Hepatitis B Virus Regulates Apoptosis and Tumorigenesis through the MicroRNA-15a-Smad7-Transforming Growth Factor Beta Pathway

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ABSTRACT

Hepatitis B virus (HBV) infection causes chronic hepatitis in hundreds of millions of people worldwide, which can eventually lead to hepatocellular carcinoma (HCC). Previously, we found that HBV mRNAs can absorb microRNA-15a (miR-15a) to affect apoptosis through the Bcl-2 pathway. We asked whether HBV could inhibit apoptosis and promote tumorigenesis through different pathways. In this study, we found that the transforming growth factor β (TGF- β) pathway-inhibitory factor Smad7 is a novel target of miR-15a. We demonstrated that HBV can upregulate the level of Smad7 by downregulating miR-15a. Furthermore, we examined the level of Smad7 in liver samples from HBV-infected HCC patients and found that HBV mRNAs are positively correlated with the level of Smad7. By taking the approach of using immunoblotting and luciferase reporter assays, we revealed that HBV can abrogate TGF- β signaling via upregulating Smad7. By using annexin V staining and caspase 3/7 activity assays, we found that HBV can inhibit TGF- β -induced apoptosis of HepG2 cells. We also showed that HBV can promote tumor growth in BALB/c nude mice through upregulating the expression of Smad7. In conclusion, we demonstrated that HBV can upregulate Smad7 expression and inhibit TGF- β signaling, which makes the cells resistant to TGF- β -induced apoptosis and promotes tumorigenesis.

IMPORTANCE

Hepatitis B virus (HBV) infection causes chronic hepatitis, which can eventually lead to hepatocellular carcinoma (HCC). TGF- β signaling is closely linked to liver fibrosis, cirrhosis, and subsequent HCC progression and plays a unique role in the pathogenesis of HCC. At the early stage of tumor formation, TGF- β functions as a tumor suppressor that inhibits cell proliferation and induces apoptosis. Previously, we found that HBV mRNAs can sponge off miR-15a to affect apoptosis through the Bcl-2 pathway. In this study, we identified that the TGF- β -inhibitory factor Smad7 is a novel target of miR-15a. We reveal that HBV can abrogate TGF- β signaling via upregulating Smad7, inhibit TGF- β -induced apoptosis, as well as promote tumor development. Our study provides evidence to support the idea that viral RNAs can exert their functions as competing endogenous RNAs (ceRNAs) toward microRNA and participate in important cellular processes.

Hepatitis B virus (HBV) infection remains a major public health concern, with >350 million people being chronically infected worldwide (1). Chronic HBV infection is related to the occurrence and development of hepatocellular carcinoma (HCC), which is the third leading cause of cancer mortality (2–4).

HBV is an enveloped, partially double-stranded DNA virus with a genome size of 3.2 kb, and it replicates through an RNA intermediate form (pre-C/C, pre-S, S, and X mRNAs) by reverse transcription. The mRNAs of HBV encode several viral proteins, including the polymerase, core, HBe, pre-S1, S2, S, and X proteins (5). HBV infection has been reported to play an important role in regulating hepatocyte apoptosis for persistent survival (6–8). MicroRNAs (miRNAs) are small, noncoding, single-stranded RNA molecules that are involved in the regulation of target gene expression in multiple cellular processes. It has been reported that HBx promotes tumorigenesis by downregulating microRNA-148a (miR-148a) (9) and induces aberrant DNA methylation by downregulating miR-101 (10). Also, HBV can promote cell proliferation and tumor formation by downregulating miR-122 (11). Previously, we found that HBV inhibits apoptosis by directly sponging miR-15a and upregulating Bcl-2 expression (12). It has been reported that miR-15a can enhance prostate cancer progression and promote cell growth and survival by targeting Bcl-2, CCND1, and WNT3A (13). In this report, we identified that miR-15a can reg-

ulate the level of Smad7 mRNA and enhance the transforming growth factor β 1 (TGF- β 1) signaling pathway.

TGF- β 1 not only inhibits cell proliferation but also induces apoptosis in hepatocytes, myeloid cells, and epithelial cells (14). Smad proteins have been identified as key signal transducers in TGF- β 1-dependent growth inhibition (15). When activated by TGF- β , the TGF- β type I receptor phosphorylates Smad2, which leads to the association of Smad2 with Smad4. Smad2/Smad4 then translocates to the nucleus to promote downstream gene transcription. Smad7 acts as an inhibitor of TGF- β signaling by interacting with the TGF- β type I receptor to prevent the phosphorylation and activation of Smad2 (16). It has been reported that

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Smad7 can block TGF- β -induced growth inhibition and inhibit the apoptosis of FET cells, which may enhance the tumorigenicity of FET cells (14).

TGF- β signaling is closely linked to liver fibrosis, cirrhosis, and subsequent HCC progression and plays a unique role in the pathogenesis of HCC (17). At the early stage of tumor formation, TGF- β functions as a tumor suppressor that inhibits cell proliferation and induces apoptosis, while at the later stage of tumor progression, tumor cells lose their response to TGF- β (18, 19). However, the exact function of TGF- β signaling in chronic HBV infection and HBV-related HCC is still unclear. In this study, we identified that Smad7, as an inhibitory factor of the TGF- β pathway, is a novel target of miR-15a. We further demonstrate that HBV mRNAs can interfere with TGF- β signaling through upregulating Smad7 expression by eliminating miR-15a, inhibit TGF- β -induced apoptosis, and facilitate tumor formation.

MATERIALS AND METHODS

Patients and human specimens. Liver tissues from 40 patients with HCC were collected from the 302 Hospital of PLA. The patients were hospitalized during June 2012 to July 2013. The clinical characteristics of enrolled subjects were listed in a previous study (12). Written informed consent was provided by all study participants. Patient samples were assigned arbitrary identification numbers based on the order of enrollment in our study. The study protocol was approved by the ethics committee of the 302 Hospital of PLA.

HBV transgenic mice. HBV transgenic BALB/c mice were purchased from the Transgenic Engineering Lab, Infectious Disease Center (Guangzhou, China), and were described in our previous study (12).

Plasmid constructs. The Smad7 3' untranslated region (UTR) (nucleotides [nt] 1608 to 1627) (GenBank accession no. [NM_001190821](#)) was amplified by PCR, and the PCR product was cloned into the dual-luciferase reporter pmirGLO vector (Promega, USA) and named pGLO-Smad7-3'UTR. pGLO-Smad7-3'UTR-mut, with mutations in the seed region of the miR-15a-binding site, was generated by site-directed mutagenesis. p3TP-luc was purchased from Addgene (plasmid 11767) (20). Smad7 and Smad2 were cloned into pFLAG-CMV2 (Sigma, USA) and pCMV-Myc (Clontech, USA), respectively. pHBV1.3, carrying the full-length HBV genome, and its mutant pHBV1.3-mut, which cannot absorb miR-15a, were generated as described previously (12).

Reagents and antibodies. The chemically synthesized miR-15a inhibitor, mimic, and nonspecific controls were purchased from RiboBio Co., Ltd. (Guangzhou, China). The following reagents and antibodies were obtained as indicated: the rabbit anti-Smad7 antibodies were produced by immunizing rabbits with His-Smad7 (amino acids [aa] 1 to 200) or His-Smad7 (aa 201 to 426), and the rabbit anti-human poly(ADP-ribose) polymerase (PARP) antibody (Cell Signaling, USA), the anti-FLAG antibody (Sigma, USA), the anti-Myc antibody (Santa Cruz, USA), the rabbit anti-human Smad2/3 antibody (Santa Cruz, USA), the phosphor-Smad2 (Ser465/467) rabbit monoclonal antibody (MAb) (Santa Cruz, USA), the goat anti-human lamin B antibody (Santa Cruz, USA), the rabbit anti-human β -actin antibody (Santa Cruz, USA), and the horseradish peroxidase-conjugated secondary antibodies (Jackson Laboratory, USA) were purchased.

Cell culture and transfection. The human hepatoma cell line HepG2 and the human normal liver cell line L-02 were obtained from the ATCC (Manassas, VA, USA). The HepG2-C5 and HepG2-4D14 cell lines, in which the HBV genome was integrated, were kindly provided by Dongping Xu (302 Hospital of PLA). Transfections were performed by using Lipofectamine 2000 reagent (Invitrogen, USA).

RNA extraction and real-time PCR. Total RNA was extracted from cells or frozen tissues by using TRIzol reagent (Invitrogen, USA) according to the manufacturer's instructions. The RNA was reverse transcribed, and real-time PCR was performed by using SYBR green real-time PCR

master mix (Toyobo, Japan). Relative expression was quantified by using the comparative threshold cycle (C_T) method.

Luciferase assay. HepG2 cells were cotransfected with pGLO-Smad7-3'UTR, pGLO-Smad7-3'UTR-mut, and miR-15a for 24 h. The cell lysates were harvested for a dual-luciferase assay according to the manufacturer's instructions (Promega, USA). Three independent repeats were performed. For analyzing the activation of TGF- β signaling, the TGF- β reporter p3TP-luc and miR-15a were cotransfected into HepG2 cells for 24 h, and the cell lysates were harvested for luciferase assays.

Immunoblotting. Protein samples were subjected to SDS-PAGE and blotted with the indicated antibodies. After samples were washed with phosphate-buffered saline (PBS)-Tween (PBST) three times, the horseradish peroxidase-conjugated secondary antibody was added for 1 h. Protein bands were visualized by using Enlight Western blotting reagents (Engreen Biosystem, China).

Caspase activity assay. HepG2 and L-02 cells were plated into a 96-well plate and treated as indicated. The cell lysates were harvested for caspase activity assays with Caspase-Glo3/7 reagents (Promega, USA). The luminescence of each sample was measured with a plate-reading luminometer.

Flow cytometry. HepG2 cells were transfected with pHBV1.3, pHBV1.3-mut, or pcDNA3.1 for 24 h and then treated with TGF- β 1 (10 μ g/ml) for 48 h. The cells were subjected to annexin V and propidium iodide (PI) staining for flow cytometry analysis.

Construction of HepG2-4D14-shSmad7 cell lines. The lentiviral plasmids for expressing short hairpin RNAs (shRNAs) targeting Smad7 or control shRNA and the lentiviral packaging system were purchased from Sigma-Aldrich (gene identification no. 4092). Lentiviruses for each shRNA were produced according to the manufacturer's instructions. HepG2-4D14 cells were infected with the lentiviruses in the presence of Polybrene (8 μ g/ml) for 48 h. The cells were then selected with puromycin (1 μ g/ml) and named HepG2-4D14-shSmad7 or HepG2-4D14-shNC (negative control).

Cell viability analysis. HepG2, HepG2-4D14, and HepG2-4D14-shSmad7 cells were seeded into 96-well plates and treated with or without TGF- β (10 μ g/ml) for the indicated times. The cells were then harvested and subjected to a viability assay using Cell Counting Kit-8 (CCK-8).

Tumorigenesis. A total of 10^7 cells (HepG2, HepG2-4D14, or HepG2-4D14-shSmad7 cells) were resuspended in PBS and injected subcutaneously into the left upper flank regions of 6- to 8-week-old BALB/c nude mice (5 for each group). After 30 days, the mice were sacrificed, the tumors were dissected, and the size of the tumors was measured and calculated as follows: tumor volume (mm^3) = $(L \times W^2)/2$, where L is the long axis and W is the short axis. All animals received humane care, and the study of mice was permitted by the Research Ethics Committee of the Institute of Microbiology (approval no. PZ 2MCAS2013002).

Immunofluorescence. Tumor tissues were fixed with 4% paraformaldehyde in PBS for 8 h at 4°C. After fixation, the tissues were embedded in paraffin, cut with a microtome (Leica) to 5 μ m, and affixed onto the slides. The slides were treated with 3% H_2O_2 for 30 min at 25°C and with 0.01 M sodium citrate (pH 6.0) for antigen retrieval and permeabilized with pre-cooled acetone for 10 min. The slides were then blocked with 10% goat serum for 1 h at 25°C and incubated with rabbit anti-Smad7 antibody overnight at 4°C. After being washed 3 times in PBST, the slides were incubated with Alexa Fluor 488 goat anti-rabbit IgG(H+L) for 1 h at 25°C. The sections were counterstained with 4',6-diamidino-2-phenylindole (DAPI), and images were acquired with a Nikon Eclipse 80i instrument.

Statistical analysis. Differences between each group were determined by using Student's t test. A P value of <0.05 was considered significant. The degree of association between variables was determined by Spearman's nonparametric correlation.

RESULTS

MiR-15a downregulates Smad7 expression by targeting its 3' UTR. In our previous study, we found that HBV can downregulate the level of miR-15a, which has been reported to act as a putative tumor suppressor by targeting Bcl-2, CCND1, and WNT3A (13).

FIG 1 MiR-15a downregulates Smad7 expression by targeting its 3' UTR. (A) HepG2 cells were transfected with miR-15a or a randomized oligonucleotide as a control for 48 h. Total RNA was extracted and quantified by real-time PCR for the expression of predicted target genes. (B) HepG2 cells were transfected with miR-15a or a randomized oligonucleotide as a control for 48 h. Cell lysates were harvested for immunoblotting with rabbit anti-human Smad7 antibody. (C) The predicted miR-15a-binding sequences are located in the 3' UTR of Smad7 mRNA. The matched nucleotides are indicated with a line. (D) Mutations on the 3' UTR of Smad7 were made in the predicted region of miR-15a-binding sites (named Smad7-3' UTR-mut). The mutated nucleotides are shown in boldface italic type. (E) HepG2 cells were cotransfected with the miR-15a mimic and pGLO-Smad7-3'UTR or pGLO-Smad7-3'UTR-mut. The cell lysates were harvested for dual-luciferase assays. The relative luciferase activity was quantified. **, $P < 0.01$.

To further understand the significance of the HBV/miR-15a pathway, we tried to identify other targets of miR-15a. By analysis with TargetScan at the miRBase website (<http://www.mirbase.org/>), several genes, such as the DEDD, CDC25A, Smad7, E2F3, E2F7, and PLAGL1 genes, were predicted to be the targets of miR-15a. To confirm the above-mentioned predictions, we transfected HepG2 cells with miR-15a and examined the expression of these genes with CCND1 as a positive control. As shown in Fig. 1A, the mRNA levels of CDC25A, Smad7, and E2F7 were significantly decreased in HepG2 cells transfected with miR-15a. We chose Smad7 for further study since it is an important regulator of TGF- β signaling. First, we examined the protein level of Smad7 in miR-15a-transfected cells. The immunoblotting data showed that the protein level of Smad7 was significantly reduced in miR-15a-transfected cells (Fig. 1B). We then looked for the potential miR-15a-targeting site on Smad7 by using TargetScan. There is a putative miR-15a-complementary region located in the Smad7 mRNA 3' UTR (nt 1608 to 1627) (Fig. 1C). Therefore, we generated a Smad7 3'-UTR luciferase reporter (pGLO-Smad7-3'UTR) and its mutant (pGLO-Smad7-3'UTR-mut), in which mutations in the

miR-15a-binding site were made as indicated in the legend of Fig. 1D. We transfected them with or without miR-15a into HepG2 cells. The cell lysates were harvested for dual-luciferase assays. The data showed that miR-15a can reduce the relative luciferase activity of pGLO-Smad7-3'UTR but not that of pGLO-Smad7-3'UTR-mut (Fig. 1E). These results suggested that Smad7 is a novel target of miR-15a that can downregulate the expression of Smad7 by targeting its 3' UTR.

HBV upregulates Smad7 expression by downregulating miR-15a in hepatocytes. In a previous study, we found that HBV causes a reduction in the level of miR-15a. We wonder whether HBV could upregulate Smad7 expression by downregulating miR-15a. First, we confirmed that the expression level of miR-15a in HBV transgenic HepG2-C5 and HepG2-4D14 cells was much lower than that in HepG2 cells (Fig. 2A). We then analyzed the levels of Smad6 and Smad7 mRNAs and the amounts of Smad6 and Smad7 proteins in HepG2, HepG2-C5, and HepG2-4D14 cells. The data showed that both Smad7 mRNA (Fig. 2B, left) and Smad7 protein (Fig. 2B, right) levels in HepG2-C5 and HepG2-4D14 cells were significantly higher than those in HepG2 cells,

FIG 2 HBV upregulates Smad7 expression by downregulating miR-15a in hepatocytes. (A and B) Total mRNAs of HepG2, HepG2-C5, and HepG2-4D14 cells were extracted and subjected to real-time PCR for miR-15a (A) and Smad6 and Smad7 mRNAs (B, left), and the cell lysates from the above-described cell lines were harvested for immunoblotting with Smad6 and Smad7 antibodies (B, right). (C and D) HepG2 and L-02 cells were transfected with pHBV1.3, pHBV1.3-mut, or pcDNA3.1 as the control for 48 h. The amount of Smad7 mRNA was quantified by real-time PCR (C), and the protein level of Smad7 was detected by immunoblotting (D). **, $P < 0.01$.

while there was no obvious difference in Smad6 expression levels in the above-described cells. Furthermore, we transfected pHBV1.3 or pHBV1.3-mut into HepG2 and L-02 cells and analyzed the amounts of Smad7 mRNA and protein. The data showed that the amounts of both Smad7 mRNA (Fig. 2C) and protein (Fig. 2D) were upregulated in pHBV1.3-transfected cells compared to those in control cells and pHBV1.3-mut-transfected cells. These results suggested that HBV can upregulate the expression of Smad7.

HBV upregulates Smad7 expression by downregulating miR-15a *in vivo*. To further confirm that HBV can upregulate the expression of Smad7 *in vivo*, we examined the amount of Smad7 mRNA in HBV transgenic BALB/c mice by real-time PCR. The data showed that the relative amount of Smad7 mRNA in liver tissue of HBV transgenic BALB/c mice was 2-fold higher than that in control mice ($P < 0.01$) (Fig. 3A). We next examined the correlation between miR-15a and Smad7 mRNA in liver tissue samples from HCC patients who were all chronic HBV carriers. As shown in Fig. 3B, there was a negative correlation between miR-15a and Smad7 mRNA levels. We then analyzed the levels of Smad7 mRNA and HBV transcripts in liver tissue samples from HCC patients by real-time PCR. The data indicated that there was a positive correlation between the levels of HBV mRNAs and the level of Smad7 mRNA in liver tissue samples of HCC patients (Fig. 3C). In addition, we collected the liver tumor and adjacent non-tumorous liver tissues (ANLTs) from 5 patients and examined

Smad7 protein levels. The data showed that the level of Smad7 protein in tumors was higher than that in adjacent nontumorous liver tissues of each patient (Fig. 3D, top). More importantly, the level of Smad7 protein was positively correlated with the amount of HBV mRNA in patients (Fig. 3D).

MiR-15a enhances TGF- β signaling by downregulating Smad7. It is known that Smad7 acts as an antagonist of TGF- β signaling (16). Therefore, we examined whether miR-15a could enhance TGF- β signaling by downregulating Smad7. HepG2 cells were transfected with an miR-15a mimic, an miR-15a inhibitor, or a randomized oligonucleotide as a control and the TGF- β luciferase reporter p3TP-luc, followed by treatment with TGF- β 1. The cell lysates were harvested for luciferase assays. As shown in Fig. 4A, the miR-15a mimic enhanced the activation of the TGF- β luciferase reporter, while the miR-15a inhibitor inhibited the activation of TGF- β signaling. We then transfected HepG2 cells with pFLAG-CMV2-Smad7 and treated the cells with TGF- β 1. We analyzed the amount of Smad2 in total cell extracts and in the nucleus by immunoblotting. The data showed that the amount of Smad2 in the nucleus in Smad7-overexpressing cells was lower than that in control cells, while the total amounts of Smad2 were similar in both groups (Fig. 4B). We checked the amount of Smad2 in miR-15a mimic-transfected cells. As shown in Fig. 4C, miR-15a enhanced the translocation of Smad2 into the nucleus in cells treated with TGF- β 1. However, the overexpression of Smad7 counteracted the effect of miR-15a on the nuclear

FIG 3 HBV upregulates Smad7 expression by downregulating miR-15a *in vivo*. (A) Total RNAs extracted from liver tissues of HBV transgenic BALB/c mice ($n = 3$) or control BALB/c mice ($n = 3$) were subjected to real-time PCR to detect the amount of Smad7 mRNA. (B) Total RNAs from liver specimens of HCC patients were extracted and subjected to real-time PCR for miR-15a and Smad7 mRNA. The correlation between miR-15a and Smad7 mRNA in samples from HCC patients was analyzed by a Spearman rank test. Correlation coefficient (R) values were calculated. (C) Total RNAs from liver specimens of HCC patients were extracted and subjected to real-time PCR for Smad7 mRNA and HBV transcripts. The correlation between HBV transcripts and Smad7 mRNA in HCC patient samples was analyzed by a Spearman rank test. R values are also shown. (D) Protein levels of Smad7 were detected by immunoblotting (top), and the relative amount of HBV mRNA was detected by real-time PCR (bottom) in tumor tissues (T) and adjacent nontumorous liver tissues (ANLTs). **, $P < 0.01$.

translocation of Smad2 (Fig. 4D). These data indicated that miR-15a enhanced TGF- β signaling and promoted Smad2 translocation into the nucleus through downregulating the expression of Smad7.

HBV inhibits TGF- β signaling by downregulating miR-15a. Based on the above-described results, which demonstrated that HBV could upregulate the expression of Smad7, we sought to determine whether HBV could inhibit TGF- β signaling. HepG2 cells were transfected with pHBV1.3 or pHBV1.3-mut together with the TGF- β reporter p3TP-luc and then treated with TGF- β 1. The cells were harvested for luciferase assays and immunoblotting. As shown in Fig. 5A, the relative luciferase activity in pHBV1.3-transfected cells was significantly reduced compared with that in control or pHBV1.3-mut-transfected cells treated

with TGF- β 1. Consistently, the amount of Smad2 in the nucleus was greatly reduced in pHBV1.3-transfected cells compared to that in control or pHBV1.3-mut-transfected cells (Fig. 5B). To further determine whether HBV upregulates the level of Smad7 by downregulating miR-15a, we transfected HepG2 cells with pHBV1.3 with or without the miR-15a mimic, followed by treatment with TGF- β 1. The cell lysates were harvested for immunoblotting with the indicated antibodies. As shown in Fig. 5C, the level of Smad7 was increased in pHBV1.3-transfected cells but returned to the original level in pHBV1.3- and miR-15a-cotransfected cells, suggesting that miR-15a can counteract the effect of pHBV1.3 on the upregulation of Smad7. Consistently, as shown in Fig. 5D, the amount of Smad2 in the nucleus was significantly lower in pHBV1.3-transfected cell than that in control cells, but in

FIG 4 MiR-15a enhances TGF- β signaling by downregulating Smad7. (A) HepG2 cells were transfected with an miR-15a mimic, an miR-15a inhibitor, or a randomized oligonucleotide as a control, together with the TGF- β luciferase reporter p3TP-luc and renilla luciferase plasmid pRL-TK. The cell lysates were harvested for firefly luciferase and renilla luciferase activity assays. The relative luciferase activity was quantified. (B) HepG2 cells were transfected with pcDNA3.1 or pCMV-FLAG-Smad7 for 24 h and then treated with TGF- β 1 for 24 h. The total cell lysate and the nuclear fraction were subjected to immunoblotting with the indicated antibodies. (C) HepG2 cells were transfected with the miR-15a mimic or a randomized oligonucleotide as a control for 24 h and then treated with TGF- β 1 for 24 h. Immunoblotting was performed as described above. (D) HepG2 cells were transfected with the miR-15a mimic with or without FLAG-Smad7 for 24 h and then treated with TGF- β 1 for 24 h. Immunoblotting was performed as indicated. The relative amount of Smad2 in the nucleus was quantified. *, $P < 0.05$.

pHBV1.3- and miR-15a-cotransfected cells, it went back to the same level as that in control cells. We also checked the amount of phosphorylated Smad2 in pHBV1.3- and pCMV-Myc-Smad2-transfected HepG2 cells treated with TGF- β 1. The data showed that the level of phosphorylated Smad2 in pHBV1.3- and pCMV-Myc-Smad2-cotransfected cells was much lower than that in pCMV-Myc-Smad2-transfected cells (Fig. 5E), which suggested that HBV could downregulate the level of phosphorylated Smad2 in HepG2 cells.

HBV makes HepG2 cells resistant to TGF- β 1-induced apoptosis. As HBV can downregulate TGF- β signaling through the miR-15a/Smad7 pathway, we wondered whether there was any correlation between HBV and TGF- β -induced apoptosis. First, we tested whether TGF- β could induce apoptosis in HepG2 cells by checking the cleavage of PARP-1. As shown in Fig. 6A, PARP-1 cleavage occurred obviously in TGF- β 1-treated cells. We then compared PARP-1 cleavage in HepG2, HepG2-C5, and HepG2-4D14 cells treated with TGF- β 1. The data indicated that PARP-1 cleavage in HepG2-C5 and HepG2-4D14 cells was much weaker than that in HepG2 cells, which means that HepG2-C5 and HepG2-4D14 cells are resistant to apoptosis induced by TGF- β 1 (Fig. 6B). Furthermore, we transfected HepG2 cells with pHBV1.3 and pHBV1.3-mut and treated the cells with TGF- β 1. As shown in Fig. 6C, PARP-1 cleavage was reduced in pHBV1.3-transfected

cells compared to that in control cells, while there was no obvious reduction in pHBV1.3-mut-transfected cells. Next, we transfected HepG2 cells with pHBV1.3 with or without miR-15a and treated the cells with TGF- β 1. The data showed that PARP-1 cleavage was reduced in pHBV1.3-transfected cells compared to that in control cells, while in pHBV1.3- and miR-15a-cotransfected cells, PARP-1 cleavage was recovered to a certain level (Fig. 6D). We also examined caspase 3/7 activity in HepG2 and L-02 cells, which were transfected with pHBV1.3, pHBV1.3-mut, or the control plasmid and treated with TGF- β 1. The data showed that the level of caspase 3/7 activity in pHBV1.3-transfected cells was lower than those in control cells and pHBV1.3-mut-transfected cells (Fig. 6E). Finally, we performed flow cytometry analysis with annexin V and PI staining to monitor apoptotic cells. As shown in Fig. 6F, after TGF- β 1 treatment, the percentage of apoptotic cells in pHBV1.3-transfected cells was 3-fold lower than that in control cells, while the percentage of apoptotic cells in pHBV1.3-mut-transfected cells was also reduced but not as dramatically as in pHBV1.3-transfected cells. These results suggested that HBV could inhibit TGF- β -induced apoptosis through the miR-15a/Smad7 pathway.

HBV promotes tumorigenesis through inhibiting TGF- β signaling by upregulating Smad7. It has been reported that TGF- β 1 signaling is involved in cell proliferation and tumorigenesis (21). We wondered whether HBV could promote tumorigen-

FIG 5 HBV inhibits TGF- β signaling by downregulating miR-15a. (A) HepG2 cells were transfected with pHBV1.3, pHBV1.3-mut, or pcDNA3.1 as the control, together with the TGF- β luciferase reporter p3TP-luc and renilla luciferase plasmid pRL-TK. The cell lysates were harvested for firefly luciferase and renilla luciferase activity assays. The relative luciferase activity was quantified. (B) HepG2 cells were transfected with pHBV1.3 or pcDNA3.1 as the control, with or without the miR-15a mimic, for 24 h and then treated with TGF- β 1 for 24 h. The total cell lysate and nuclear fraction were subjected to immunoblotting with the indicated antibodies. (C) HepG2 cells were transfected with pHBV1.3 or pHBV1.3-mut for 24 h and then treated with TGF- β 1 for 24 h. Immunoblotting was performed as indicated. (D) HepG2 cells were transfected with pHBV1.3 with or without the miR-15a mimic for 24 h and then treated with TGF- β 1 for 24 h. The cell lysates were subjected to immunoblotting. (E) HepG2 cells were transfected with pHBV1.3 and Myc-Smad2 for 24 h and then treated with TGF- β 1 for 24 h. Immunoblotting was performed as described above. The relative amount of Smad2 in the nucleus was quantified. **, $P < 0.01$.

esis through inhibiting TGF- β signaling. To address this question, we first measured the cell viabilities of HepG2 and HepG2-4D14 cells treated with TGF- β 1 for the indicated times. As shown in Fig. 7A, the viability of HepG2-4D14 cells is better than that of HepG2 cells after TGF- β 1 treatment. We then injected HepG2, HepG2-C5, and HepG2-4D14 cells subcutaneously into nude mice and measured the size of tumors at different time points. The data showed that HepG2-C5 and HepG2-4D14 cells formed larger tumors in nude mice than did HepG2 cells (Fig. 7B). We dissected the tumors from each group and measured the levels of miR-15a and Smad7. As shown in Fig. 7C, the tumors generated from HepG2-4D14 cells expressed lower levels of miR-15a (Fig. 7C, left) and higher levels of Smad7 mRNA and protein (middle and right) than did the tumors generated from HepG2 cells. In order to confirm the function of Smad7 in tumorigenesis of HBV-positive cells, we generated the Smad7 knockdown cell lines shSmad7#1 and shSmad7#2, which were derived from HepG2-4D14 cells. The efficiency of Smad7 knockdown in the above-described cell lines was determined by immunoblotting (Fig. 7D, bottom). The data from the cell viability assays showed that the viability in Smad7

knockdown cells was reduced compared to that in control cells treated with TGF- β (Fig. 7D, top). The results from tumor formation assays with nude mice showed that the tumors generated from shSmad7#1 and shSmad7#2 cells were obviously smaller than those generated from control cells (Fig. 7E), which suggested that Smad7 facilitated the tumorigenesis of HepG2-4D14 cells. In conclusion, we revealed that HBV can inhibit apoptosis and promote tumorigenesis through inhibiting TGF- β signaling by up-regulating Smad7.

DISCUSSION

HCC is among the most frequent forms of cancer, and its incidence is increasing worldwide because of hepatitis B and C virus infection (22). HBV infection accounts for more than 60% of HCC patients in developing countries and as much as 80% of all HCC cases in China (23, 24). However, there are still some unanswered questions regarding the mechanism of HBV infection causing HCC. In this study, we proposed that HBV mRNAs act as “ceRNAs” (competing endogenous RNAs) for miR-15a to regu-

FIG 6 HBV makes HepG2 cells resistant to TGF-β-induced apoptosis. (A) HepG2 cells were treated with or without TGF-β1 for 48 h. The cell lysates were harvested for immunoblotting with PARP-1 antibody or β-actin as an internal control. (B) HepG2, HepG2-C5, and HepG2-4D14 cells were treated with or without TGF-β1 for 48 h. The cell lysates were harvested for immunoblotting with the indicated antibodies. (C) HepG2 cells were transfected with pHBV1.3 or pHBV1.3-mut for 24 h and then treated with TGF-β1 for 24 h. The cell lysates were harvested for immunoblotting with the indicated antibodies. (D) HepG2 cells were transfected with pHBV1.3 or pHBV1.3-mut for 24 h and then treated with TGF-β1 for 24 h. The cell lysates were harvested for immunoblotting with the indicated antibodies. (E) HepG2 (left) and L-02 (right) cells were transfected with pHBV1.3 or pcDNA3.1 for 24 h and then treated with TGF-β1 for 24 h. The cell lysates were harvested and subjected to a caspase 3/7 activity assay. (F) HepG2 cells were transfected with pHBV1.3, pHBV1.3-mut, or pcDNA3.1 as a control for 24 h and then treated with TGF-β1 for 48 h. (Left) The cells were subjected to annexin V and PI staining for flow cytometry analysis. (Right) The percentage of apoptotic cell was calculated. *, $P < 0.05$; **, $P < 0.01$.

late TGF-β signaling, which contributes to the development of HBV-related HCC. Recently, ceRNAs have been characterized by their ability to compete for miRNA binding and alleviate the repressive effect of miRNAs on their mRNA targets (25). More and more ceRNAs have been identified and found to participate in miRNA-dependent cross talk and produce robust networks (26–30). Deregulation of ceRNAs may lead to cancer (31). For example, Hmga2 can

FIG 7 HBV promotes tumorigenesis through inhibiting TGF- β signaling by upregulating Smad7. (A) HepG2 and HepG2-4D14 cells were treated with TGF- β 1 for 12 h, 24 h, and 48 h. Cell viability was detected by a CCK-8 assay. (B and C) HepG2, HepG2-C5, and HepG2-4D14 cells were injected subcutaneously into BALB/c nude mice (5 mice/group). (B, left) The size of tumors was measured at the indicated times after injection. (Right) At day 30, the tumors were dissected and photographed. (C, left) The RNAs of tumors generated from HepG2 and HepG2-4D14 cells were subjected to real-time PCR for miR-15a and Smad7 mRNA. (Right) Immunofluorescence analysis of tumor tissues was performed with Smad7 antibody or rabbit IgG as a negative control. (D) Smad7 knockdown HepG2-4D14 cells (HepG2-4D14 shSmad7#1 [shSmad7#1 in short] and HepG2-4D14 shSmad7#2 [shSmad7#2 in short]) or control (HepG2-4D14 sh-NC [sh-NC in short]) cells were generated as described in Materials and Methods. The cells were treated with TGF- β 1 for the indicated times. (Top) Cell viability was detected by a CCK-8 assay. (Bottom) The protein level of Smad7 in cells was checked by immunoblotting. (E) HepG2-4D14 sh-NC, HepG2-4D14 shSmad7#1, or HepG2-4D14 shSmad7#2 cells were injected subcutaneously into BALB/c nude mice. (Top) The size of tumors was measured at the indicated times after injection. The results are presented as means $\pm$ standard deviations from 5 mice for each group. (Bottom) The tumors were dissected at day 30 and photographed.

operate as a ceRNA for the miRNA let-7 to promote lung cancer progression (32). Based on the mechanism of ceRNAs competing for miRNA binding, we proposed that HBV mRNAs may act as “ceRNAs” of miR-15a to regulate TGF- β signaling. It has been suggested HBV mRNAs acting as ceRNAs may represent the viral strategy of HBV to exploit its compact genome for the development of HBV-related HCC. In a previous study, we showed that HBV mRNAs (nt 1368 to 1383) can function as miR-15a sponges and increase the expression level of Bcl-2, which is one of the miR-15a targets. Here, we identified that Smad7 is a novel target of miR-15a. HBV mRNAs reduced the miR-15a expression level and increased the level of Smad7 to downregulate TGF- β signaling. TGF- β is known to be a multifunctional factor that plays a critical role in various aspects of liver pathogenesis, including chronic HBV/hepatitis C virus (HCV) infection, cirrhosis, and tumorigenesis (33, 34). TGF- β signaling could have cytostatic effects during liver damage as well as carcinogenesis. In cultured hepatocytes, TGF- β can trigger apoptosis and an epithelial-mesenchymal transition (EMT) via the induction of reactive oxygen species (ROS) and the proapoptotic protein BIM (15, 35). We found that HBV can inhibit TGF- β signaling through upregulating Smad7. This may explain why the HBV transgenic cell line HepG2-4D14 is less sensitive to TGF- β -induced apoptosis.

HBV has been found to downregulate other miRNAs to control various signaling pathways. For example, HBV mRNA can reduce the level of miR-122 by direct absorption to promote hepatocellular carcinoma tumor growth (11), and the HBx protein modulates the tumor suppressor miR-520b, accelerates hepatocarcinogenesis, and represses miR-148a to inhibit the AKT/ERK/FOXO4/ATF5 pathway (9, 36). It has been reported that miR-15a plays important roles in apoptosis, cell proliferation, and tumorigenesis by targeting different mRNAs in various cancers (13, 37, 38). In this study, we identified that HBV can inhibit TGF- β signaling by downregulating miR-15a and upregulating Smad7. It is worth further investigating whether there is any cross talk between the HBV/miR15a/Smad7-regulated TGF- β pathway and other pathways, such as the HBV/miR-122/PBF or HBx/p53 signaling pathway, to promote cell proliferation and tumorigenesis. In addition, we confirmed that levels of other miRNAs, such as miR-422a, miR-210, and miR-7-5p, were also reduced, as reported previously for HBV transgenic cells (39). However, the correlation of HBV infection with the expression of these miRNAs as well as its biological significance need to be further studied.

In conclusion, we demonstrate that HBV mRNAs act as ceRNAs of miR-15a and regulate TGF- β signaling in the development of HBV-related HCC. Our study provides evidence to support the idea that viral RNAs can exert their functions as regulatory factors toward miRNA. We propose that there may be more viral RNAs serving as ceRNAs involved in various aspects of cellular processes. However, this needs further investigation.

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REFERENCES

- Neuveut C, Wei Y, Buendia MA. 2010. Mechanisms of HBV-related hepatocarcinogenesis. *J Hepatol* 52:594–604. <http://dx.doi.org/10.1016/j.jhep.2009.10.033>.
- Whittaker S, Marais R, Zhu AX. 2010. The role of signaling pathways in the development and treatment of hepatocellular carcinoma. *Oncogene* 29:4989–5005. <http://dx.doi.org/10.1038/onc.2010.236>.
- Williams R. 2006. Global challenges in liver disease. *Hepatology* 44:521–526. <http://dx.doi.org/10.1002/hep.21347>.
- You H, Ding W, Rountree CB. 2010. Epigenetic regulation of cancer stem cell marker CD133 by transforming growth factor-beta. *Hepatology* 51:1635–1644. <http://dx.doi.org/10.1002/hep.23544>.
- Protzer U, Maini MK, Knolle PA. 2012. Living in the liver: hepatic infections. *Nat Rev Immunol* 12:201–213. <http://dx.doi.org/10.1038/nri3169>.
- Arzberger S, Hosel M, Protzer U. 2010. Apoptosis of hepatitis B virus-infected hepatocytes prevents release of infectious virus. *J Virol* 84:11994–12001. <http://dx.doi.org/10.1128/JVI.00653-10>.
- Du J, Liang X, Liu Y, Qu Z, Gao L, Han L, Liu S, Cui M, Shi Y, Zhang Z, Yu L, Cao L, Ma C, Zhang L, Chen Y, Sun W. 2009. Hepatitis B virus core protein inhibits TRAIL-induced apoptosis of hepatocytes by blocking DR5 expression. *Cell Death Differ* 16:219–229. <http://dx.doi.org/10.1038/cdd.2008.144>.
- Hu L, Chen L, Yang G, Li L, Sun H, Chang Y, Tu Q, Wu M, Wang H. 2011. HBx sensitizes cells to oxidative stress-induced apoptosis by accelerating the loss of Mcl-1 protein via caspase-3 cascade. *Mol Cancer* 10:43. <http://dx.doi.org/10.1186/1476-4598-10-43>.
- Xu X, Fan Z, Kang L, Han J, Jiang C, Zheng X, Zhu Z, Jiao H, Lin J, Jiang K, Ding L, Zhang H, Cheng L, Fu H, Song Y, Jiang Y, Liu J, Wang R, Du N, Ye Q. 2013. Hepatitis B virus X protein represses miRNA-148a to enhance tumorigenesis. *J Clin Invest* 123:630–645. <http://dx.doi.org/10.1172/JCI64265>.
- Wei X, Xiang T, Ren G, Tan C, Liu R, Xu X, Wu Z. 2013. MiR-101 is down-regulated by the hepatitis B virus x protein and induces aberrant DNA methylation by targeting DNA methyltransferase 3A. *Cell Signal* 25:439–446. <http://dx.doi.org/10.1016/j.cellsig.2012.10.013>.
- Li C, Wang Y, Wang S, Wu B, Hao J, Fan H, Ju Y, Ding Y, Chen L, Chu X, Liu W, Ye X, Meng S. 2013. Hepatitis B virus mRNA-mediated miR-122 inhibition upregulates PTTG1-binding protein, which promotes hepatocellular carcinoma tumor growth and cell invasion. *J Virol* 87:2193–2205. <http://dx.doi.org/10.1128/JVI.02831-12>.
- Liu N, Zhang J, Jiao T, Li Z, Peng J, Cui Z, Ye X. 2013. Hepatitis B virus inhibits apoptosis of hepatoma cells by sponging the microRNA 15a/16 cluster. *J Virol* 87:13370–13378. <http://dx.doi.org/10.1128/JVI.02130-13>.
- Bonci D, Coppola V, Musumeci M, Addario A, Giuffrida R, Memeo L, D'Urso L, Pagliuca A, Biffoni M, Labbaye C, Bartucci M, Muto G, Peschle C, De Maria R. 2008. The miR-15a-miR-16-1 cluster controls prostate cancer by targeting multiple oncogenic activities. *Nat Med* 14:1271–1277. <http://dx.doi.org/10.1038/nm.1880>.
- Halder SK, Beauchamp RD, Datta PK. 2005. Smad7 induces tumorigenicity by blocking TGF-beta-induced growth inhibition and apoptosis. *Exp Cell Res* 307:231–246. <http://dx.doi.org/10.1016/j.yexcr.2005.03.009>.
- Yamamura Y, Hua X, Bergelson S, Lodish HF. 2000. Critical role of Smads and AP-1 complex in transforming growth factor-beta-dependent apoptosis. *J Biol Chem* 275:36295–36302. <http://dx.doi.org/10.1074/jbc.M006023200>.
- Hayashi H, Abdollah S, Qiu Y, Cai J, Xu YY, Grinnell BW, Richardson MA, Topper JN, Gimbrone MA, Jr, Wrana JL, Falb D. 1997. The MAD-related protein Smad7 associates with the TGFbeta receptor and functions as an antagonist of TGFbeta signaling. *Cell* 89:1165–1173. [http://dx.doi.org/10.1016/S0092-8674\(00\)80303-7](http://dx.doi.org/10.1016/S0092-8674(00)80303-7).
- Giannelli G, Mazzocca A, Fransvea E, Lahn M, Antonaci S. 2011. Inhibiting TGF-beta signaling in hepatocellular carcinoma. *Biochim Biophys Acta* 1815:214–223. <http://dx.doi.org/10.1016/j.bbcan.2010.11.004>.
- Jakowlew SB. 2006. Transforming growth factor-beta in cancer and metastasis. *Cancer Metastasis Rev* 25:435–457. <http://dx.doi.org/10.1007/s10555-006-9006-2>.
- Heldin CH, Landstrom M, Moustakas A. 2009. Mechanism of TGF-beta signaling to growth arrest, apoptosis, and epithelial-mesenchymal transition. *Curr Opin Cell Biol* 21:166–176. <http://dx.doi.org/10.1016/j.ccb.2009.01.021>.
- Wrana JL, Attisano L, Carcamo J, Zentella A, Doody J, Laiho M, Wang XF, Massague J. 1992. TGF beta signals through a heteromeric protein

- kinase receptor complex. *Cell* 71:1003–1014. [http://dx.doi.org/10.1016/0092-8674\(92\)90395-S](http://dx.doi.org/10.1016/0092-8674(92)90395-S).
21. Fischer AN, Fuchs E, Mikula M, Huber H, Beug H, Mikulits W. 2007. PDGF essentially links TGF- β signaling to nuclear beta-catenin accumulation in hepatocellular carcinoma progression. *Oncogene* 26:3395–3405. <http://dx.doi.org/10.1038/sj.onc.1210121>.
 22. Llovet JM, Burroughs A, Bruix J. 2003. Hepatocellular carcinoma. *Lancet* 362:1907–1917. [http://dx.doi.org/10.1016/S0140-6736\(03\)14964-1](http://dx.doi.org/10.1016/S0140-6736(03)14964-1).
 23. Jemal A, Bray F, Center MM, Ferlay J, Ward E, Forman D. 2011. Global cancer statistics. *CA Cancer J Clin* 61:69–90. <http://dx.doi.org/10.3322/caac.20107>.
 24. Yang P, Li QJ, Feng Y, Zhang Y, Markowitz GJ, Ning S, Deng Y, Zhao J, Jiang S, Yuan Y, Wang HY, Cheng SQ, Xie D, Wang XF. 2012. TGF- β -miR-34a-CCL22 signaling-induced Treg cell recruitment promotes venous metastases of HBV-positive hepatocellular carcinoma. *Cancer Cell* 22:291–303. <http://dx.doi.org/10.1016/j.ccr.2012.07.023>.
 25. Cesana M, Daley GQ. 2013. Deciphering the rules of ceRNA networks. *Proc Natl Acad Sci U S A* 110:7112–7113. <http://dx.doi.org/10.1073/pnas.1305322110>.
 26. Poliseno L, Salmena L, Zhang J, Carver B, Haveman WJ, Pandolfi PP. 2010. A coding-independent function of gene and pseudogene mRNAs regulates tumour biology. *Nature* 465:1033–1038. <http://dx.doi.org/10.1038/nature09144>.
 27. Cesana M, Cacchiarelli D, Legnini I, Santini T, Sthandier O, Chinappi M, Tramontano A, Bozzoni I. 2011. A long noncoding RNA controls muscle differentiation by functioning as a competing endogenous RNA. *Cell* 147:358–369. <http://dx.doi.org/10.1016/j.cell.2011.09.028>.
 28. Franco-Zorrilla JM, Valli A, Todesco M, Mateos I, Puga MI, Rubio-Somoza I, Leyva A, Weigel D, Garcia JA, Paz-Ares J. 2007. Target mimicry provides a new mechanism for regulation of microRNA activity. *Nat Genet* 39:1033–1037. <http://dx.doi.org/10.1038/ng2079>.
 29. Hansen TB, Jensen TI, Clausen BH, Bramsen JB, Finsen B, Damgaard CK, Kjems J. 2013. Natural RNA circles function as efficient microRNA sponges. *Nature* 495:384–388. <http://dx.doi.org/10.1038/nature11993>.
 30. Memczak S, Jens M, Elefsinioti A, Torti F, Krueger J, Rybak A, Maier L, Mackowiak SD, Gregersen LH, Munschauer M, Loewer A, Ziebold U, Landthaler M, Kocks C, Le Noble F, Rajewsky N. 2013. Circular RNAs are a large class of animal RNAs with regulatory potency. *Nature* 495:333–338. <http://dx.doi.org/10.1038/nature11928>.
 31. De Giorgio A, Krell J, Harding V, Stebbing J, Castellano L. 2013. Emerging roles of competing endogenous RNAs in cancer: insights from the regulation of PTEN. *Mol Cell Biol* 33:3976–3982. <http://dx.doi.org/10.1128/MCB.00683-13>.
 32. Kumar MS, Armenteros-Monterroso E, East P, Chakravorty P, Matthews N, Winslow MM, Downward J. 2014. HMGA2 functions as a competing endogenous RNA to promote lung cancer progression. *Nature* 505:212–217. <http://dx.doi.org/10.1038/nature12785>.
 33. Marotta F, Vangieri B, Cecere A, Gattoni A. 2004. The pathogenesis of hepatocellular carcinoma is multifactorial event. Novel immunological treatment in prospect. *Clin Ter* 155:187–199.
 34. Massague J. 2008. TGF β in cancer. *Cell* 134:215–230. <http://dx.doi.org/10.1016/j.cell.2008.07.001>.
 35. Ramesh S, Qi XJ, Wildey GM, Robinson J, Molkentin J, Letterio J, Howe PH. 2008. TGF β -mediated BIM expression and apoptosis are regulated through SMAD3-dependent expression of the MAPK phosphatase MKP2. *EMBO Rep* 9:990–997. <http://dx.doi.org/10.1038/embor.2008.158>.
 36. Zhang W, Lu Z, Kong G, Gao Y, Wang T, Wang Q, Cai N, Wang H, Liu F, Ye L, Zhang X. 2014. Hepatitis B virus X protein accelerates hepatocarcinogenesis with partner survivin through modulating miR-520b and HBXIP. *Mol Cancer* 13:128. <http://dx.doi.org/10.1186/1476-4598-13-128>.
 37. Xin C, Buhe B, Hongting L, Chuanmin Y, Xiwei H, Hong Z, Lulu H, Qian D, Renjie W. 2013. MicroRNA-15a promotes neuroblastoma migration by targeting reversion-inducing cysteine-rich protein with Kazal motifs (RECK) and regulating matrix metalloproteinase-9 expression. *FEBS J* 280:855–866. <http://dx.doi.org/10.1111/febs.12074>.
 38. Ofir M, Hacohen D, Ginsberg D. 2011. MiR-15 and miR-16 are direct transcriptional targets of E2F1 that limit E2F-induced proliferation by targeting cyclin E. *Mol Cancer Res* 9:440–447. <http://dx.doi.org/10.1158/1541-7786.MCR-10-0344>.
 39. Liu Y, Zhao JJ, Wang CM, Li MY, Han P, Wang L, Cheng YQ, Zoulim F, Ma X, Xu DP. 2009. Altered expression profiles of microRNAs in a stable hepatitis B virus-expressing cell line. *Chin Med J* 122:10–14.