptomycin, 10 nM dexamethasone, and 10 nM insulin), diluted to 1 × 10⁶ cells/ml, and seeded onto 12-well plates (Costar, USA). The cells were transfected with vehicle vector or Ad-HGF at a multiplicity of infection (MOI) of 100. After incubation for 2 h, viruses were removed by rinsing twice with PBS, and then cultured for 48 h in serum-free medium. The conditioned medium was collected and analyzed by ELISA (Bio-source, USA).

Rats were given 1 × 10⁸ plaque-forming units (PFU) of vehicle vector or Ad-HGF by intravenous injection, and plasma was collected 5, 7, 11 and 14 days later and analyzed by ELISA.

B. Animal Model

Adult male Wistar rats (30; body weight 200–280g) were obtained from the Academy of Military Medical Sciences (Beijing, China) and housed under conventional clean conditions. All animals received humane care and the experimental protocol was approved by the Committee of Laboratory Animals according to the guidelines of the Institute of Radiation Medicine (Beijing, China).

Six rats were used as the control group (group A). On the first day of the study, the other 24 rats were each given a single subcutaneous injection of CCL4 (2.3 (v/v) mixture of CCL4 (400 mL/L) and olive oil) at a CCL4 dose of 5mL/kg body weight, and a single subcutaneous injection of the CCL4/oil mixture at a CCL4 dose of 3mL/kg body weight twice a week for eight weeks. The 24 CCL4-treated rats were divided at random into four groups of 6 (groups B – E). Group B was the 5 × 10⁷ PFU vehicle-infused group. Group C was the 1 × 10⁷ PFU Ad-HGF-infused group. Group D was the 2.5 × 10⁸ PFU Ad-HGF-infused group. Group E was the 5 × 10⁸ PFU Ad-HGF-infused group. After injection of CCL4/oil for four weeks, adenoviruses were infused through the tail vein each two days, five times totally. The model schedule and treatment are shown in Figure 1.

C. Biochemical Assays

Blood samples were taken at the time of sacrifice. Serum biochemical parameters glutamic oxaloacetic transaminase (GOT), glutamate pyruvate transaminase (GPT), total protein (TP), albumin (ALB), and total bilirubin (TBil) were determined by an automated analyzer in the Clinical Laboratory in Beijing Cancer Hospital.
D. Hydroxyproline Content

The hydroxyproline content of liver samples was measured with a hydroxyproline assay kit (Nanjing Jiancheng Bioengineering Institute, China). Briefly, liver samples (30–40 mg) were homogenized in distilled water. The homogenate was hydrolyzed by incubation in 10 mol/L HCl at 110°C for 18 h. The hydrolysates were dried by speed vacuum centrifugation for 3–5 h and then dissolved in 0.2 mol/L citric acid, 0.2 mol/L glacial acetic acid, 0.4 mol/L sodium acetate, 0.85 mol/L sodium hydroxide, pH 6.0. Hydroxyproline in the hydrolysates was measured as described (Ren et al., 2007).

E. Histological and Immunohistochemical Staining

Liver tissue sections were stained with hematoxylin and eosin (H&E) for histopathological examination or with Sirius red for collagen determination (Duan et al., 2003). The expression of TGF-β1 was detected by immunohistochemical staining. The paraffin sections of left median lobes were incubated with 30 ml/L H2O2 in methanol at 37°C for 10 min to quench endogenous peroxidase activity. The sections were blocked at room temperature for 20 min, then incubated with antibodies against TGF-β1 (Santa Cruz, CA) overnight at 4°C. The sections were washed with PBS then treated with DAB to stain.

F. Statistical Analysis

All data are expressed as mean ± SD. Differences between mean values of groups were tested for statistical significance (*P ≤0.05) by the Kruskal-Wallis test using the SAS system.

III. Results

A. HGF expression in vitro and in vivo

Currently, the clinical use of adenovirus is limited to local application; systemic administration leads to redundant transgene expression in the liver (Yao et al., 2007). However, this characteristic may be used to treat liver disease. Using an MOI of 100, primary cultured rat hepatocytes were transfected with vehicle vector or Ad-HGF. The expression of the HGF transgene was determined by ELISA (Figure 2). HGF secretion was increased about 44-fold and reached a concentration of 8.8 ng/ml in the culture supernatant of Ad-HGF infected cells.

To elucidate HGF expression in vivo, normal rats were injected intravenously with 1 × 10⁷ PFU of vehicle or Ad-HGF. A substantial amount of HGF in the plasma of rats after two weeks was detected by ELISA (Figure 2). The concentration of plasma HGF increased about 5-fold and exceeded 50 ng/ml for at least 10 days in the Ad-HGF–injected rats.

B. Ad-HGF alleviate hepatosis caused by CCl4 Injection

Repeated administration of CCl4 has been shown to cause hepatocyte destruction and liver dysfunction, resulting in the manifestation of chronic liver injury (Nagano et al., 2007). Figure 3 shows the morphological changes of the livers of virus–injected rats. The surface of the liver was coarse and pale in the model group, while it was glossy and ruddy in the normal and the 5 × 10⁷ PFU Ad-HGF infused group. As shown in Table 1, the injection of CCl4 resulted in a significant increase in GOT/GPT from 175.9/56.4 U/L in the normal control group to 469.7/110.3 U/L in the model group, and was reduced significantly, to 336.1/80.4 U/L, in the 5 × 10⁷ PFU Ad-HGF infused group compared with the model group (*P <0.05). The TP, ALB and Tbil levels in serum were determined to evaluate liver function. The levels of TP and ALB in rat serum decreased to 49.6 g/L and 22.9 g/L, respectively, in the model group from 60.3 g/L and 29.3 g/L in the normal group. The TP and ALB levels in the liver injury rats recovered to 55.3 g/L and 27.7 g/L after administration of 5 × 10⁷ PFU Ad-HGF (*P <0.05). The level of Tbil also recovered from 28.1 μmol/L in the model group to 15.2 μmol/L in the 5 × 10⁷ PFU Ad-HGF treated group (*P <0.05). The histological sections showed significant necrosis and inflammation in the model group, while 5 × 10⁷ PFU Ad-HGF was effectively able to relieve the histopathological changes of hepatic cells injured by administration of CCl4 (Figure 4).
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Figure 2. Expression of HGF in vitro and in vivo. (A) Primary rat hepatocytes were infected at MOI of 100, and HGF expression was measured by ELISA in the culture supernatant 48 h post infection. (B) The rats were given $1 \times 10^8$ PFU of vehicle vector or Ad-HGF intravenously, and the plasma HGF was determined by ELISA. Values are expressed as mean ± SD.

Figure 3. Morphology of livers in virus injected rats. The livers were excised and photographed. Note the morphological changes and the smaller volume of livers treated with Ad-HGF versus livers treated with vehicle alone.
Table 1. Effects of Ad-HGF on liver function. Glutamic oxaloacetic transaminase (GOT) glutamate pyruvate transaminase (GPT), and total bilirubin (TBil) were markedly reduced, whereas total protein (TP) and albumin (ALB) were increased significantly in the Ad-HGF-injected group. Values are expressed as mean ± SD (n = 6).

*P <0.05, **P <0.01 versus with CCl₄ + 5 × 10⁹ PFU in the vehicle group.

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>GOT (U/L)</th>
<th>GPT (U/L)</th>
<th>TP (g/L)</th>
<th>ALB (g/L)</th>
<th>TBil (μmol/L)</th>
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<tr>
<td>Normal</td>
<td>6</td>
<td>175.9±11.9**</td>
<td>56.4±6.0**</td>
<td>60.3±5.1**</td>
<td>29.3±1.0**</td>
<td>8.3±1.0**</td>
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<tr>
<td>CCl₄ + 5×10⁹PFU vehicle</td>
<td>6</td>
<td>469.7±83.5</td>
<td>110.3±16.8</td>
<td>49.6±3.0</td>
<td>22.9±2.6</td>
<td>28.1±1.5</td>
</tr>
<tr>
<td>CCl₄ + 1×10⁹PFU Ad-HGF</td>
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<td>102.2±16.4</td>
<td>52.7±6.9</td>
<td>23.4±1.7</td>
<td>19.9±3.0**</td>
</tr>
<tr>
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<td>96.0±12.6</td>
<td>53.3±6.6</td>
<td>25.4±1.8</td>
<td>18.1±2.9**</td>
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<tr>
<td>CCl₄ + 5×10⁹PFU Ad-HGF</td>
<td>6</td>
<td>336.1±61.0*</td>
<td>80.4±5.8**</td>
<td>55.3±4.7*</td>
<td>27.7±1.9</td>
<td>15.2±3.2**</td>
</tr>
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</table>

Figure 4. Attenuation of liver fibrosis by HGF gene therapy. Extensive interstitial collagen stain was noticeable in liver sections of the rat injected with vehicle and pseudolobule formations were found in this group. The collagen content and TGF-β1 expression were decreased significantly with increasing dose of Ad-HGF. (A) Histological sections stained with H&E (original magnification, × 100). (B) Collagen content determined by staining with Sirius red (original magnification, × 100). (C) TGF-β1 expression, determined by staining with antibody to TGF-β1 (original magnification, × 400).
C. Ad-HGF attenuate the fibrosis induced by chronic liver injury

Hepatic fibrosis is the result of the wound-healing response of the liver to chronic injury. In acute liver injury, parenchymal cells regenerate and replace the necrotic and apoptotic cells. This process is associated with an inflammatory response and a limited deposition of extracellular matrix (ECM). If the injury is chronic and persistent, however, liver regeneration eventually fails, and hepatocytes are substituted with abundant ECM. HGF is a potent activator of latent hepatic collagenases that promote the degradation of ECM. In order to evaluate the effect of Ad-HGF on hepatic fibrosis, rats were injected with various doses of Ad-HGF. Figure 4 shows representative micrographs of liver tissue sections stained with Sirius red. In rats treated with vehicle alone, extensive deposition of collagen was observed (Figure 3 B); however, it was markedly reduced after administration of Ad-HGF. In addition, biochemical methods were used to determine the hydroxyproline content of the hydrolysates extracted from liver tissue. This assay is based on the observation that essentially all of the hydroxyproline in animal tissues is found in collagen. As shown in Figure 5, the quantitative assay of liver hydroxyproline content revealed an increase in the liver extracts at 8 weeks after the injection of CCl₄, suggesting increased hepatic fibrosis; while that in the 2.5 × 10⁵ PFU and 5 × 10⁶ PFU Ad-HGF injected groups was lower than that in vehicle injected group (*P <0.05). The results indicated the high dose of Ad-HGF was more effective for fibrosis reduction than low dose.

D. Measurement of TGF-β1 Expression

TGF-β is the most intensively studied regulator of the ECM and has an important role in the development of liver fibrosis (Clouthier et al., 1997). There are three isoforms of TGF-β in mammals, TGF-β1, TGF-β2, and TGF-β3, which all have similar biological activity; however, liver fibrosis is attributed primarily to the TGF-β1 isofrom (Gorelik and Flavell, 2002). As TGF-β1 is critical to elicit liver fibrosis, we asked whether HGF would alter the expression of TGF-β1 in the liver under fibrosis conditions. We examined the expression of protein TGF-β1 in the liver by immunohistochemical staining. As shown in Figure 4 C, CCl₄ induced a dramatic increase in hepatic expression of TGF-β1, while the level of TGF-β1 was extremely low in normal liver. Treatment with Ad-HGF largely suppressed its induction by CCl₄.

IV. Discussion

Liver fibrosis is a process of liver cirrhosis, which is associated with most chronic liver diseases and thought to be reversible; nevertheless, there is no reliable treatment in current clinical practice to reverse this pathological process. There is growing evidence that several growth factors participate in this process. Hepatic injury can induce a transient high concentration of HGF in the liver, which is the stress reaction to trauma and has a crucial role in liver regeneration. There are two variants of HGF, an active single-chain protein (scHGF), which is cleaved proteolytically at the Arg-Val-Val site to form an active two-chain HGF (tcHGF). The two variants have the ability to bind the HGF receptor c-Met, but only tcHGF can activate the c-Met tyrosine kinase domain (Shima et al., 1994). It has been hypothesized that scHGF activation is blocked in cirrhotic liver, which induced the retarded regeneration (Xue et al., 2003). Chang-Goo Huh reported that loss of c-Met appeared not to be detrimental to hepatocyte function under physiological conditions, but enhanced Fas-induced apoptosis (Huh et al., 2004). c-Met tyrosine kinase can upregulate many downstream signal pathways, such as extracellular signal-regulated kinase/mitogen-activated protein kinase (ERK1/2-MAPK), phosphatidylinositol 3-kinase (PI,K), sphingosine kinase-sphingosine 1 phosphate-endothelial differentiation gene (SPK-S1P-DEG), which mediate the multifunction of HGF (Duan et al., 2004). Two different mechanisms have been proposed to explain the antifibrotic effects of HGF. One is proposed to promote the activity of hepatic collagenase and accelerate the degradation of the ECM. The other is proposed to be a reduction of protein levels of procollagens and TGF-β1 (Xue et al., 2003). TGF-β1 is a crucial factor in the liver cirrhosis process and a potent growth inhibitor of hepatocytes, which could serve as a chemotaxtant for fibroblasts and monocytes/macrophages, and induce these cells to secrete pro-inflammatory and fibrogenic cytokines; for example, TNF-α, IL-1β, and TGF-β1 itself. Ueki and colleagues reported that HGF could antagonize TGF-β1 directly, thus improving liver regeneration (Chen et al., 2005).

![Figure 5. Total tissue hydroxyproline content.](image)
The hydroxyproline content was determined by biochemical assay. Data were expressed as micrograms of hydroxyproline per milligram of tissue and presented as mean ± SD (n=6).

*P<0.05 versus with CCl₄ + 5×10⁶ PFU vehicle group.
In addition, injuries associated with hypoxia and neovascularization are related to the progression of liver fibrosis (Chaparro et al., 2007). Neovascularization includes angiogenesis and vasculogenesis, representing formation of blood vessels by distinction of endothelial progenitor cells. HGF has been recognized as one of the most efficient angiogenetic factors and endothelial chemoattractants, which also mobilizes endothelial progenitor cells and bone marrow stem cells to participate in injury repair and in vasculogenesis (Purdie et al., 2002; Van Belle et al., 1998). Therefore, HGF should be one of the most efficient growth factors, with the ability to promote liver regeneration and rivalry fibrosis. Unfortunately, the half-life of the HGF protein is very short in the blood circulation. Accordingly, gene therapy is needed urgently to apply in liver disease. Adenovirus, which is in general use in gene therapy, is utilized to treat many diseases. In earlier work, Ad-HGF was proven to cure myocardial ischemia efficiently, which has been applied in clinical research in China PR.

Yao et al. showed the level of gene expression in liver induced by intravenous injection of adenovirus is significantly higher than that in other organs (Yao et al., 2007), which indicates the strong probability of systemic administration of adenovirus for liver disease gene therapy. In this study, we proved systemic administration of 1 × 10⁷ PFU of Ad-HGF could upregulate the plasma HGF level significantly and maintain it for at least 10 days. That means Ad-HGF therapy effect may be through both hepatocytes’ autocrine HGF and paracrine HGF from circulation. However this dose of Ad-HGF cannot attenuate the extent of CCl₄-induced liver fibrosis. The 5 × 10⁷ PFU of Ad-HGF injected intravenously recovered the serum levels of GOT, GPT, TP, ALB, and TBil significantly, which indicated the chronic injury induced by CCl₄ was repaired. The Hyp content may represent hepatic collagens, which are major extracellular matrix proteins in hepatic fibrosis. In this study, the Hyp content was decreased, which coincided with the reduction of collagen deposition observed by staining with H&E and with Sirius red. Immunohistochemical staining showed the expression of TGF-β1 was attenuated in the gene therapy group. In histological sections, we observed the pseudolobule formation in the control group, which was reduced significantly in the Ad-HGF-injected group. There was no significant change of liver function or collagen deposition (data not shown), indicating that Ad-HGF therapy may be ineffective against severe liver cirrhosis. The starting effective dose in rat models is 5 × 10⁷ PFU, so the counterpart in humans should exceed 1 × 10¹³ PFU. This high dose may carry clinical risk to some extent. It is therefore important to consider means to reduce the dose of adenovirus administered as well as ways to alter the tropism of adenovirus; for instance, utilizing albumin as a promoter and enhancer to control the specificity of gene expression.

In conclusion, adenovirus gene therapy is a convenient and efficient way to express high levels of exogenous HGF in vivo. Here, we evaluated intravenous injection of Ad-HGF as a method to transfect the HGF gene and maintain the serum level of HGF that would be suitable for treating chronic liver injury. It will be important to examine the feasibility of systemic therapy with 5 × 10⁵ PFU of Ad-HGF, which could decrease the amount of collagen deposited and accelerate normalization of liver function. Thus, Ad-HGF systemic therapy is a potential therapeutic method for liver fibrosis in a clinical setting.

Acknowledgements
This project was supported by Chinese National High-Tech R&D Program, No. 2003AA216081.

References


