

# HBV induces liver fibrosis via the TGF- $\beta$ 1/miR-21-5p pathway

WENTING LI<sup>1,2\*</sup>, XIAOLAN YU<sup>2,3\*</sup>, XILIU CHEN<sup>4\*</sup>, ZHENG WANG<sup>5</sup>,  
MING YIN<sup>2,6</sup>, ZONGHAO ZHAO<sup>1,2</sup> and CHUANWU ZHU<sup>7</sup>

<sup>1</sup>3rd Liver Unit, Department of Infectious Disease, Anhui Provincial Hospital, Hefei, Anhui 230001;

<sup>2</sup>The First Affiliated Hospital of USTC, Division of Life Sciences and Medicine, University of Science and Technology of China, Hefei, Anhui 230000; <sup>3</sup>Department of Ear-Nose-Throat, Anhui Provincial Hospital, Hefei, Anhui 230001;

<sup>4</sup>Department of Infectious Diseases, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, Hubei 430022; <sup>5</sup>Department of Respiratory and Critical Medicine,

People's Hospital of Zhengzhou University, Zhengzhou, Henan 450003; <sup>6</sup>Intensive Care Unit, Department of Infectious Disease, Anhui Provincial Hospital, Hefei, Anhui 230001; <sup>7</sup>Department of Hepatology,

The Affiliated Infectious Diseases Hospital of Soochow University, Suzhou, Jiangsu 215131, P.R. China

Received March 14, 2020; Accepted December 8, 2020

DOI: 10.3892/etm.2020.9600

**Abstract.** MicroRNA (miR)-21-5p is a newly discovered factor that mediates TGF- $\beta$ 1 signaling. The present study was designed to investigate the role of TGF- $\beta$ 1/miR-21-5p in hepatitis B virus (HBV)-induced liver fibrosis. HBV-infected sodium taurocholate co-transporting polypeptide (NTCP)-transfected Huh7.5.1 cells were co-cultured with LX2 cells to simulate HBV infection in the present study. A total of 29 patients with chronic HBV infection were enrolled. Cells were transfected with miR-21-5p mimic or inhibitor with or without TGF- $\beta$ 1 stimulation. The demographic, biochemical and virological data from the 29 patients were analyzed and liver tissues were collected. miR-21-5p levels and the mRNA and protein expression of  $\alpha$ -smooth muscle actin (SMA), collagen type 1  $\alpha$  1 (CoL1A1), tissue inhibitor of metalloproteinase (TIMP)-1 and Smad from liver cells or tissues were detected by quantitative PCR analysis and western blotting, respectively. Cell viability was observed, and the liver fibrosis score was evaluated. The association between miR-21-5p and liver fibrosis was evaluated by correlation analysis. HBV infection upregulated TGF- $\beta$ 1/miR-21-5p mRNA expression in NTCP-Huh7.5.1 cells compared with mock infection ( $P < 0.05$ ). TGF- $\beta$ 1 incubation significantly increased miR-21-5p levels, as well as the mRNA and protein expression of  $\alpha$ -SMA, CoL1A1 and TIMP-1, and reduced Smad7 expression in LX2

cells compared with the normal group, and these effects were counteracted by miR-21-5p inhibitor ( $P < 0.05$ ). miR-21-5p overexpression also contributed to TGF- $\beta$ 1-induced  $\alpha$ -SMA, CoL1A1 and TIMP-1 expression in LX2 cells ( $P < 0.05$ ). Co-culture with HBV-infected NTCP-Huh7.5.1 cells upregulated TGF- $\beta$ 1/miR-21-5p activity and CoL1A1 expression in LX2 cells compared with normal control, which were significantly reduced by miR-21-5p inhibitor ( $P < 0.05$ ). miR-21-5p levels were significantly correlated with the liver fibrosis score ( $r = 0.888$ ;  $P < 0.05$ ). These data demonstrated that HBV induced liver fibrosis via the TGF- $\beta$ 1/miR-21-5p pathway and suggested that miR-21-5p may be an effective anti-fibrosis target.

## Introduction

Liver fibrosis has long been considered as a healing response to various chronic liver injuries, including viral hepatitis, immune compounds and toxic agents, among others (1). Hepatitis B virus (HBV) remains the most important cause of liver fibrosis in China, which is an endemic area (2). During the life cycle, HBV generates covalently closed circular DNA (cccDNA), which acts as the model of HBV replication (3). Additionally, cccDNA integrates into the genome of hepatocyte, which makes it hard to eliminate HBV from infected cells (3). A recent study from China also showed that Persistent Low Level of Hepatitis B Virus (LLV) widely existed in HBV infected patients despite antiviral therapy (4). Moreover, despite the widespread use of strong antiviral treatments, patients with HBV infection still inevitably progress to liver fibrosis, or even liver cirrhosis (5). However, the mechanism underlying HBV-induced liver fibrosis remains poorly understood.

Over the past decades, accumulative studies have focused on the cascades of the TGF- $\beta$ 1 pathway, which takes part in multiple biological progresses, including cell activation (6), tissue differentiation (7), fibrosis (8) and cancer progression (9). As expected, TGF- $\beta$ 1 has a strong association with HBV replication and HBV related liver diseases (10,11). TGF- $\beta$ 1 has been demonstrated to serve important roles in

*Correspondence to:* Professor Chuanwu Zhu, Department of Hepatology, The Affiliated Infectious Diseases Hospital of Soochow University, 10 Guangqian Road, Suzhou, Jiangsu 215131, P.R. China  
E-mail: Wt19911002mgh@163.com

\*Contributed equally

**Key words:** hepatitis B virus, liver fibrosis, microRNA-21-5p, TGF- $\beta$ 1

hepatic stellate cell (HSC) activation, which is a key event in liver fibrosis (11,12). Accumulating evidence suggested that HBV infection may promote TGF- $\beta$ 1 production by hepatocytes, which in turn activates HSCs and accelerates liver fibrosis (13-15). HSCs is the predominant cell type responsible for liver fibrosis. In physiological conditions, quiescent-like HSCs exhibit necessary effects on lipid metabolism, maintaining the balance of extracellular matrix (ECM) production and degradation (16). When liver injury occurs, HSCs are activated by cytokines including TGF- $\beta$ 1 secreted by a variety of cells in the liver (17). These activated HSCs produce excessive ECM deposited in liver tissue disrupting liver structure leading to liver fibrogenesis (17).

MicroRNAs (miRNAs) are a group of small and non-coding RNAs, which regulates gene expression by binding to specific mRNA targets. miRNAs can promote degradation and/or translational inhibition of target gene by post-transcription modification (18). Interestingly, a big cluster of miRNAs has been demonstrated to accelerate HSC activation, fibrogenesis and cancer by augmenting fibrosis-associated signaling pathways including transforming growth factor- $\beta$ /Smad (19). It was reported that microRNA (miR/miRNA)-21-5p acts as a key mediator in the TGF- $\beta$ 1 signaling pathway through assisting in the inhibition of Smad7 expression (20,21). However, the role of the TGF- $\beta$ 1/miR-21-5p pathway in HSC activation and liver fibrosis remains to be elucidated. The present study was designed to evaluate the role of the TGF- $\beta$ 1/miR-21-5p pathway in liver fibrosis induced by HBV infection.

## Materials and methods

**Patients.** Liver tissue samples were collected from 29 patients with HBV infection. These patients were antiviral treatment-naïve and were admitted to the Department of Infectious Disease of the Anhui Provincial Hospital (Hefei, China) between Jan 2018 and December 2019. All patients were diagnosed with chronic hepatitis B according to the guidelines of the Chinese Medical Association (22). Inclusion criteria is the positive of S antigen for at least 6 months. Exclusion criteria includes co-infection with other viruses such as HCV/HIV/EBV/CMV; suffering from alcohol, drug or autoimmune related hepatitis; cancer and other disease receiving drugs which may affect host immune system. A total of 5 patients without HBV infection were enrolled as controls. The patients in the present study underwent liver biopsy. All data from medical records of these patients were collected at Anhui Provincial Hospital. All patients provided written informed consent for the present study, and the study protocol was approved by the Ethics Committee of Anhui Provincial Hospital.

**Biochemical examinations and HBV DNA quantification.** Serum alanine transaminase (ALT), aspartate aminotransferase (AST), total bilirubin (TBil) and albumin (ALB) levels were measured by routine laboratory tests at the Department of Laboratory Science of Anhui Provincial Hospital (Hefei, China). In addition, serology tests were performed on the ARCHITECT i2000SR immunoassay analyzer (Abbott Pharmaceutical Co., Ltd.) and serum HBV DNA levels were measured using the COBAS TaqMan HBV test (Roche Diagnostics).

**Histological assessment of liver fibrosis.** Liver tissues were collected from patients by liver biopsy using a Bard biopsy needle (18G, 910 cm; BD Biosciences) as previously described (23). Liver tissue samples were fixed for 24 h at room temperature in 10% formalin, embedded in paraffin and cut into 5- $\mu$ m sections.

Hematoxylin and eosin (HE) and Masson's staining were performed to assess the alterations in liver architecture and the presence of liver fibrosis. The liver fibrosis score was evaluated by two independent pathologists in a blinded manner according to the METAVIR system (24) as follows: S<sub>0</sub>, no collagen deposition; S<sub>1</sub>, minimal collagen deposition; S<sub>2</sub>, extended fibrosis; S<sub>3</sub>, bridging fibrosis and S<sub>4</sub>, cirrhosis. Smad7 expression in liver tissue was observed by immunohistochemistry (IHC) and calculated using the following formula: Positive index (PI)=mean optical density x positive area percentage.

**Cell culture and viral stocks.** The human HSC line LX2, and Huh7.5.1 and HepAD38 hepatocellular carcinoma cells, were maintained in our laboratory. Cells were grown in DMEM (HyClone; Cytiva) supplemented with 10% FBS (Gibco; Thermo Fisher Scientific, Inc.) with 100 U/ml penicillin and 100  $\mu$ g/ml streptomycin under a humidified atmosphere at 37°C with 5% CO<sub>2</sub> (v/v). HBV viral stock was collected from the supernatant of HepAD38 cells and stored at -20°C. HBV copies were quantified by quantitative PCR (qPCR) analysis. Entecavir (ETV) (Sigma-Aldrich; Merck KGaA) was diluted into DMEM at 20  $\mu$ M as the working concentration. Cells maintained in DMEM without HBV were used as the mock group (mock infection). Human TGF- $\beta$ 1 was purchased from R&D System (240-B-010/CF) and was diluted into DMEM at 10 ng/ml as the working concentration.

**Bioinformatics analysis.** TargetScan (<http://www.targetscan.org>), PicTar (<http://pictar.mdc-berlin.de/>), miRanda (<http://microrna.sanger.ac.uk>) and KEGG Pathway analysis ([www.genome.jp/kegg](http://www.genome.jp/kegg)) to explore the target genes for miR-21-5p.

**Plasmid preparation and transfection.** Sequences of miR-21-5p were collected from miRBase. miR-21-5p mimic, inhibitor (antagomir-21) and their corresponding negative control were all obtained from Guangzhou RiboBio Co., Ltd.

U6 small nucleolar RNA (Guangzhou RiboBio Co., Ltd.) was used as the endogenous control. Plasmids were amplified and purified using the HiSpeed Plasmid Midi Kit (cat. no. 12643; Qiagen, Inc.) and stored in ddH<sub>2</sub>O at 1  $\mu$ g/ $\mu$ l before use. All plasmids were transfected into cells using Lipofectamine<sup>®</sup> LTX with Plus<sup>™</sup> reagent (Invitrogen; Thermo Fisher Scientific, Inc.).

**Co-culture system.** A co-culture system containing sodium taurocholate co-transporting polypeptide (NTCP)-Huh7.5.1 and LX2 cells were used in the present study. First, pCMV-NTCP (RC210241; OriGene Technologies, Inc.) was transfected into Huh 7.5.1 cells to construct NTCP-Huh7.5.1 cells. Then, NTCP-Huh7.5.1 cells were infected with HBV viral stock (MOI=1.0) (25) and LX2 cells were transfected with miR-21-5p mimic inhibitor or the negative control, respectively. After 72 h, HBV-infected NTCP-Huh7.5.1 cells

Table I. List of primers used in reverse transcription-quantitative PCR analysis.

Target	Species	Forward primer (5'-3')	Reverse primer (5'-3')
$\alpha$ -SMA	Human	AAAAGACAGCTACGTGGGTGA	GCCATGTTCTATCGGGTACTTC
TIMP-1	Human	ACTTCCACAGGTCCCACAAC	GCTAAGCTCAGGCTGTTCCA
CoL1A1	Human	CAGCCGCTTCACCTACAGC	TCAATCACTGTCTTGCCCCA
miR-21-5p	Human	TAGCTTATCAGACTGATGTTGA	CTGAAGTCGCCATGCAGATA
Smad7	Human	GCTCCCATCCTGTGTGTTAA	TAGGTGTCAGCCTAGGATGGT
U6	Human	CTCGCTTCGGCAGCACA	AACGCTTACGAATTTGCGT
GAPDH	Human	ACCTTCCCCATGGTGTCTGA	GCTCCTCTGTTTCGACAGTCA

SMA, smooth muscle actin; CoL1A1, collagen type 1  $\alpha$  1; miR, microRNA.

were seeded in a 12-well plate at a density of  $2 \times 10^5$  cells/well and LX2 cells were plated on 0.4- $\mu$ M 12-well Transwell inserts (Costar; Corning, Inc.). After overnight maintenance at 37°C, LX2 Transwells were loaded into NTCP-Huh7.5.1 Transwells. After another 72 h of incubation, the cells were harvested for mRNA and protein analysis.

**Reverse transcription (RT)-qPCR analysis.** Total RNA was extracted from liver tissues and cells using TRIzol<sup>®</sup> reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. Subsequently, first-strand cDNA was reverse-transcribed from RNA using the PrimeScript RT reagent (Takara Bio, Inc.) according to the manufacturer's instructions. qPCR was performed with SYBR Green Assay kit (Thermo Fisher Scientific, Inc.) on an ABI 7500 PRISM system (Applied Biosystems; Thermo Fisher Scientific, Inc.). The following thermocycling conditions were used for the qPCR: Initial denaturation at 95°C for 3 min, followed by 40 cycles of 94°C for 20 sec, 60°C for 30 sec and 72°C for 20 sec. The expression of each mRNA was calculated against U6/GAPDH and quantified using the  $2^{-\Delta\Delta C_q}$  method (26). The primers used in the present study are listed in Table I.

**Western blot assay.** Total protein was collected from cultured cells using a Pierce BCA Protein Assay kit (Thermo Fisher Scientific, Inc.). Equal amounts (20  $\mu$ g) of protein were separated by SDS-PAGE 4-12% Bis-Tris gradient gels and transferred to PVDF membranes. Subsequently, membranes were blocked with 5% BSA (Sigma-Aldrich; Merck KGaA) dissolved in TBS-Tween 20 (TBS-T; 0.05% Tween-20) for 2 h at room temperature. The membranes were incubated with primary antibodies overnight at 4°C including mouse anti- $\alpha$ -smooth muscle actin ( $\alpha$ -SMA; cat. no. ab7817; Abcam; 1:1,000), rabbit anti-tissue inhibitor of metalloproteinase (TIMP)-1 (cat. no. ab211926; Abcam; 1:1,000), rabbit anti-collagen type 1  $\alpha$  1 (CoL1A1; cat. no. 84336; Cell Signaling Technology, Inc. 1:400), rabbit anti-Smad7 (cat. no. ab216428; Abcam; 1:1,000) and rabbit anti- $\beta$ -actin (cat. no. 4967; Cell Signaling Technology, Inc.; 1:3,000). The blots were then incubated with HRP-labeled goat-anti-rabbit IgG (cat. no. NA934V) and sheep-anti-mouse IgG (NA931) secondary antibodies (Amersham; Cytiva) diluted in 5% BSA at room temperature (25°C) for 2 h. The blots were washed with TBS-T for 40 min visualization using ECL

(cat. no. NCI4106; Beijing Solarbio Science & Technology Co., Ltd.) for 10 min. Protein expression was calculated with  $\beta$ -actin as a reference using Image J software (v1.8.0; National Institutes of Health).

**Statistical analysis.** All data in the present study are expressed as the mean  $\pm$  SD. All experiments were repeated three times. Data analyses were performed by t-test or one-way ANOVA with Tukey's post-hoc test with SPSS version 17.0 (SPSS, Inc.). The correlation between miR-21-5p and liver fibrosis stage was assessed by Spearman's correlation coefficient.  $P < 0.05$  was considered to indicate a statistically significant difference.

## Results

**HBV replication enhances TGF- $\beta$ 1 expression in hepatocellular carcinoma cells.** To observe the effects of HBV replication on TGF- $\beta$ 1 expression, NTCP-Huh7.5.1 cells were incubated with HBV stock from the supernatant of HepAD38 cells. Entecavir (ETV) was used to inhibit HBV replication in HBV-infected NTCP-Huh7.5.1 cells. The supernatant from HBV-infected NTCP-Huh7.5.1 cells was collected at different timepoints for HBV detection. At 72 h after incubation, cells were harvested to examine TGF- $\beta$ 1 mRNA expression by qPCR analysis.

As shown in Fig. 1A, NTCP-Huh7.5.1 cells can stably support HBV replication; HBV DNA levels in the supernatant of HBV-infected NTCP-Huh7.5.1 cells were  $4.6 \times 10^4$  copies/ml at 72 h after infection and remained at a stable level over a further 72 h, while ETV treatment significantly reduced HBV DNA levels in the supernatant of HBV-infected NTCP-Huh7.5.1 cells ( $P < 0.05$ ). Furthermore, HBV DNA levels of total cell DNA from HBV-infected NTCP-Huh7.5.1 cells were significantly higher compared with mock infection, which was significantly reduced by ETV treatment (Fig. 1B;  $P < 0.05$ ).

Moreover, HBV-infected NTCP-Huh7.5.1 cells showed significantly higher TGF- $\beta$ 1 mRNA and miR-21-5p levels compared with NTCP-Huh7.5.1 cells that underwent the mock infection, while ETV treatment significantly reduced TGF- $\beta$ 1 mRNA and miR-21-5p levels compared with HBV-infected NTCP-Huh7.5.1 cells (Fig. 1C and D;  $P < 0.05$ ). HBV infection and ETV treatment did not affect NTCP-Huh7.5.1 cell viability (Fig. 1E).

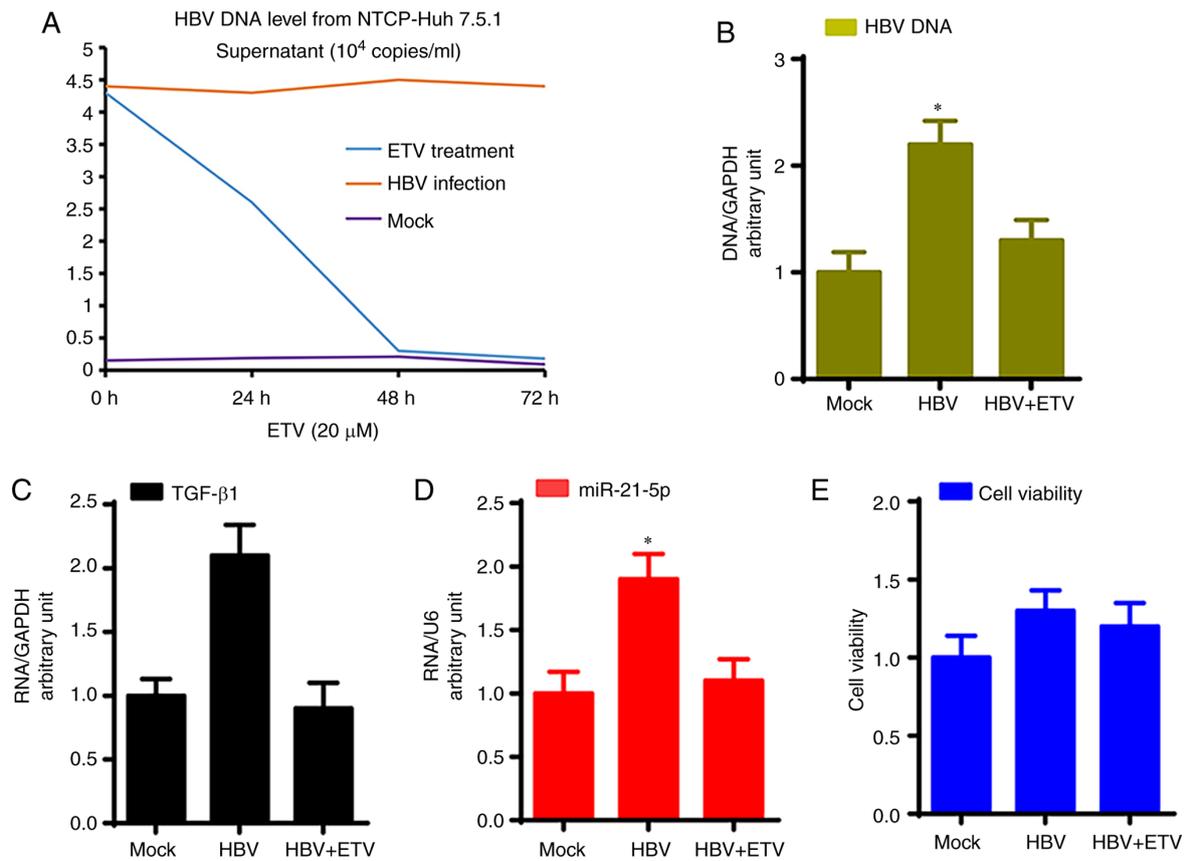


Figure 1. HBV replication enhances TGF- $\beta$ 1/miR-21-5p activity in NTCP-Huh7.5.1 cells. HBV DNA levels in (A) supernatant and (B) NTCP-Huh7.5.1 cells by quantitative PCR analysis. HBV DNA levels varied between 4-5 $\times$ 10<sup>4</sup> copies/ml, and ETV treatment significantly reduced HBV DNA levels both in supernatant and NTCP-Huh7.5.1 cells ( $P < 0.05$ ). (C) HBV replication upregulated the mRNA expression of TGF- $\beta$ 1 compared with the control group, which was reduced by ETV ( $P < 0.05$ ). (D) HBV-infected NTCP-Huh7.5.1 cells exhibited higher miR-21-5p levels compared with mock infection, which were significantly decreased by ETV ( $P < 0.05$ ). (E) HBV replication and ETV treatment did not affect the viability of NTCP-Huh7.5.1 cells ( $P > 0.05$ ). \* $P < 0.05$  vs. Mock. HBV, hepatitis B virus; NTCP, sodium taurocholate co-transporting polypeptide; ETV, entecavir; miR, microRNA.

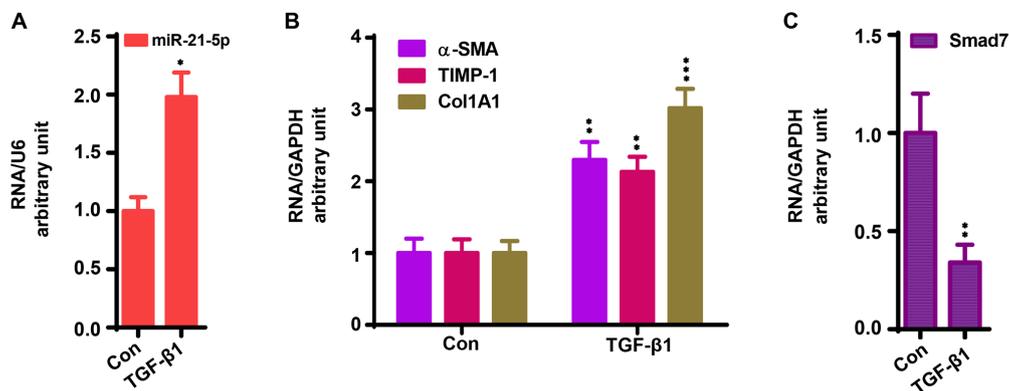


Figure 2. TGF- $\beta$ 1 stimulation activates LX2 cells via miR-21-5p. Treatment for 72 h with TGF- $\beta$ 1 (10 ng/ml) significantly increased (A) miR-21-5p and (B)  $\alpha$ -SMA, TIMP-1 and Col1A1 mRNA expression based on qPCR analysis in LX2 cells compared with controls ( $P < 0.05$ ). By contrast, (C) TGF- $\beta$ 1 treatment significantly reduced Smad7 mRNA expression based on qPCR analysis in LX2 cells compared with controls ( $P < 0.05$ ). \* $P < 0.05$ , \*\* $P < 0.01$  and \*\*\* $P < 0.001$  vs. controls. SMA, smooth muscle actin; Col1A1, collagen type 1  $\alpha$  1; TIMP, tissue inhibitor of metalloproteinase; qPCR, quantitative PCR; Con, control; miR, microRNA.

TGF- $\beta$ 1 induces liver fibrosis gene expression in LX2 cells through miR-21-5p. TGF- $\beta$ 1 was reported to be the key cytokine in the process of HSC activation and liver fibrosis (17). The present study investigated the role of miR-21-5p in TGF- $\beta$ 1-induced liver fibrosis. Following incubation with 10 ng/ml TGF- $\beta$ 1 for 72 h, LX2 cells were collected for qPCR

assay as previously reported (27). As shown in Fig. 2A and B, TGF- $\beta$ 1 treatment significantly enhanced the mRNA expression of miR-21-5p,  $\alpha$ -SMA, TIMP-1 and Col1A1, while this treatment significantly decreased the mRNA expression of Smad7 (Fig. 2C) compared with the control group ( $P < 0.05$ ).

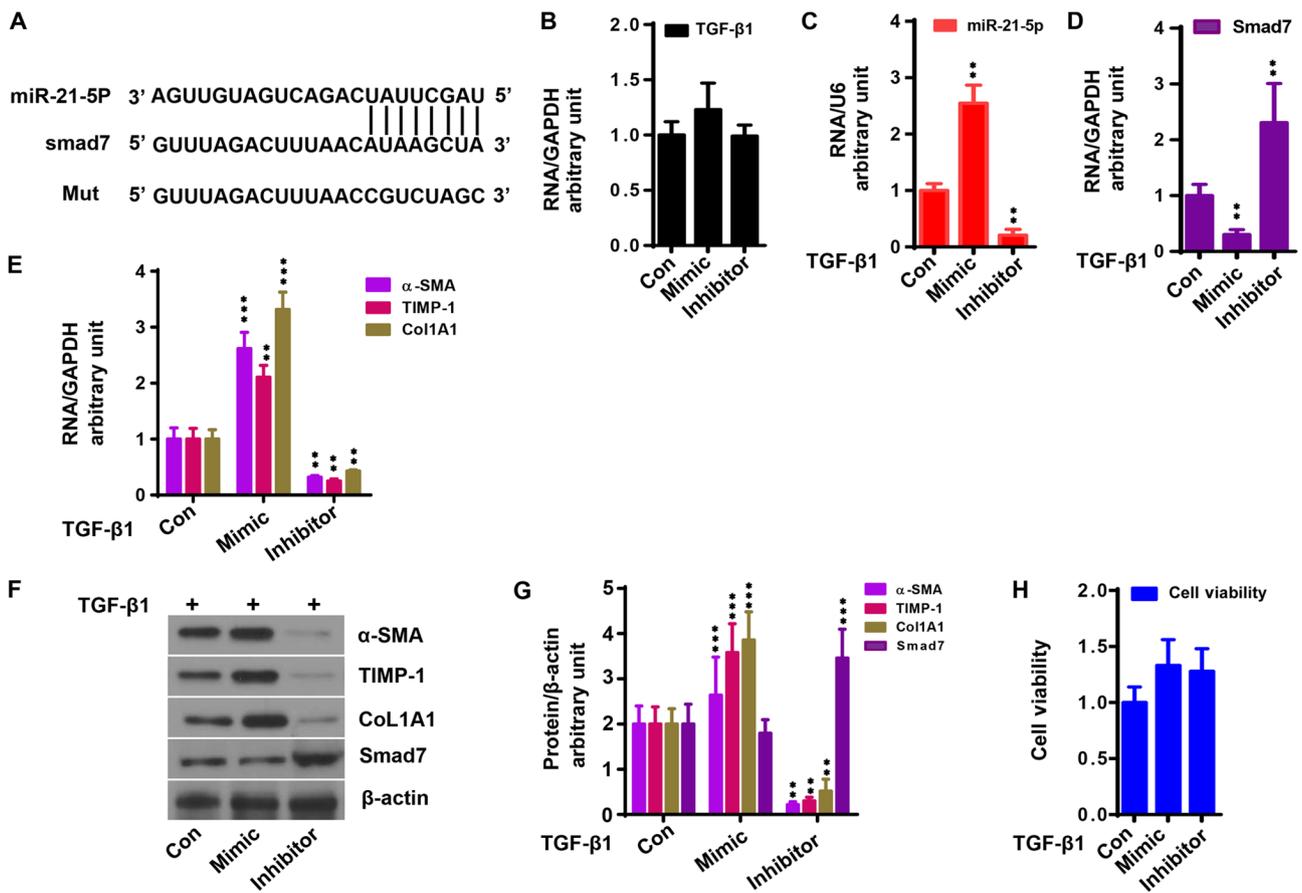


Figure 3. miR-21-5p upregulates TGF-β1 activation in LX2 cells. Control, mimic and inhibitor of miR-21-5p were transfected into LX2 cells with TGF-β1 (10 ng/ml) stimulation. (A) miR-21-5p directly binds to the Smad7 3'-untranslated region. (B) miR-21-5p mimic had no effect on TGF-β1 mRNA expression. miR-21-5p mimic (C) enhanced miR-21-5p expression and (D) reduced Smad7 mRNA expression induced by TGF-β1 based on qPCR analysis. (E) miR-21-5p mimic enhanced α-SMA, TIMP-1 and Col1A1 mRNA expression in LX2 cells induced by TGF-β1 based on qPCR analysis, which was reduced by miR-21-5p inhibitor (P<0.05). (F) Protein expression of Smad7, α-SMA, TIMP-1 and Col1A1 in LX2 cells. (G) Relative protein expression of Smad7, α-SMA, TIMP-1 and Col1A1 in LX2 cells to β-actin. (H) miR-21-5p mimic and inhibitor had no effect on cell viability (P>0.05). \*\*P<0.01 and \*\*\*P<0.001 vs. control. SMA, smooth muscle actin; Col1A1, collagen type 1 α 1; TIMP, tissue inhibitor of metalloproteinase; qPCR, quantitative PCR; miR, microRNA; Con, control.

In addition, antagomiR-21 was used to knock down miR-21-5p expression to further investigate the role of miR-21-5p in TGF-β1-induced liver fibrosis. LX2 cells were transfected with antagomiR-21 or miR-21 mimic together with TGF-β1. As shown in Fig. 3A, miR-21-5p binds to the Smad7 3'-untranslated region (UTR). miR-21-5p had no effect on TGF-β1 expression (Fig. 3B). miR-21 mimic enhanced the upregulation of miR-21-5p induced by TGF-β1, which was counteracted by antagomiR-21 (Fig. 3C; P<0.05). By contrast, miR-21 mimic aggravated the downregulation of Smad7 gene expression induced by TGF-β1, which was alleviated by antagomiR-21 (Fig. 3D, F and G; P<0.05). miR-21 mimic significantly promoted TGF-β1-induced liver fibrosis gene expression, which was significantly reduced by antagomiR-21 (Fig. 3E-G; P<0.05). Neither miR-21 mimic nor antagomiR-21 affected the viability of LX2 cells (Fig. 3H).

**HBV replication contributes to liver fibrosis by upregulating TGF-β1/miR-21-5p.** To investigate the mechanism underlying HBV-induced liver fibrosis, the interaction between HBV-infected NTCP-Huh7.5.1 cells and LX2 cells was observed in a co-culture model (Fig. 4A) as previously described (27). LX2 cells were transfected with antagomiR-21 or miR-21 mimic prior to co-culture. As a result, HBV-infected NTCP-Huh7.5.1

cell co-culture significantly increased the mRNA and protein expression of Col1A1 in LX2 cells compared with the control group, which was enhanced by miR-21 mimic and reduced by antagomiR-21 compared with HBV-infected NTCP-Huh7.5.1 cell co-culture (Fig. 4C, E and F; P<0.05). As expected, miR-21-5p expression exhibited a similar trend, while Smad7 expression exhibited an opposite trend compared with Col1A1 (Fig. 4D-F; P<0.05). LX2 cell viability was not affected by miR-21 mimic, antagomiR-21 or HBV transfection (Fig. 4H).

**General and clinical data of patients with HBV.** The present study included 29 patients with HBV infection to assess the effect of HBV infection on miR-21; 5 patients without HBV infection were enrolled as controls. The demographic, biological and virological data are shown in Table II. The data demonstrated that patients with HBV infection had higher serum levels of ALT and AST as well as HBV DNA levels, compared with the control group (P<0.05). Furthermore, the liver fibrosis score of HBV-infected patients was higher compared with the control group (P<0.05).

**IHC staining and liver fibrosis assessment.** The liver fibrosis score was evaluated using HE and Masson's staining. As

Table II. Clinical, biochemical and laboratory data of patients.

Characteristic	Control (n=5)	Patients (n=29)	P-value
Sex (M/F)	4/1	13/16	>0.05
Age (years)	38.2 $\pm$ 19.4	42.0 $\pm$ 12.3	>0.05
Liver fibrosis (S:1/2/3/4)	4/1/0/0	19/4/4/2	<0.05
ALT (IU/L)	13.4 $\pm$ 3.4	24.0 $\pm$ 23.4	<0.05
AST (IU/L)	16.0 $\pm$ 2.3	25.0 $\pm$ 10.1	<0.05
HBV DNA (log copy/ml)	0	4.0 $\pm$ 2.3	<0.05

Data are presented as the mean  $\pm$  SD. M, male; F, female; ALT, alanine aminotransferase; AST, aspartate aminotransferase; HBV, hepatitis B virus.

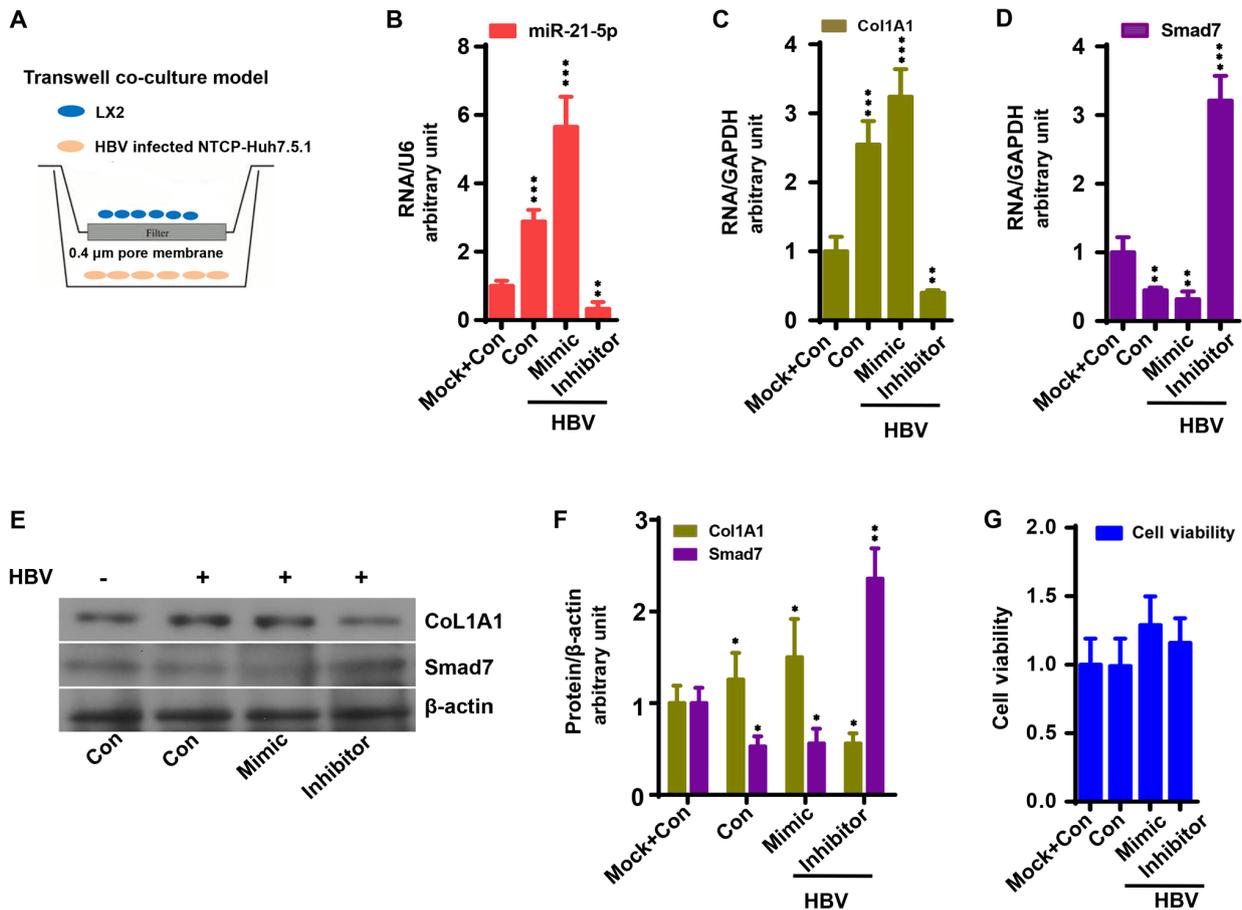


Figure 4. miR-21-5p accelerates the activation of LX2 cells co-cultured with HBV-infected NTCP-Huh7.5.1 cells. (A) Co-culture system. miR-21-5p mimic enhanced (B) miR-21-5p and (C) Col1A1 mRNA expression in LX2 cells co-cultured with HBV-infected NTCP-Huh7.5.1 cells, which was reduced by miR-21-5p inhibitor ( $P < 0.05$ ). (D) miR-21-5p mimic reduced Smad7 mRNA expression in LX2 cells co-cultured with HBV-infected NTCP-Huh7.5.1 cells based on quantitative PCR analysis, which was increased by miR-21-5p inhibitor ( $P < 0.05$ ). (E) Protein expression of Smad7 and Col1A1 in LX2 cells. (F) Relative protein expression of Smad7,  $\alpha$ -SMA, TIMP-1 and Col1A1 in LX2 cells to  $\beta$ -actin. (G) miR-21-5p mimic and inhibitor had no effect on cell viability ( $P > 0.05$ ). \* $P < 0.05$ , \*\* $P < 0.01$  and \*\*\* $P < 0.001$  vs. control. HBV, hepatitis B virus; NTCP, sodium taurocholate co-transporting polypeptide; Col1A1, collagen type 1  $\alpha$  1; Con, control; miR, microRNA.

shown in Fig. 5A, liver tissue samples with no fibrosis ( $S_{0-1}$ ) exhibited normal lobular architecture with central veins and radiating hepatic cords. Liver tissue samples with mild fibrosis ( $S_2$ ) exhibited disordered architecture and bridging fibrosis. In liver tissue samples with severe fibrosis ( $S_{3-4}$ ), normal structure was not visible, while a large number of pseudolobules was readily identifiable.

IHC staining was also used to assess Smad7 protein expression in liver tissue. As shown in Fig. 5A, Smad7 expression was significantly lower in liver tissues with severe fibrosis compared with mild fibrosis ( $P < 0.05$ ), while mild fibrosis was associated with lower Smad7 expression compared with liver tissue with no fibrosis ( $P < 0.05$ ).

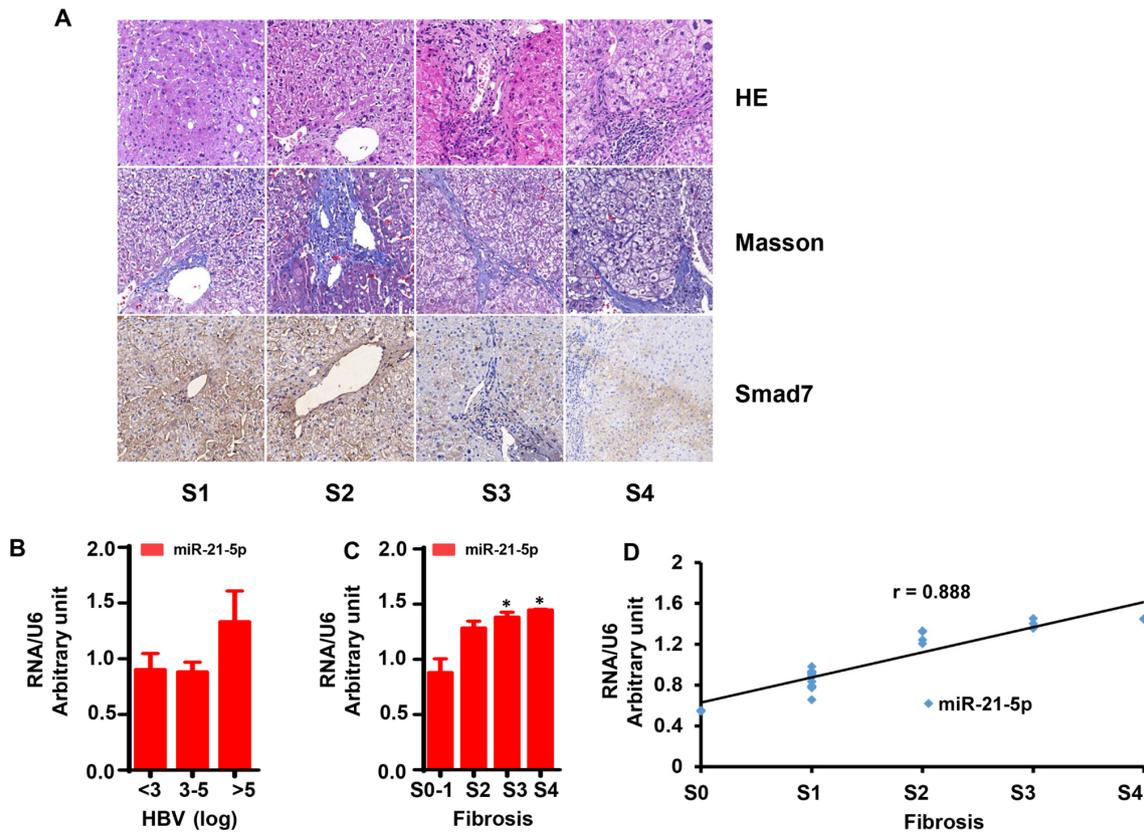


Figure 5. Expression of miR-21-5p and Smad7 in liver tissue. (A) Liver pathology was evaluated by hematoxylin and eosin and Masson's staining, and the degree of liver fibrosis was scored as S<sub>1</sub>-S<sub>4</sub>. (A) Smad7 expression was measured by immunohistochemical staining (magnification, x200). Expression was evaluated using the following formula: Positive index=mean optical density x positive area percentage. Smad7 expression decreased with the progression of liver fibrosis. miR-21-5p expression was measured by quantitative PCR analysis. (B) There was no significant difference in miR-21-5p expression in the liver tissue of patients with various serum HBV DNA levels. (C) Liver miR-21-5p expression was higher in severe fibrosis compared with mild fibrosis (P<0.05). (D) Liver miR-21-5p level exhibited a strong positive correlation with liver fibrosis score (r=0.888; P<0.05). \*P<0.05 vs. control. miR, microRNA; HBV, hepatitis B virus.

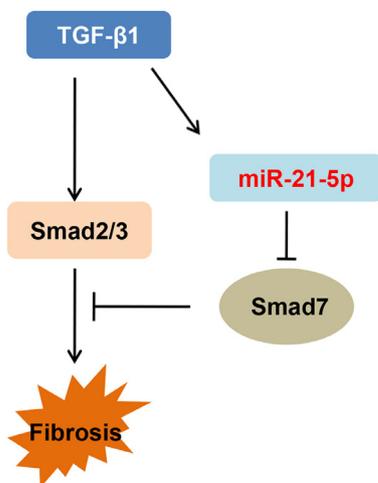


Figure 6. Proposed model through which HBV infection induces liver fibrosis via the TGF-β1/miR-21-5p pathway. HBV infection enhances TGF-β1 production and secretion in hepatocytes, in turn activating hepatic stellate cells and inhibiting Smad7 expression through miR-21-5p. HBV, hepatitis B virus; miR, microRNA.

*mRNA expression of miR-21-5p and fibrosis genes in liver tissues.* The mRNA expression of miR-21-5p in liver tissue was detected by qPCR. The results demonstrated that liver tissues

with mild fibrosis (S<sub>2</sub>) had low mRNA levels of miR-21-5p compared with severe fibrosis (S<sub>3,4</sub>), and its levels were even lower in liver tissue with no fibrosis (S<sub>0,1</sub>) (P<0.05).

Patients in the present study were divided into two series as follows: Series 1, HBV DNA levels (log copies/ml) ≤3, 3-5 and ≥5 and series 2, liver fibrosis score S<sub>0,1</sub>, S<sub>2</sub>, S<sub>3</sub> and S<sub>4</sub>. As shown in Fig. 5B, miR-21 levels exhibited no significant difference between patients with HBV DNA >5 log and those with HBV DNA <5 log. Of note, the miR-21 levels in patients with severe fibrosis was significantly higher compared with patients with mild fibrosis (Fig. 5C; P<0.05).

*Correlation between miR-21-5p and liver fibrosis.* Correlation analysis revealed that miR-21-5p exhibited a strong positive correlation with liver fibrosis score (Fig. 5D; r=0.888; P<0.05). However, there was no significant correlation between miR-21-5p and HBV DNA levels (r=-0.158; P>0.05).

**Discussion**

There is a consensus that liver fibrosis is the healing response of the liver to chronic injuries, including hepatitis, immune compounds and drugs (28). Generally, hepatitis B remains a major health concern worldwide, infecting ~350 million patients (29). HBV also ranks first among

the causes of liver fibrosis in China, infecting ~7% of the entire population (30). At present, although anti-HBV treatment has limited success, chronic HBV infection gradually leads to the development of liver fibrosis and even liver cancer (31,32).

HBV causes liver damage by engaging hepatocytes, macrophages and HSCs in a complicated process that remains poorly understood (33-35). The main obstacle in HBV research is the lack of cell models of HBV infection. The present study condensed HBV stock from the HepAD38 cell line, a derivative of hepatoblastoma cells. HepAD38 can stably produce HBV particles, and have been widely used in scientific research (25). However, this cell line could not stimulate the identification and entry of HBV particles (25,36). NTCP has been demonstrated to be the receptor for HBV (25,36,37). The present study constructed a NTCP-Huh7.5.1 cell line by transfecting NTCP into Huh7.5.1 cells, a derivative of hepatocellular carcinoma cells. Using this cell line, the present study was able to stimulate the natural process of HBV infection. As a result, HBV levels in the supernatant of HBV-infected NTCP-Huh7.5.1 cells remained stable at  $1 \times 10^4$  copies/ml, indicating that NTCP-Huh7.5.1 cells supported HBV infection stably.

Among the various cytokines implicated in liver fibrosis, TGF- $\beta$ 1 has been proven to be the most important (38). It was demonstrated that TGF- $\beta$ 1 initiates signaling by binding to its receptors on the surface of LX2 cells, resulting in LX2 activation and ECM production (39). Interestingly, it was previously suggested that TGF- $\beta$ 1 can be secreted by hepatocytes and macrophages, in turn activating HSCs (40). Accumulating evidence indicated that TGF- $\beta$ 1 mainly transduces signals through Smad (41,42). We have also reported that Smad assisted  $\beta$ -catenin transport to the nucleus of HSCs, initiating fibrosis gene expression (43). We also demonstrated that HBV infection promoted TGF- $\beta$ 1 production in hepatocellular carcinoma cells (26). The present study demonstrated that HBV-infected NTCP-Huh7.5.1 cells enhanced TGF- $\beta$ 1 production by 2.0-fold compared with NTCP-Huh7.5.1 cells with mock infection.

miRNAs are a group of non-coding RNAs that play important roles in regulating the expression of genes involved in chronic hepatitis B, liver fibrosis and liver cancer (44-46). Wang *et al.* (21) reported that miR-21-5p was a key mediator in the TGF- $\beta$ 1/Smad pathway through inhibiting Smad7 expression in the process of spinal fibrosis by targeting the AUAAGCUA sequence in the Smad7 3'-UTR, which was consistent with other reports (46). To the best of our knowledge, no study has revealed the interaction between miR-21-5p and fibrosis genes such as  $\alpha$ -SMA, TIMP-1 and Col1A1. The present study sought to investigate the role of miR-21-5p in the TGF- $\beta$ 1/Smad pathway in HBV-induced liver fibrosis. As expected, TGF- $\beta$ 1 stimulation upregulated miR-21-5p expression in LX2 cells by 2.1-fold compared with the control group, and increased the expression of liver fibrosis genes, such as  $\alpha$ -SMA, TIMP-1 and Col1A1. Moreover, the TGF- $\beta$ 1-induced liver fibrosis gene expression in LX2 cells was enhanced by miR-21-5p expression and inhibited by miR-21-5p knock-down, indicating that miR-21-5p acts as the key mediator of TGF- $\beta$ 1-induced liver fibrosis. The present study also examined the interaction between miR-21-5p and Smad7 and observed that inhibition of miR-21-5p overturned the inhibitory effects of TGF- $\beta$ 1 on Smad7. These results suggested

that miR-21-5p accelerates liver fibrosis by inhibiting Smad7 activation (Fig 6).

Liver fibrosis is characterized by excessive deposition of ECM in the liver, with ensuing destruction of the liver architecture (28). HSCs are the main sources of ECM, and their activation has been considered as a key event in liver fibrosis (40). In this process, cytokines released from injured hepatocytes initiates HSC activation (41). In the present study, hepatocyte-HSC interaction was stimulated using a co-culture system, as previously reported (27). It was demonstrated that co-culture with HBV-infected NTCP-Huh7.5.1 cells significantly enhanced Col1A1 expression in LX2 cells compared with mock infection, which was reduced by miR-21-5p over-expression, suggesting that miR-21-5p inhibited HBV-induced liver fibrosis.

In addition, the correlation between miR-21-5p and liver fibrosis was examined. Results from 29 patients indicated that liver miR-21-5p expression exhibited a strong positive correlation with liver fibrosis ( $r=0.888$ ;  $P<0.05$ ). Furthermore, miR-21-5p levels were evaluated according to the HBV DNA levels, and it was observed that liver miR-21-5p levels did not differ significantly between patients with HBV DNA  $>5$  log and those with HBV levels  $<5$  log. There was no significant positive correlation between miR-21-5p and HBV DNA levels ( $r=-0.158$ ).

In conclusion, the results of the present study revealed that HBV leads to liver fibrosis by regulating the TGF- $\beta$ 1/miR-21-5p pathway. Therefore, miR-21-5p may be a novel target for the treatment of HBV-induced liver fibrosis.

#### Acknowledgements

Not applicable.

#### Funding

The study was supported by the Natural Science Foundation of Anhui Province (grant no. 1508085MH172) and the Fundamental Research Funds for the Central Universities (grant no. WK9110000048).

#### Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

#### Authors' contributions

WL, XY and XC performed the experiments and drafted the manuscript. MY participated in the liver biopsy and specimen storage. ZZ participated in the liver inflammation and fibrosis assessment. CZ was involved in research design, literature review and data examination. ZW participated in the statistical analysis. All authors read and approved final manuscript.

#### Ethics approval and consent to participate

The present study was approved by the Ethics Committee of Anhui Provincial Hospital (approval no. 2019-ky026).

## Patient consent for publication

Not applicable.

## Competing interests

The authors declare that they have no competing interests.

## References

- Zhang K, Shi Z, Zhang M, Dong X, Zheng L, Li G, Han X, Yao Z, Han T and Hong W: Silencing lncRNA Lfar1 alleviates the classical activation and pyroptosis of macrophage in hepatic fibrosis. *Cell Death Dis* 11: 132, 2020.
- Su QD, Zhang S, Wang F, Liu H, Zhang GM, Zheng H, Qiu F, Sun XJ, Liang XF, Bi SL, *et al*: Epidemiological distribution of hepatitis B virus genotypes in 1-29-year-olds in the mainland of China. *Vaccine* 38: 8238-8246, 2020.
- Mohd-Ismail NK, Lim Z, Gunaratne J and Tan YJ: Mapping the interactions of HBV cccDNA with host factors. *Int J Mol Sci* 20: 4276, 2019.
- Sun Y, Wu X, Zhou J, Meng T, Wang B, Chen S, Liu H, Wang T, Zhao X, Wu S, *et al*: Persistent low level of hepatitis B virus promotes fibrosis progression during therapy. *Clin Gastroenterol Hepatol* 18: 2582-2591.e6, 2020.
- Dai Y, Che F, Jiang X, Cui D, Zhou H, Xu X, Sun C and Cheng J: Clinical characteristics and association analysis of persistent low-level HBsAg expression in a physical examination population with HBV infection. *Exp Ther Med* 19: 19-32, 2020.
- Kim J, Kang W, Kang SH, Park SH, Kim JY, Yang S, Ha SY and Paik YH: Proline-rich tyrosine kinase 2 mediates transforming growth factor-beta-induced hepatic stellate cell activation and liver fibrosis. *Sci Rep* 10: 21018, 2020.
- Clayton SW, Ban GI, Liu C and Serra R: Canonical and non-canonical TGF- $\beta$  signaling regulate fibrous tissue differentiation in the axial skeleton. *Sci Rep* 10: 21364, 2020.
- Chang CJ, Lin CF, Lee CH, Chuang HC, Shih FC, Wan SW, Tai C and Chen CL: Overcoming interferon (IFN)- $\gamma$  resistance ameliorates transforming growth factor (TGF)- $\beta$ -mediated lung fibroblast-to-myofibroblast transition and bleomycin-induced pulmonary fibrosis. *Biochem Pharmacol* 183: 114356, 2020.
- Derynck R, Turley SJ and Akhurst RJ: TGF $\beta$  biology in cancer progression and immunotherapy. *Nat Rev Clin Oncol* 18: 9-34, 2021.
- Kori M and Arga KY: Pathways involved in viral oncogenesis: New perspectives from virus-host protein interactomics. *Biochim Biophys Acta Mol Basis Dis* 1866: 165885, 2020.
- Ho CH, Chang TT and Chien RN: Telbivudine on IgG-associated hypergammaglobulinemia and TGF- $\beta$ 1 hyperactivity in hepatitis B virus-related liver cirrhosis. *PLoS One* 14: e0225482, 2019.
- Xu Y, Sun X, Zhang R, Cao T, Cai SY, Boyer JL, Zhang X, Li D and Huang Y: A positive feedback loop of TET3 and TGF- $\beta$ 1 promotes liver fibrosis. *Cell Rep* 30: 1310-1318.e5, 2020.
- Kim JY, Kim KM, Yang JH, Cho SS, Kim SJ, Park SJ, Ahn SG, Lee GH, Yang JW, Lim SC, *et al*: Induction of E6AP by microRNA-302c dysregulation inhibits TGF- $\beta$ -dependent fibrogenesis in hepatic stellate cells. *Sci Rep* 10: 444, 2020.
- Zhang Y, Li J, Wang S, Yang F, Zhou Y, Liu Y, Zhu W and Shi X: HBx-associated long non-coding RNA activated by TGF- $\beta$  promotes cell invasion and migration by inducing autophagy in primary liver cancer. *Int J Oncol* 56: 337-347, 2020.
- Li MH, Chen QQ, Zhang L, Lu HH, Sun FF, Zeng Z, Lu Y, Yi W and Xie Y: Association of cytokines with hepatitis B virus and its antigen. *J Med Virol*: Jul 14, 2020 (Epub ahead of print).
- Martin N, Ziegler DV, Parent R and Bernard D: Hepatic stellate cell senescence in liver tumorigenesis. *Hepatology*: Sep 15, 2020 (Epub ahead of print).
- Zhang J, Jiang N, Ping J and Xu L: TGF- $\beta$ 1-induced autophagy activates hepatic stellate cells via the ERK and JNK signaling pathways. *Int J Mol Med*: Nov 3, 2020 (Epub ahead of print).
- Wang X, He Y, Mackowiak B and Gao B: MicroRNAs as regulators, biomarkers and therapeutic targets in liver diseases. *Gut*: Oct 30, 2020 (Epub ahead of print).
- Ezhilarasan D: MicroRNA interplay between hepatic stellate cell quiescence and activation. *Eur J Pharmacol* 885: 173507, 2020.
- Salimi S, Noorbakhsh F, Faghihzadeh S, Ghaffarpour S and Ghazanfari T: Expression of miR-15b-5p, miR-21-5p, and SMAD7 in lung tissue of sulfur mustard-exposed individuals with long-term pulmonary complications. *Iran J Allergy Asthma Immunol* 18: 332-339, 2019.
- Wang W, Liu R, Su Y, Li H, Xie W and Ning B: MicroRNA-21-5p mediates TGF- $\beta$ -regulated fibrogenic activation of spinal fibroblasts and the formation of fibrotic scars after spinal cord injury. *Int J Biol Sci* 14: 178-188, 2018.
- Chinese Society of Hepatology, Chinese Medical Association; Chinese Society of Infectious Diseases: The guideline of prevention and treatment for chronic hepatitis B: A 2019 update. *Chin J Infect Dis* 37: 711-736, 2019.
- Zhu CL, Li WT, Li Y and Gao RT: Serum levels of tissue inhibitor of metalloproteinase-1 are correlated with liver fibrosis in patients with chronic hepatitis B. *J Dig Dis* 13: 558-563, 2012.
- Bedossa P and Poynard T: An algorithm for the grading of activity in chronic hepatitis C. The METAVIR cooperative study group. *Hepatology* 24: 289-293, 1996.
- Duan X, Li S, Holmes JA, Tu Z, Li Y, Cai D, Liu X, Li W, Yang C, Jiao B, *et al*: MicroRNA 130a regulates both hepatitis C virus and hepatitis B virus replication through a central metabolic pathway. *J Virol* 92: e02009-17, 2018.
- Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. *Methods* 25: 402-408, 2001.
- Li W, Yu X, Zhu C, Wang Z, Zhao Z, Li Y and Zhang Y: Notum attenuates HBV-related liver fibrosis through inhibiting Wnt 5a mediated non-canonical pathways. *Biol Res* 52: 10, 2019.
- Kisseleva T and Brenner D: Molecular and cellular mechanisms of liver fibrosis and its regression. *Nat Rev Gastroenterol Hepatol*: Oct30, 2020 (Epub ahead of print).
- Liu L, Zhu J, Yang J, Li X, Yuan J, Wu J and Liu Z: GP73 facilitates hepatitis B virus replication by repressing the NF- $\kappa$ B signaling pathway. *J Med Virol*, February 20, 2020 (Online ahead of print).
- Wang T, Dai Y, Lu W, Zhou H, Chen Y, Xu X, Sun C and Cheng J: An epidemiological survey of HBV infection and low-level HBsAg in military camps in eastern China. *Medicine (Baltimore)* 97: e12201, 2018.
- Li Z, Hu Y, Wang H, Wang M, Gu X, Ping Y, Zeng Q, Li H, Yan J and Yu Z: Predictors for the progression of hepatic cirrhosis to hepatocellular carcinoma under long-term antiviral therapy. *Eur J Gastroenterol Hepatol* 32: 447-453, 2020.
- Yotsuyanagi H, Takano T, Tanaka M, Amano K, Imamura M, Ogawa K, Yasunaka T, Yasui Y, Hayashi K, Tanaka Y, *et al*: Hepatitis B virus-related hepatocellular carcinoma in young adults: Efficacy of nationwide selective vaccination. *Hepatology Res* 50: 182-189, 2020.
- Duriez M, Mandouri Y, Leckaby B, Wang H, Schnuriger A, Redelsperger F, Guerrero CI, Lefevre M, Fauveau V, Ahodantin J, *et al*: Alternative splicing of hepatitis B virus: A novel virus/host interaction altering liver immunity. *J Hepatol* 67: 687-699, 2017.
- Yuan L, Jiang J, Liu X, Zhang Y, Zhang L, Xin J, Wu K, Li X, Cao J, Guo X, *et al*: HBV infection-induced liver cirrhosis development in dual-humanised mice with human bone mesenchymal stem cell transplantation. *Gut* 68: 2044-2056, 2019.
- Bility MT, Cheng L, Zhang Z, Luan Y, Li F, Chi L, Zhang L, Tu Z, Gao Y, Fu Y, *et al*: Hepatitis B virus infection and immunopathogenesis in a humanized mouse model: Induction of human-specific liver fibrosis and M2-like macrophages. *PLoS Pathog* 10: e1004032, 2014. Yan H, Zhong G, Xu G, He W, Jing Z, Gao Z, Huang Y, Qi Y, Peng B, Wang H, *et al*: Sodium taurocholate cotransporting polypeptide is a functional receptor for human hepatitis B and D virus. *Elife* 1: e00049, 2012.
- Donkers JM, Appelman MD and van de Graaf SFJ: Mechanistic insights into the inhibition of NTCP by myrcludex B. *JHEP Rep* 1: 278-285, 2019.
- Tian F, Liu Y, Gao J, Yang N, Shang X, Lv J, Ba D, Zhou X, Zhang C and Ma X: Study on the association between TGF- $\beta$ 1 and liver fibrosis in patients with hepatic cystic echinococcosis. *Exp Ther Med* 19: 1275-1280, 2020.
- Tao L, Xue D, Shen D, Ma W, Zhang J, Wang X, Zhang W, Wu L, Pan K, Yang Y, *et al*: MicroRNA-942 mediates hepatic stellate cell activation by regulating BAMB1 expression in human liver fibrosis. *Arch Toxicol* 92: 2935-2946, 2018.
- Dewidar B, Meyer C, Dooley S and Meindl-Beinker AN: TGF- $\beta$  in hepatic stellate cell activation and liver fibrogenesis—updated 2019. *Cells* 8: 1419, 2019.

40. Frangianni N: Transforming growth factor- $\beta$  in tissue fibrosis. *J Exp Med* 217: e20190103, 2020.
41. Marvin DL, Heijboer R, Ten Dijke P and Ritsma L: TGF- $\beta$  signaling in liver metastasis. *Clin Transl Med* 10: e160, 2020.
42. Li W, Zhu C, Chen X, Li Y, Gao R and Wu Q: Pokeweed antiviral protein down-regulates Wnt/ $\beta$ -catenin signalling to attenuate liver fibrogenesis in vitro and in vivo. *Dig Liver Dis* 43: 559-566, 2011.
43. Sagnelli E, Potenza N, Onorato L, Sagnelli C, Coppola N and Russo A: Micro-RNAs in hepatitis B virus-related chronic liver diseases and hepatocellular carcinoma. *World J Hepatol* 10: 558-570, 2018.
44. Zhi SC, Chen SZ, Li YY, Li JJ, Zheng YH and Yu FX: Rosiglitazone inhibits activation of hepatic stellate cells via up-regulating Micro-RNA-124-3p to alleviate hepatic fibrosis. *Dig Dis Sci* 64: 1560-1570, 2019.
45. Sendi H, Mehrab-Mohseni M, Russo MW, Steuerwald N, Jacobs C, Clemens MG and Bonkovsky HL: Baseline hepatic levels of miR-29b and claudin are respectively associated with the stage of fibrosis and HCV RNA in hepatitis C. *Clin Exp Gastroenterol Hepatol* 1: 105, 2019.
46. Li Q, Li B, Li Q, Wei S, He Z, Huang X, Wang L, Xia Y, Xu Z, Li Z, *et al*: Exosomal miR-21-5p derived from gastric cancer promotes peritoneal metastasis via mesothelial-to-mesenchymal transition. *Cell Death Dis* 9: 854, 2018.



This work is licensed under a Creative Commons Attribution-NonCommercial-NoDerivatives 4.0 International (CC BY-NC-ND 4.0) License.