Recombinant human histidine triad nucleotide-binding protein 1 attenuates liver fibrosis induced by carbon tetrachloride in rats

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Abstract. It is currently thought that the transforming growth factor-β (TGF-β)/Smad signaling pathway acts as a central pathway leading to liver fibrosis, and that the aberrant Wnt/β-catenin signaling pathway also plays a vital role in the development of liver fibrosis. There is evidence that the histidine triad nucleotide-binding protein 1 (Hint1) was capable of inhibiting these two pathways. However, little data regarding the effects of Hint1 on liver fibrosis exists. Thus, we sought to investigate whether the recombinant human Hint1 protein (rhHint1) was capable of attenuating liver fibrosis induced by carbon tetrachloride (CCl_4) in rats and the possible underlying mechanism(s) of action. In the present study, purified rhHint1 was obtained using genetic engineering technology. Liver fibrosis was induced in male Sprague-Dawley (SD) rats by the subcutaneous injection of CCl_4. The rats were randomly divided into the normal control, the liver fibrosis model and the rhHint1 (doses, 50 and 100 μg/kg)-treated groups. Following four weeks of treatment, the rhHint1-treated rats exhibited significantly reduced liver fibrosis upon histopathological analysis and lower levels of hydroxyproline. Furthermore, rhHint1 inhibited the expression of α-smooth muscle actin (α-SMA) in the liver tissues. Additionally, rhHint1 lowered the gene expression levels of TGF-β1/Smad3 and β-catenin/cyclin D1, whereas it increased the gene expression levels of Smad7. In conclusion, the results of this study indicated that rhHint1 is capable of attenuating CCl_4-induced liver fibrosis by simultaneously targeting multiple pathogenic pathways, which may be developed as a new treatment for liver fibrosis.

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Introduction

Liver fibrosis is considered to be a model of the wound-healing response to chronic liver injury (1). Multiple etiologies of liver injury may lead to fibrosis, such as viral infection, alcohol abuse, drug toxicity and autoimmune hepatopathy (2). Liver fibrosis is characterized by the over accumulation of extracellular matrix proteins (ECM), particularly collagen types I and III (3). The activation of hepatic stellate cells (HSCs) is crucial in the pathogenesis of liver fibrogenesis. Transforming growth factor-β (TGF-β) is widely acknowledged as a crucial factor in the acceleration of the process of liver fibrosis, and is mostly activated by HSCs through the TGF-β/Smad signaling pathway, thereby causing liver fibrosis (4-6). Advanced liver fibrosis results in cirrhosis, portal hypertension and liver failure, for which no effective medical treatments are currently available (7). However, liver fibrosis may be reversed in the early stages by the intervention of external factors (8). Thus, there is a great need for developing novel therapies to treat liver fibrosis.

In recent years, the majority of therapeutic strategies for liver fibrosis have been focused on the TGF-β1/Smad signaling pathway; however, the inhibition of TGF-β1 expression signaling by various approaches, such as by using monoclonal antibodies to target TGF-β1, soluble TGF-β receptors or an ALK5 (the TGF-β type I receptor) inhibitor only partially relieve liver fibrosis (9,10). These results indicated that blocking TGF-β1 alone may miss other potentially major therapeutic targets for the treatment of liver fibrosis.

Wnt/β-catenin is an evolutionarily conserved cellular signaling system essential for various biological processes, such as embryonic development, homeostasis self-renewal and the pathogenesis of a variety of human diseases (11). Studies have demonstrated that the aberrant Wnt/β-catenin signaling pathway plays a vital role in the development of liver fibrosis (12-14). Our previous studies have suggested that β-catenin may aggravate hepatic fibrosis induced by TGF-β1 *in vitro* (15). Furthermore, there is cross-talk between Wnt/β-catenin signaling and TGF-β signaling in the fibrosis process (16,17).

The histidine triad nucleotide-binding protein 1 (Hint1), a member of the evolutionary highly conserved HIT protein superfamily, is a haplo-insufficient tumor suppressor (18,19). A variety of studies have demonstrated that the Hint1 protein

has an inhibitory role in several pathways, such as the TGF-β/Smad and Wnt/β-catenin pathways (20-23). However, little data regarding the effects of Hint1 on liver fibrosis exists. Herein, we hypothesize that Hint1 is a promising therapeutic target for liver fibrosis. In this study, we used genetic engineering technology to acquire the recombinant human Hint1 protein (rhHint1), and subsequently investigated the effects of rhHint1 on carbon tetrachloride (CCl_4)-induced liver fibrosis and the possible underlying mechanism.

Materials and methods

Purification of the rhHint1 protein. Human Hint1 cDNA was obtained from total RNA of LoVo cells (China Center for Type Culture Collection, Wuhan, Hubei, China) by reverse transcription-polymerase chain reaction (RT-PCR). The recombinant PET28a-Hint1 expression vector was constructed and transformed into *E. coli* (DE3). The activated engineering strain PET28a-Hint1/(DE3) was subsequently inoculated into a lysogeny broth (LB) medium (with kanamycin) at 37˚C, whilst being agitated at 200 rpm. After 12 h, isopropyl β-D-1-thiogalactopyranoside (IPTG; Yuanye Biological Technology, Shanghai, China) was added to induct for 16 h with a final IPTG concentration of 0.1 mmol/l. Purification was accomplished using immobilized metal (Ni^{2+}) affinity column chromatography (GE Healthcare, Madison, WI, USA). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Coomassie brilliant blue staining confirmed the purity of the rhHint1.

Animals and treatment. In total, 55 adult male Sprague-Dawley (SD) rats (200±20 g) were obtained from the Experimental Animal Center of Tongji Medical College of Huazhong University of Science and Technology (Wuhan, Hubei, China; certificate no. 4209800073). The rats were provided with standard feed and water *ad libitum* and individually housed at a constant temperature (18-20˚C) and humidity (60-70%) with a 12 h light/dark cycle. All experimental procedures were approved by the Institutional Animal Ethics Committee of Tongji Medical College, Huazhong University of Science and Technology and also performed according to the Laboratory Animal Care and Usage Manual of the university.

We used the liver fibrosis modeling method according to Yao *et al* (24). Following a week of acclimatizing to the standard conditions, the rats (n=45, in addition, 10 rats were selected as the normal control group) were subcutaneously administered 50% CCl_4 (CCl_4: olive oil, 1:1) at 0.3 ml/100 g of body weight, twice a week for eight weeks. After four weeks, the model rats were randomly divided into three experimental groups: i) fibrotic model group (given CCl_4 only); ii) CCl_4 + low dose rhHint1 (50 μg/kg); iii) CCl_4 + high dose rhHint1 (100 μg/kg). The dose of rhHint1 was selected based on our previous studies (25). All rats in the rhHint1-treated groups were administered their respective doses by an i.p. injection simultaneously with the CCl_4 injection for four continuous weeks. The rats of the normal control and model groups were i.p. injected with the same dose of sterilized saline. The rats were anesthetized using 10% chloral hydrate (0.4 ml/100 g) 24 h after the final treatment, and were subsequently sacrificed and a section of the right hepatic lobe was removed. A section of the liver was fixed in 10% buffered formalin and the remaining tissues were stored at -80˚C until required.

Histopathological analysis. The liver tissues were fixed and embedded in paraffin, 5-μm sections were stained with hematoxylin & eosin (HE) for routine histology and Masson's trichrome stained to detect collagen. Serial sections were examined under a microscope (Olympus, Tokyo, Japan) and photographed. Quantitative analysis of the fibrous area was calculated using Image-Pro plus 6.0 imaging software using five microscopic fields for each specimen.

Immunohistochemistry. For immunohistochemical analysis, 5-μm sections were deparaffinized in xylene and rehydrated in alcohol. Antigen retrieval was achieved by a 500-W microwave, and the sections were heated in citric saline for 15 min. After blocking endogenous peroxidases with 3% hydrogen peroxide for 10 min, sections were treated with 5% BSA in order to block the non-specific binding of antibodies and subsequently incubated with the anti-α-SMA antibody, 1:100 dilution (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) overnight at 4˚C. After washing in PBS, sections were incubated with the appropriate peroxidase-conjugated secondary antibody (goat anti-mouse IgG; Boster Biological Technology, Wuhan, China) for 50 min at 4˚C. Positive staining was visualized using a DAB enhancer (Guge Biotechnology, Wuhan, Hubei, China) and washed with water prior to counterstaining with hematoxylin. Quantitative analysis of the immunopositive cell area was performed with the Image-Pro plus 6.0 imaging software with five microscopic fields for each specimen.

Detection of hydroxyproline in liver tissue. Liver tissue (80 mg) samples were subjected to base hydrolysis to determine the levels of hydroxyproline. It was measured using the Hydroxyproline Alkaline Hydrolysis test kit according to the manufacturer's instructions (Jiancheng Bioengineering Institute, Nanjing, Jiangsu, China). The hydroxyproline levels were expressed as μg/g of wet liver tissue.

Quantitative polymerase chain reaction (qPCR) analysis. Total RNA of the liver tissues were extracted using TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. mRNA was converted using a cDNA First Chain Synthesis kit (Fermentas, Foster City, CA, USA). Quantitative assessment of cDNA was performed with a SYBR Green Master Mix kit (Fermentas) using an ABI prism 7900 Sequence detector (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. β-actin was used as an internal control. The following primers were used: TGF-β1: 5'-GTCAACTGTGGAGCAACACG-3' (forward), 5'-ACTGAAGCGAAAGCCCTGTA-3' (reverse); Smad3: 5'-TGATCCCTCCAATTCAGAGC-3' (forward), 5'-GTTGGGAGACTGGACGAAAA-3' (reverse); Smad7: 5'-TGTGTCCAAGAGCCCTCCCT-3' (forward), 5'-CACGCCATCCACTTCCCTT-3' (reverse); β-catenin: 5'-CGACTAAGCAGGAAGGGATG-3' (forward), 5'-ATGGCAGGCTCGGTAATG-3' (reverse); cyclin D1: 5'-TGCTGGCGAAGGTTTAGG-3' (forward), 5'-GAGCGGCGGCAAGAATGT-3' (reverse); β-actin: 5'-CACGATGGAGGGGCCGGACTCATC-3' (forward),

5'-TAAAGACCTCTATGCCAACACAGT-3' (reverse). The relative gene expression levels were expressed as fold changes following normalization with β-actin and calculated using the comparative Ct method formula, $2^{-\Delta\Delta Ct}$.

Statistical analysis. Experiments were repeated a minimum of three times, and the values were expressed as the means ± SD. Statistical analysis was performed using one-way analysis of variance (ANOVA) for multiple group comparisons or the Student's t-test for two group comparisons. The data were analyzed using SPSS 17.0 software. P<0.05 was considered to indicate a statistically significant difference.

Results

Identification of rhHint1. SDS-PAGE and Coomassie brilliant blue staining were used to confirm and identify the purity of rhHint1. The protein was >95% pure as judged by 12% SDS-PAGE with an expected molecular mass of 14 kDa (Fig. 1).

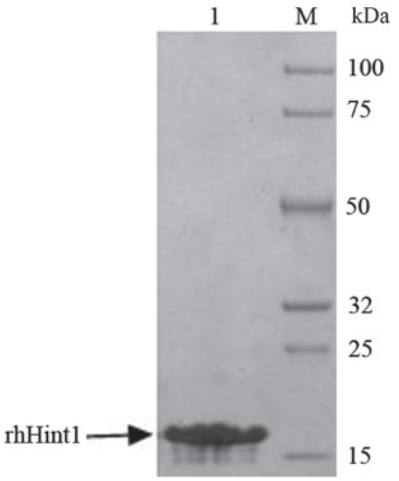

Figure 1. Purity detection of rhHint1 by Coomassie brilliant blue staining. Line M, Marker; L1, rhHint1. rhHint1, recombinant human histidine triad nucleotide-binding protein 1.

Effect of rhHint1 on the histological changes in the liver. In this study, routine histological analysis and collagen fiber examination of the livers in SD rats used HE staining and Masson's trichrome staining, respectively. Representative images of the liver morphology are shown in Fig. 2A and B. For the HE staining, when compared with the normal control group, there were prominent hepatic steatosis, necrosis, formation of regenerative nodules and fibrotic septa caused by CCl_4 treatment. However, in the rhHint1 treatment group, the rats presented with hepatic steatosis, fewer fibrotic septa and inflammatory infiltrate when compared with the model group (Fig. 2A). With Masson's trichrome staining, there was a limited quantity of visible collagen fiber (blue) in the normal control group, whereas, it was found that a greater quantity of collagen fiber was present with thickening of the partial compartments in the model group. Furthermore, it was found that a medium quantity of collagen fiber was present and extended to the peripheral region without distinct compartment formation in the groups treated with rhHint1 and the anti-fibrotic effect was more marked with an increase in the dose (Fig. 2B). The quantitative analysis of the fibrous area percentage showed that collagen deposition was significantly lower in the rhHint1-treated group compared with the model group (P<0.01; Fig. 2C).

Effect of rhHint1 on the level of hydroxyproline in the liver. As the hydroxyproline level in the liver tissue was parallel to the extent of fibrosis, next we detected the liver hydroxyproline content in each group. In this study, we observed that liver fibrosis induced by CCl_4 caused a significant rise in the levels of hydroxyproline (P<0.01), whereas treatment with rhHint1 significantly decreased the level of the hydroxyproline compared with the fibrotic model group (P<0.01), and administration with rhHint1 (100 μg/kg) caused a more significant decrease in the levels of hydroxyproline than rhHint1 (50 μg/kg; P<0.01; Fig. 2D).

Effect of rhHint1 on the activation of HSCs in the liver. To evaluate the effect of rhHint1 treatment on the activation of HSCs *in vivo*, we detected the expression of α-SMA in the liver by immunohistochemical staining and qPCR. As shown in Fig. 3A and B, only a few cells in the liver section from the normal control group were recognized by anti-α-SMA. The administration of CCl_4 produced a marked increase in the number of cells recognized by anti-α-SMA (P<0.01). rhHint1 treatment significantly reduced the number of cells labeled with anti-α-SMA (P<0.01), but there was no statistical significance between the rhHint1 (50 μg/kg) and rhHint1 (100 μg/kg) groups (P>0.05). Meanwhile, rhHint1 treatment decreased the α-SMA mRNA expression compared with the model group (P<0.01), and presented no statistical significance difference with an increase in the dosage (P>0.05; Fig. 3C).

Effect of rhHint1 on the expression of TGF-β1/Smad in the liver. To investigate the effect of rhHint1 treatment on the TGF-β1/Smad signaling pathway, we examined TGF-β1, Smad3 and Smad7 mRNA expression using qPCR. As shown in Fig. 4, compared with the normal control group, TGF-β1 and Smad3 mRNA expression increased markedly, whereas Smad7 mRNA levels decreased significantly (P<0.01). Compared with the model group, TGF-β1 and Smad3 mRNA levels were markedly reduced in the rhHint1-treated group, whereas Smad7 mRNA levels were significantly elevated (P<0.01). Furthermore, there was no statistically significant difference between rhHint1 (50 μg/kg) and rhHint1 (100 μg/kg) treatment in TGF-β1, Smad3 mRNA expression (P>0.05), but the high dose of rhHint1 treatment caused a significant increase in Smad7 mRNA expression compared with the low dose (P<0.01).

Effect of rhHint1 on the expression of β-catenin/cyclin D1 in the liver. To further investigate the effects of rhHint1 treatment on the Wnt/β-catenin signaling pathway, we examined β-catenin and cyclin D1 mRNA expression using qPCR (Fig. 5). The results demonstrated that β-catenin and cyclin D1 mRNA expression were elevated in the CCl_4-treated group compared with the normal control group (P<0.01), whereas β-catenin and cyclin D1 mRNA expression were significantly reduced in the rhHint1-treated group compared with the model group (P<0.05). Additionally, there was no statistical significance between the rhHint1 (50 μg/kg) and the rhHint1 (100 μg/kg) groups (P>0.05).

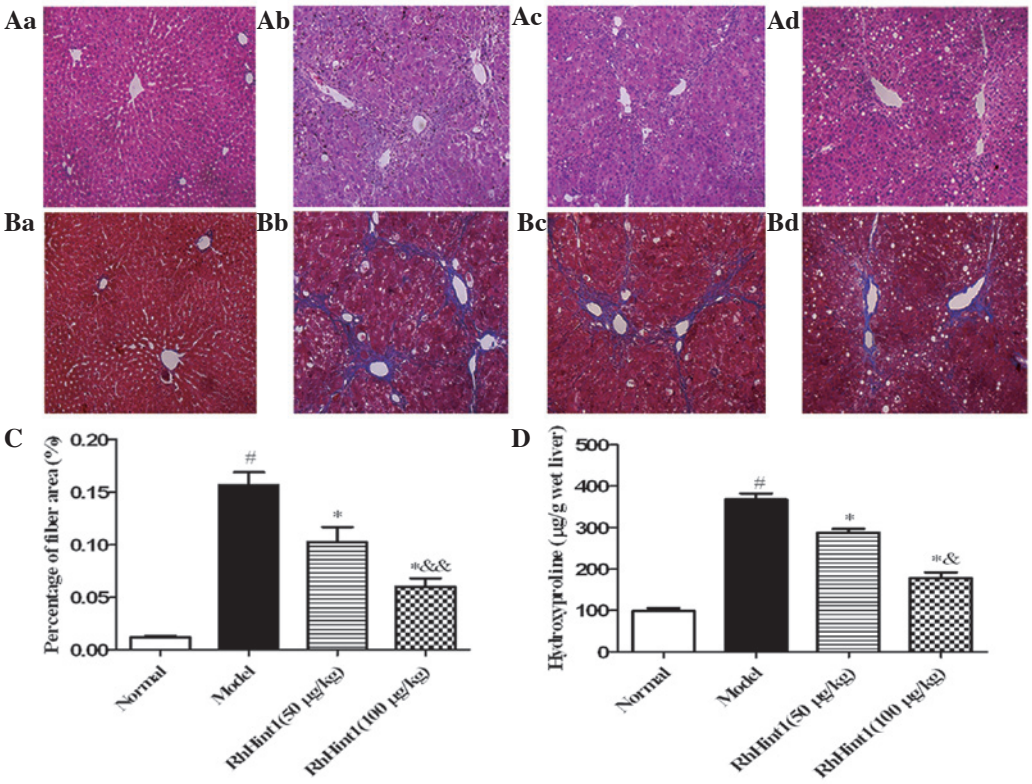

Figure 2. Effect of rhHint1 on the fibrogenesis of liver fibrosis. (A) HE staining of liver sections, (magnification, x200). (Aa) Normal control group. (Ab) Fibrosis model group. (Ac) rhHint1 (50 μg/kg) treatment group. (Ad) rhHint1 (100 μg/kg) treatment group. (B) Collagen deposition by Masson's trichrome staining of the liver sections, (magnification, x200). (Ba) Normal control group. (Bb) Fibrosis model group. (Bc) rhHint1 (50 μg/kg) treatment group. (Bd) rhHint1 (100 μg/kg) treatment group. (C) Quantitative analysis of the fibrous area percentage by Image-Pro plus 6.0. The fibrous area percentage in the rhHint1-treated group was significantly reduced compared with the model group (P<0.01). (D) Hydroxyproline levels in liver tissues. rhHint1 treatment significantly decreased the levels of hydroxyproline compared with the fibrotic model group (P<0.01). $^{\#}$P<0.01 vs. normal control group; *P<0.01 vs. model group; $^{\&}$P<0.01, $^{\&\&}$P<0.05 vs. rhHint1 (50 μg/kg) group. rhHint1, recombinant human histidine triad nucleotide-binding protein 1.

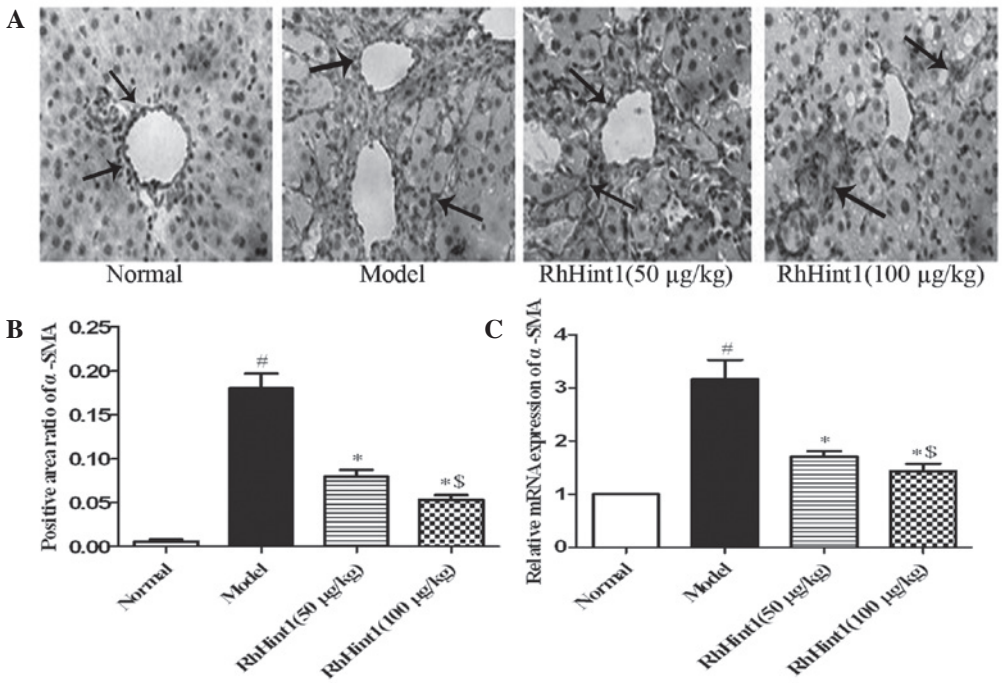

Figure 3. Effect of rhHint1 on the activation of HSCs in liver. (A) Immunohistochemical analysis of α-SMA in liver sections (magnification, x400). Black arrows indicate positive stained cells with anti-α-SMA. (B) Quantitative analysis of the positive area ratio of α-SMA by Image-Pro plus 6.0. rhHint1 treatment significantly reduced the number of cells labeled with anti-α-SMA (P<0.01). (C) qPCR was used to investigate the mRNA expression of α-SMA. rhHint1 treatment markedly decreased the α-SMA mRNA expression compared with the model group (P<0.01). $^{\#}$P<0.01 vs. normal control group, *P<0.01 vs. model group, $^{\$}$P>0.05 vs. rhHint1 (50 μg/kg) group. HSCs, hepatic stellate cells; α-SMA, α-smooth muscle actin; rhHint1, recombinant human histidine triad nucleotide-binding protein 1; qPCR, quantitative polymerase chain reaction.

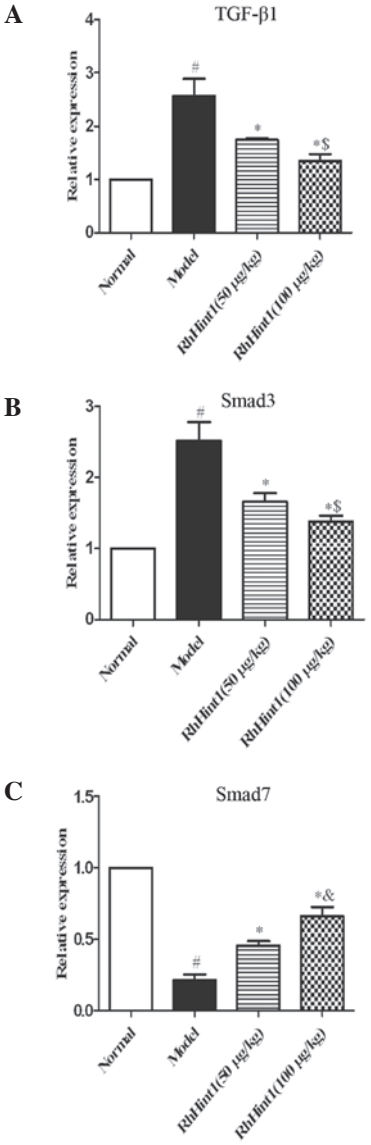

Figure 4. Effect of rhHint1 on the expression of TGF-β1/Smad in liver. qPCR was used to investigate the mRNA expression of TGF-β1. (A) Smad3; (B) Smad7 and (C) in liver tissues. TGF-β1 and Smad3 mRNA levels were markedly reduced in the rhHint1-treated group compared with the model group, whereas the Smad7 mRNA level was significantly elevated (P<0.01). #P<0.01 vs. normal control group; *P<0.01 vs. model group; &P<0.01, $P>0.05 vs. rhHint1 (50 μg/kg) group. TGF-β1, transforming growth factor-β1; rhHint1, recombinant human histidine triad nucleotide-binding protein 1; qPCR, quantitative polymerase chain reaction.

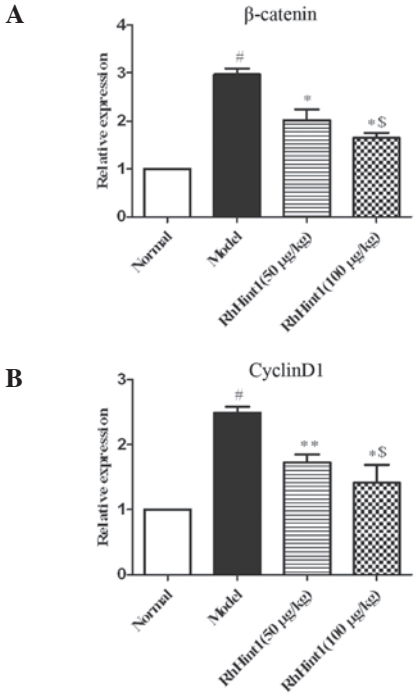

Figure 5. Effect of rhHint1 on the expression of β-catenin/cyclin D1 in the liver. qPCR was used to investigate the mRNA expression of (A) β-catenin and (B) cyclin D1 in liver tissues. β-catenin and cyclin D1 mRNA expression was significantly reduced in the rhHint1-treated group compared with the model group (P<0.05). #P<0.01 vs. normal control group; *P<0.01, **P<0.05 vs. model group; $P>0.05 vs. rhHint1 (50 μg/kg) group. rhHint1, recombinant human histidine triad nucleotide-binding protein 1; qPCR, quantitative polymerase chain reaction.

Discussion

Liver fibrosis is the progressive accumulation of ECM proteins that occurs in the majority of chronic liver diseases. Advanced liver fibrosis is associated with high morbidity and mortality (26). However, there are currently no effective anti-fibrotic therapies available for clinical use. Thus, it is essential to develop new therapeutic strategies to counteract liver fibrosis.

The increased deposition of collegen is a characteristic feature of liver fibrosis. As shown in Fig. 2, the fibrosis model group exhibited a pattern of fibrosis with the formation of regenerative nodules and fibrotic septa upon histopathological analysis. Thus, CCl_4-induced liver fibrosis in SD rats is a useful animal model for studying the anti-fibrotic effect of rhHint1. In this study, HE and Masson's trichrome staining indicated that fibrogenesis could be reduced following treatment with rhHint1. In addition, the hydroxyproline content of the livers of the rhHint1-treated group was significantly lower compared with that of the fibrotic model group. These results suggested that rhHint1 exhibited a suppressive effect on the progression of liver fibrosis induced by CCl_4 in rats.

Several studies have theorized that HSCs play a central role in the pathogenesis of liver fibrosis, and α-SMA expression is a reliable marker of the activation of HSCs *in vivo*. Furthermore, the expression of α-SMA is commonly used to quantitate the number of activated HSCs (27,28). In this study, we found that α-SMA expression was markedly reduced in the rhHint1 treatment group compared with the model group. This suggested that rhHint1 may inhibit the activation of HSCs.

The TGF-β/Smad signaling pathway is central to the development of liver fibrosis. Smad7, an inhibitory Smad, acts in a negative feedback loop to inhibit TGF-β1 activity by preventing the phosphorylation of Smad2/3 (29). The results of this study indicate that rhHint1 may decrease TGF-β1 and Smad3 gene expression by increasing Smad7 gene expression in CCl_4-induced rat liver fibrosis, therefore activating the negative feedback effects of inhibiting liver fibrosis.

A number of studies have suggested that the reactivation of the Wnt/β-catenin pathway is linked to liver fibrosis (12-14). Furthermore, Wnt/β-catenin signaling functions in a combinational manner with TGF-β signaling in liver fibrosis. Cheon *et al* (30) found that the wound phenotype imparted by a Smad3 deficiency and by the injection of TGF-β prior

to inflicting the wound is mediated in part by β-catenin in cutaneous healing, and TGF-β was unable to regulate the proliferation in β-catenin null fibroblasts. Medici *et al* (31) determined that there is a unified signaling mechanism driven by the convergence of multiple TGF-β and β-catenin-TCF signaling molecules in promoting EMT. Furthermore, Cheng *et al* (12) investigated the role of Wnt/β-catenin in HSCs and verified that nuclear β-catenin was markedly increased in culture-activated HSCs compared with quiescent HSCs. Cyclin D1 is a major regulator of the progression of cells into the proliferative stage of the cell cycle, and is also a direct downstream target gene of the Wnt/β-catenin pathway (32). In this study, we examined the effect of rhHint1 on the Wnt/β-catenin pathway and observed a significant downregulation of β-catenin and cyclin D1, suggesting that the anti-fibrotic effect of rhHint1 is mediated through the suppression of the Wnt/β-catenin pathway.

Hint1, as a novel tumor suppressor, is a member of the Hint branch of the evolutionary conserved histidine triad (HIT) protein family, which is characterized by a common His-X-His-X-His-XX motif (X, hydrophobic amino acid). The identification of the Hint1 interaction partners suggested that it might be involved in the regulation of transcription processes (33). Weiske *et al* (21) reported that Hint1 was a negative regulator of the TCF-β-catenin transcriptional activity, and repressed the expression of its downstream fibrosis-related target gene cyclin D1. Wang *et al* (23) found that the increased expression of HINT1 in HepG2 cells markedly inhibited the transcriptional activities of β-catenin, and inhibited the expression of endogenous cyclin D1 and TGF-β. To date, no treatment has been capable of completely inhibiting the progress of liver fibrosis. Herein, we present a new treatment that is a promising therapeutic strategy that may inhibit TGF-β and β-catenin.

In conclusion, the results in this study showed that rhHint1 treatment may markedly attenuate CCl_4-induced fibrosis. The primary mechanisms of this anti-fibrotic effect were mediated by suppressing collagen deposition, inhibiting the activation of HSCs and regulating the TGF-β1/Smad and Wnt/β-catenin pathways simultaneously. Furthermore, it may provide us a with new therapeutic strategy for liver fibrosis.

Acknowledgements

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