

The inhibition of microRNA-15a suppresses hepatitis B virus-associated liver cancer cell growth through the Smad/TGF- β pathway

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Abstract. In the present study, the role of microRNA-15a (miR-15a) was investigated in hepatitis B virus (HBV)-associated liver cancer. The results revealed that the expression levels of miR-15a were increased in HBV-associated liver cancer tissues compared with the levels in normal tumor-adjacent tissues. Moreover, Smad-7 protein expression in patients with HBV-associated liver cancer was higher than that in normal tumor-adjacent tissues. In addition, miR-15a expression and Smad-7 protein expression were increased in HepG2 hepatocellular carcinoma cells compared with that noted in L-02 normal hepatocytes. In HepG2 cells, miR-15a inhibition suppressed cell proliferation and increased Smad-7 protein expression. The inhibition of miR-15a was also demonstrated to decrease transforming growth factor (TGF)- β 1 protein expression and Smad-2, p-Smad-2 and Smad-4 expression levels in HepG2 cells. Furthermore, FSP1 protein expression and caspase-3/-7 activities were enhanced by miR-15a inhibition in HepG2 cells compared with the control group. Treatment with recombinant TGF- β 1 was demonstrated to activate Smad-2/-4 and FSP1 protein expression and increase caspase-3/-7 activity in HepG2 cells. Collectively, these findings demonstrate that the miR-15a/Smad-7/TGF- β pathway is important in HBV-associated liver cancer.

Introduction

Hepatitis B virus (HBV) infection is closely associated with liver cirrhosis and hepatocellular carcinoma (HCC) occurrence and development, and may lead to acute and chronic hepatitis (1). Worldwide, ~2 billion people are infected with HBV. China has a particularly high incidence of hepatitis B, accounting for ~93 million of the 350 million global HBV

carriers (2). Approximately 25 million patients develop chronic hepatitis B (3). Annually, ~1 million people die of hepatic failure, liver cirrhosis or primary HCC caused by HBV infection (4).

Primary HCC is one of the most common types of cancer in China. Approximately 90% of primary liver cancer cases are HCC and 5% are cholangiocarcinoma (cancer of the bile duct cells) (5). However, mixed liver cancer is extremely rare. The etiology and pathogenesis of the disease has not yet been fully determined (6). In China, ~90% of liver cancer patients have a background of HBV infection. Primary HCC has a high mortality rate (7). The number patients who die from liver cancer exceeds 110,000 annually (8). Liver cancer ranks as the third most common cause of cancer-associated mortality, after gastric cancer and esophageal cancer.

MicroRNAs (miRNAs) are non-protein coding gene regulators that have important regulatory effects on gene expression in eukaryotes (9). By complementary pairing in the 3' untranslated region of the protein-coding mRNA, the seed sequence of miRNAs alters the expression of the target gene; expression is primarily regulated via degradation of the target mRNA or via inhibition of its translation (10). Research has shown that miRNAs are important contributors to tumor development, migration and propagation, and may act as oncogenes or tumor suppressor genes (11). In addition, recent studies demonstrated that miRNAs play key roles in the occurrence and development of liver cancer; miRNAs can act to inhibit the gene expression of numerous important coding proteins in this process (12).

There are 8 members of the Smad protein family in mammals, and Smads play an important role in transforming growth factor (TGF)- β signaling (13). The primary function of the Smad proteins is to transduce TGF- β -induced signals within cells (14). Smad proteins, which are the direct substrates of TGF- β receptors (T β Rs), transfer the TGF- β signal from the cytoplasm to the nucleus, and regulate corresponding target gene transcription along with other transcription factors. Recent research revealed that the TGF- β /Smad pathway plays an important role in the development of liver cancer (15). In the present study, the association between microRNA-15a (miR-15a)/Smad-7/TGF- β signaling and HBV-associated liver cancer was analyzed, and the effects of miR-15a/Smad-7/TGF- β on cell proliferation and apoptosis in HBV-associated liver cancer were evaluated.

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Materials and methods

Patient samples. A total of 16 patients with HBV-associated HCC treated at the Binzhou Tuberculosis Prevention and Control Hospital (Shandong, China) were included in the present study. HCC and adjacent liver tissue samples were obtained from these patients following the acquisition of informed consent.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis. Total RNA was extracted from tissues or cells using Invitrogen TRIzol reagent (Thermo Fisher Scientific, Inc., Waltham, MA, USA) according to the manufacturer's instructions. Total RNA (2 µg) was used to synthesize cDNA using an All-in-One™ miRNA First-Strand cDNA Synthesis kit (GeneCopoeia, Inc., Rockville, MD, USA). qPCR was performed on an Applied Biosystems 7500 Real-Time PCR System (Thermo Fisher Scientific, Inc.) using the following conditions: 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec, 60°C for 30 sec, and 72°C for 40 sec. Relative expression was quantified by using the comparative quantification cycle ($2^{-\Delta\Delta C_q}$) method.

Western blot analysis. Tissue samples were incubated with 100 µl of lysis buffer, and cell lines were lysed with 100 ml of pre-cooled cell lysis buffer (both from Beyotime Institute of Biotechnology, Shanghai, China) for 30 min on ice. The protein concentration was determined by the bicinchoninic acid method (Beyotime Institute of Biotechnology) and 50 µg of total protein from each sample was separated by SDS-PAGE prior to transfer onto polyvinylidene difluoride membranes (EMD Millipore, Billerica, MA, USA). Membranes were blocked with 5% skim milk in TBS-Tween (20 mM Tris-HCl, 150 mM NaCl and 0.05% Tween-20), followed by incubation with the following mouse anti-human primary antibodies from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA): anti-Smad-7 (dilution, 1:4,000), anti-TGF-β1 (dilution, 1:2,000), anti-Smad-2 (dilution, 1:3,000), anti-phosphorylated (p)-Smad-2 (dilution, 1:3,000), anti-Smad-4 (dilution, 1:4,000), anti-fibroblast-specific protein 1 (FSP1) (dilution, 1:2,000) and anti-β-actin (dilution, 1:5,000). Membranes were then incubated with goat anti-mouse secondary antibodies conjugated to horseradish peroxidase (dilution, 1:5,000; Santa Cruz Biotechnology, Inc.) and visualized by enhanced chemiluminescence (Amersham ECL Prime Western Blotting Detection Reagent; GE Healthcare Life Sciences, Piscataway, NJ, USA).

Cell culture and transfection. HepG2 and SMMC-7721 human HCC cell lines were integrated with the HBV genome. L-02, HepG2 and SMMC-7721 cells were cultured in Gibco RPMI-1640 medium (Thermo Fisher Scientific, Inc.) containing 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.), 100 U/ml penicillin and 100 µg/ml streptomycin, at 37°C in an atmosphere of 5% CO₂. Anti-miR-15a plasmid (50 nM) or a negative control plasmid (50 nM) were transfected into cells using Invitrogen Lipofectamine 2000 reagent (Thermo Fisher Scientific, Inc.).

Cell viability analysis. HepG2 cells were seeded into 96-well plates and incubated for the indicated times. Following

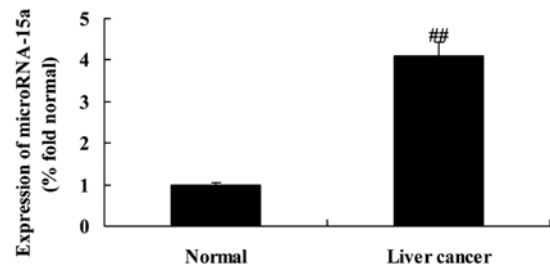


Figure 1. Expression of miR-15a in patients with HBV-associated liver cancer; ^{##}P<0.01 vs. the normal group. miR-15a, microRNA-15a; HBV, hepatitis B virus.

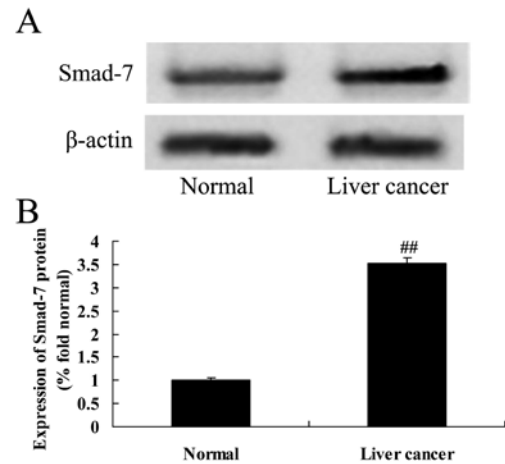


Figure 2. Expression of Smad-7 protein in HBV-associated liver cancer. (A) Expression of Smad-7 protein detected using western blotting and (B) statistical analyses of Smad-7 protein expression in patient samples of HBV-associated liver cancer; ^{##}P<0.01 vs. the normal group. HBV, hepatitis B virus.

incubation, 50 µl of MTT (0.5 mg/ml; Invitrogen; Thermo Fisher Scientific, Inc.) was added, and the plates were incubated at 37°C for 2 h. Subsequently, 100 µl of dimethyl sulfoxide (Invitrogen; Thermo Fisher Scientific, Inc.) was added to each well. Absorbance at 550 nm was assessed using a luminometer plate-reader.

Caspase-3/-7 activity. HepG2 cells were seeded into 96-well plates and incubated for the indicated times. The cell lysates were harvested and analyzed using a Caspase-3/-7 Assay kit (Promega Corporation, Madison, WI, USA). Caspase-3/-7 activity was assessed with a luminometer plate-reader at a wavelength of 490 nm.

Statistical analysis. Differences between groups were determined using Student's t-test. All data are presented as the mean ± standard deviation. P<0.05 was considered to indicate a statistically significant difference.

Results

Expression of miR-15a in HBV-induced liver cancer tissues. Our present study demonstrated that miR-15a expression in normal tissue samples (adjacent liver tissue samples) was lower than that in HBV-induced HCC tissue samples (Fig. 1).

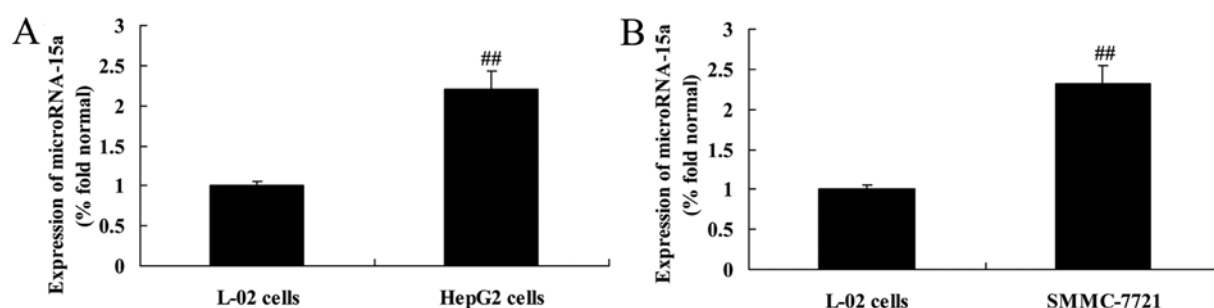


Figure 3. Expression of miR-15a in HCC and normal hepatic cell lines. Expression of miR-15a in (A) HepG2 and (B) SMMC-7721 HCC cells when compared to L-02 normal hepatocytes; ^{##}P<0.01 vs. the normal group. miR-15a, microRNA-15a; HCC, hepatocellular carcinoma.

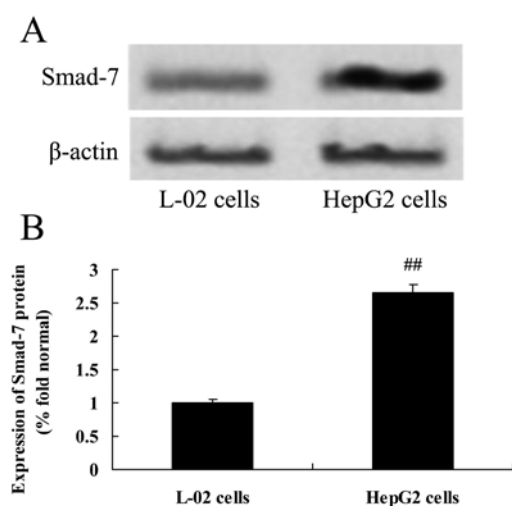


Figure 4. Expression of Smad-7 protein in HepG2 and L-02 cells. (A) Expression of Smad-7 protein detected using western blotting and (B) statistical analyses of Smad-7 protein expression in HepG2 HCC cells and L-02 normal hepatocytes; ^{##}P<0.01 vs. the normal group. HCC, hepatocellular carcinoma.

Expression of Smad-7 protein in HBV-induced liver cancer tissues. The expression of Smad-7 protein was analyzed to determine the importance of the HBV/miR-15a/Smad-7 pathway. As demonstrated in Fig. 2, the protein expression of Smad-7 in adjacent normal liver tissue samples was lower than in HBV-induced HCC tissue samples.

Expression of miR-15a in HepG2, SMMC-7721 and L-02 cells. RT-qPCR was used to analyze the expression levels of miR-15a in HepG2, SMMC-7721 and L-02 cells. The expression levels of miR-15a in HBV-infected HepG2 and SMMC-7721 cells were higher than that in the L-02 cells (Fig. 3).

Expression of Smad-7 protein in HepG2 cells. Expression of Smad-7 protein in HepG2 cells was examined using western blotting. The results indicated that, compared with the control group (L-02 cells), HBV infection was associated with significantly increased expression of Smad-7 protein in HepG2 cells (Fig. 4).

Effects of miR-15a on the viability of HepG2 cells. To further understand the effects of miR-15a on the viability of HepG2 cells, the cells were transfected with anti-miR-15a

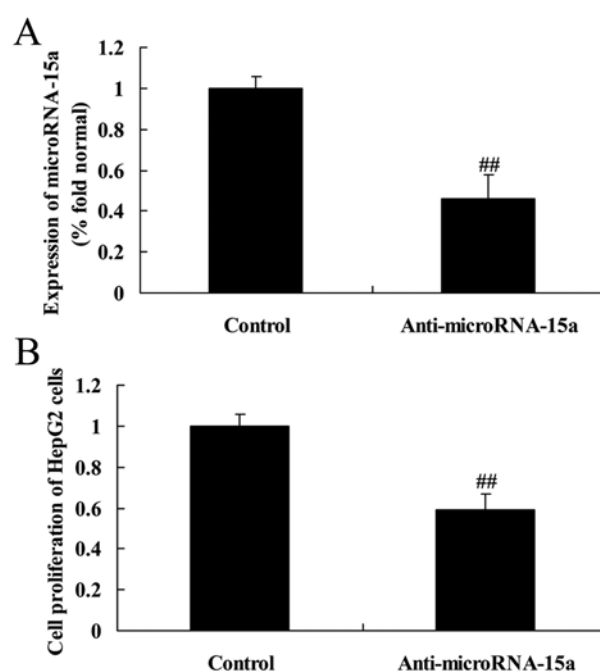


Figure 5. Effects of miR-15a on cell proliferation of HepG2 HCC cells. The effects of (A) microRNA-15a on cell proliferation of (B) HepG2 cells; ^{##}P<0.01 vs. the normal group. miR-15a, microRNA-15a; HCC, hepatocellular carcinoma.

or negative control plasmids, and the viability of HepG2 cells was examined by MTT assay. The results revealed that anti-miR-15a significantly decreased miR-15a expression compared with the control group (cells transfected with negative control plasmids) and was associated with decreased viability in HepG2 cells (Fig. 5).

Effects of miR-15a on the expression of Smad-7 protein in HepG2 cells. miR-15a was selected for further study since it is an important regulator of Smad-7 signaling. As demonstrated in Fig. 6, compared with control group (cells transfected with negative control plasmids), anti-miR-15a markedly upregulated the protein expression of Smad-7 in HBV-infected HepG2 cells (Fig. 6).

Effects of miR-15a on expression of TGF-β1 protein in HepG2 cells. Subsequently, whether TGF-β1 is a potential target of miR-15a was investigated using western blotting.

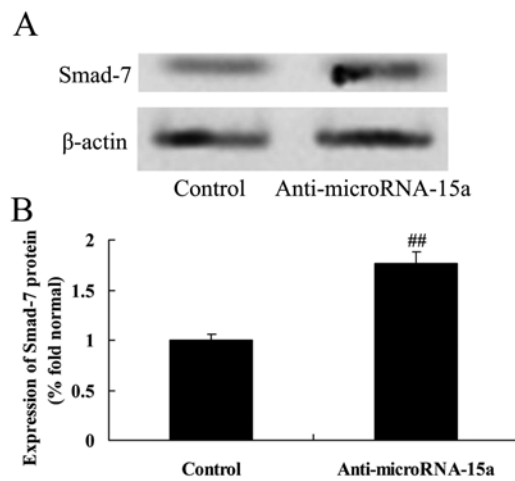


Figure 6. Effects of miR-15a on the expression of Smad-7 protein in HepG2 HCC cells. (A) Effects of miR-15a on the expression of Smad-7 protein detected using western blotting and (B) statistical analyses of Smad-7 protein expression in HepG2 cells; ^{##}P<0.01 vs. the normal group. miR-15a, microRNA-15a; HCC, hepatocellular carcinoma.

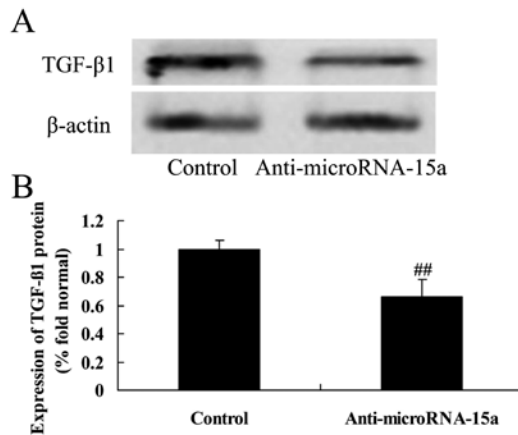


Figure 7. Effects of miR-15a on the expression of TGF-β1 protein in HepG2 HCC cells. (A) Effects of miR-15a on the expression of TGF-β1 protein detected using western blotting and (B) statistical analyses of TGF-β1 protein expression in HepG2 cells; ^{##}P<0.01 vs. the normal group. miR-15a, microRNA-15a; TGF-β1, transforming growth factor-β1; HCC, hepatocellular carcinoma.

The anti-miR-15a plasmid was transfected into HBV-infected HepG2 cells, and the results indicated that anti-miR-15a significantly suppressed the expression of TGF-β1 protein in HepG2 cells relative to the control group (cells transfected with negative control plasmids; Fig. 7).

Effects of miR-15a on the expression of Smad-2 and p-Smad-2 proteins in HepG2 cells. We confirmed whether miR-15a affected the level of Smad-2 and p-Smad-2 proteins in HepG2 cells. As demonstrated in Fig. 8, the protein expression levels of Smad-2 and p-Smad-2 in HepG2 cells transfected with anti-miR-15a was lower than that in the control group (cells transfected with negative control plasmids).

Effects of miR-15a on the expression of Smad-4 protein in HepG2 cells. The association between miR-15a and Smad-4 expression in HBV-infected HepG2 cells was examined.

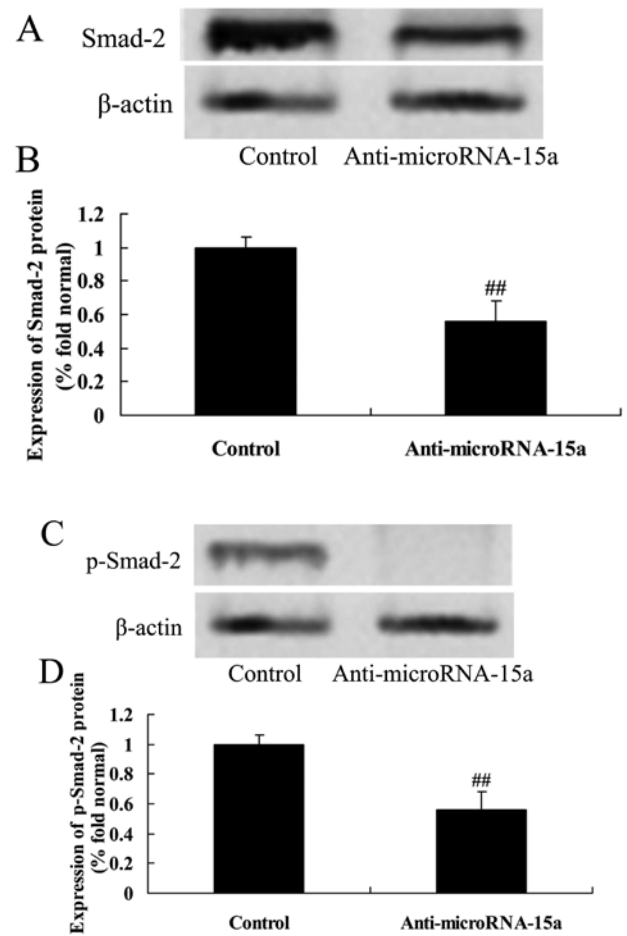


Figure 8. Effects of miR-15a on the expression of Smad-2 and p-Smad-2 protein in HepG2 HCC cells. Effects of miR-15a on the expression of (A) Smad-2 and (C) p-Smad-2 protein detected using western blotting and statistical analyses of (B) Smad-2 and (D) p-Smad-2 protein in HepG2 cells; ^{##}P<0.01 vs. the normal group. miR-15a, microRNA-15a; HCC, hepatocellular carcinoma.

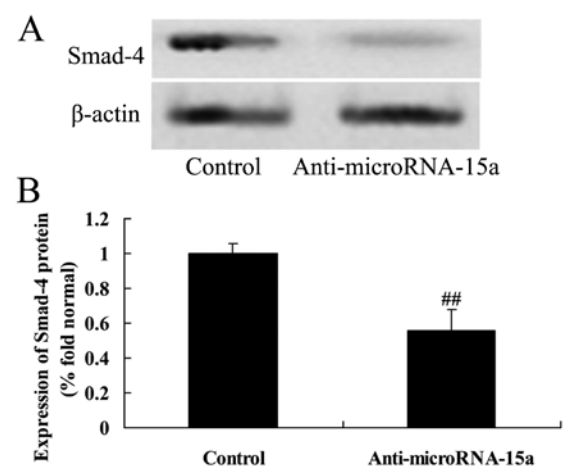


Figure 9. Effects of miR-15a on the expression of Smad-4 protein in HepG2 HCC cells. (A) Effects of miR-15a on the expression of Smad-4 protein using western blotting and (B) statistical analyses of Smad-4 protein expression in HepG2 cells; ^{##}P<0.01 vs. the normal group. miR-15a, microRNA-15a; HCC, hepatocellular carcinoma.

As demonstrated in Fig. 9, Smad-4 protein expression was markedly inhibited in HepG2 cells transfected with

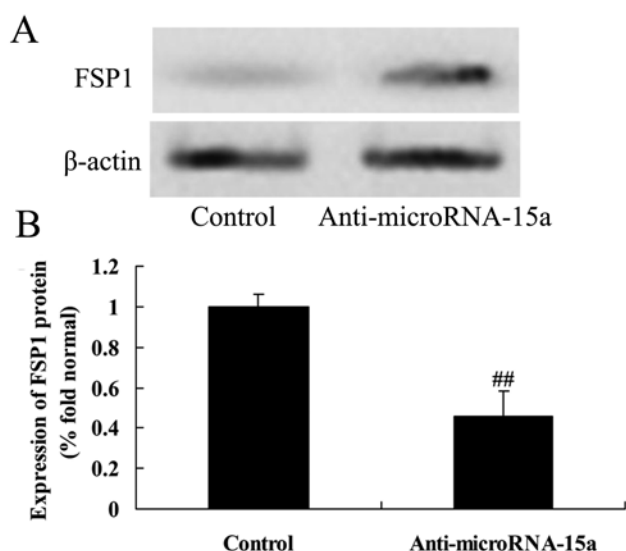


Figure 10. Effects of miR-15a on the expression of FSP1 protein in HepG2 HCC cells. (A) Effects of miR-15a on the expression of FSP1 protein detected using western blotting and (B) statistical analyses of FSP1 protein expression in HepG2 cells; ^{##}P<0.01 vs. the normal group. miR-15a, microRNA-15a; HCC, hepatocellular carcinoma.

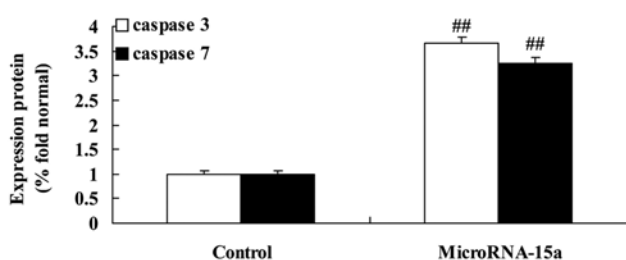


Figure 11. Effects of miR-15a on the caspase-3/-7 activity in HepG2 HCC cells. Effects of miR-15a on the caspase-3/-7 activity in HepG2 cells; ^{##}P<0.01 vs. the normal group. miR-15a, microRNA-15a; HCC, hepatocellular carcinoma.

anti-miR-15a compared with the control group (cells transfected with negative control plasmids).

Effects of miR-15a on the expression of FSP1 protein in HepG2 cells. HepG2 cells were transfected with anti-miR-15a, so, as to analyze the expression of FSP1 protein in HBV-infected HepG2 cells. The results indicated that anti-miR-15a significantly suppressed FSP1 protein expression in HBV-infected HepG2 cells compared with the control group (cells transfected with negative control plasmids; Fig. 10).

Effects of miR-15a on caspase-3/-7 activities in HepG2 cells. The association between miR-15a expression and caspase-3/-7 activities in HepG2 cells were analyzed. Consistently, the activities of caspase-3/-7 in HepG2 cells transfected with anti-miR-15a were higher than those in the control group (cells transfected with negative control plasmids), as demonstrated in Fig. 11.

Effects of TGF-β1 treatment on Smad-2/-4 and p-Smad-2 protein expression in HepG2 cells. Human recombinant TGF-β1 was purchased from BioLegend (San Diego, CA, USA). TGF-β1 (at concentrations of 0, 2, 4 or 8 ng/ml) was added into each well for 24 h of culture. As demonstrated in Fig. 12, TGF-β1 at concentrations of 4 or 8 ng/ml markedly enhanced the levels of Smad-2, p-Smad-2 and Smad-4 in HepG2 cells.

Effects of TGF-β1 treatment on FSP1 protein expression in HepG2 cells. The effects of TGF-β1 treatment on FSP1 protein expression in HepG2 cells were determined using western blotting. As demonstrated in Fig. 13, 8 ng/ml of TGF-β1 distinctly promoted FSP1 protein expression in HepG2 cells.

Effects of TGF-β1 treatment on caspase-3/-7 activities in HepG2 cells. The effect of TGF-β1 treatment on the apoptosis

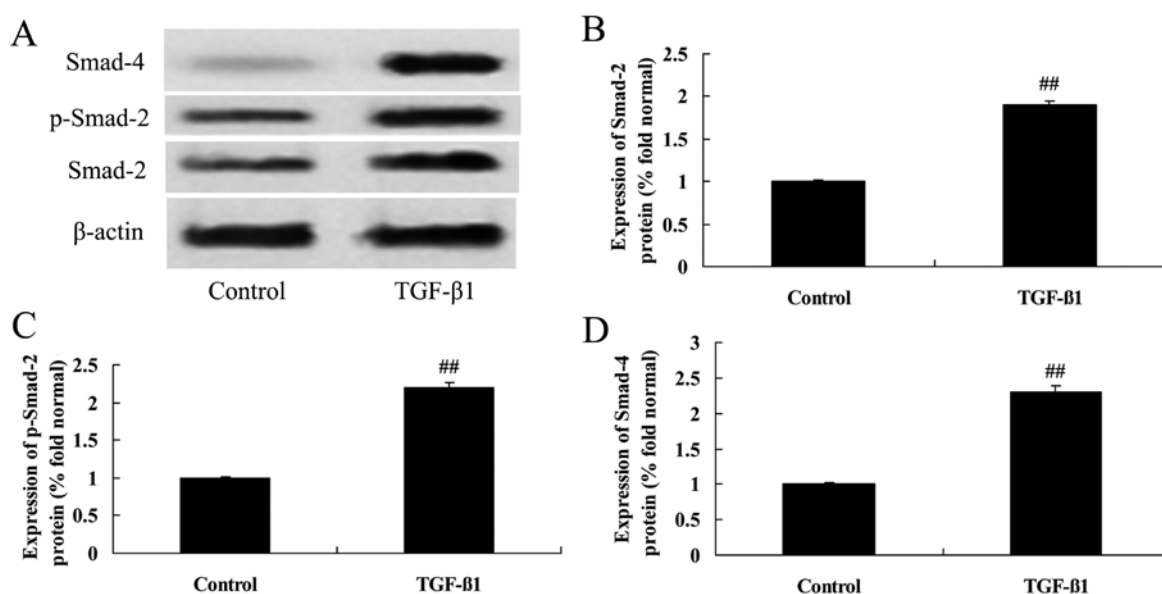


Figure 12. Effect of treatment with TGF-β1 on Smad-2/-4 protein expression in HepG2 HCC cells. (A) Effect of treatment with TGF-β1 on the expression of Smad-4, p-Smad-2 and Smad-2 proteins assessed using western blotting and (B-D) statistical analyses of Smad-2, p-Smad-2 and Smad-4 protein expression in HepG2 cells; ^{##}P<0.01 vs. the normal group. TGF-β1, transforming growth factor-β1; HCC, hepatocellular carcinoma.

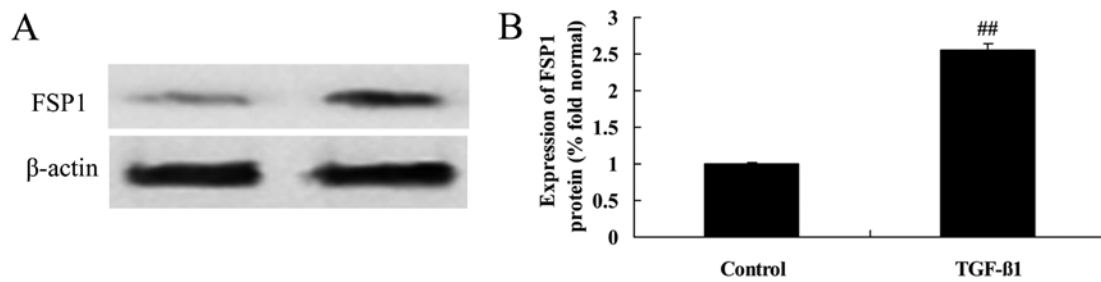


Figure 13. Effect of treatment with TGF- β 1 on FSP1 protein in HepG2 HCC cells. (A) Effect of treatment with TGF- β 1 on the expression of FSP1 protein detected using western blotting and (B) statistical analyses of FSP1 protein expression in HepG2 cells; ^{##} $P < 0.01$ vs. the normal group. TGF- β 1, transforming growth factor- β 1; HCC, hepatocellular carcinoma.

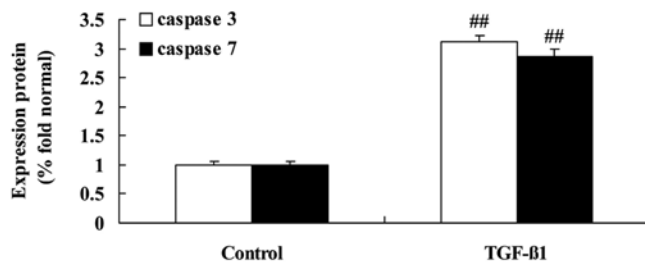


Figure 14. Effect of treatment with TGF- β 1 on caspase-3/-7 activity in HepG2 hepatocellular carcinoma cells. Effect of treatment with TGF- β 1 on the caspase-3/-7 activity in HepG2 cells; ^{##} $P < 0.01$ vs. the normal group. TGF- β 1, transforming growth factor- β 1; HCC, hepatocellular carcinoma.

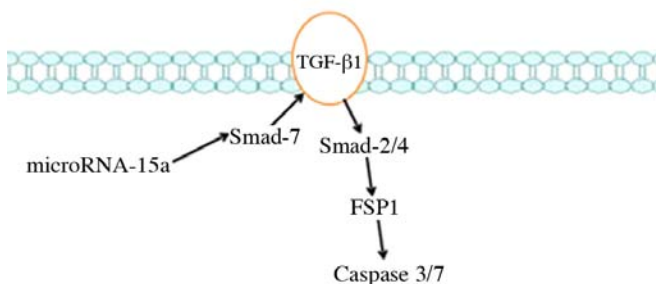


Figure 15. miR-15a/Smad-7/TGF- β 1 affects HBV-associated liver cancer. miR-15a, microRNA-15a; TGF- β 1, transforming growth factor- β 1; HBV, hepatitis B virus.

of HepG2 cells was analyzed by assessing caspase activity. As demonstrated in Fig. 14, TGF- β 1 at concentrations of 4 or 8 ng/ml activated caspase-3/-7 activities in HepG2 cells, indicating the induction of apoptosis.

Discussion

Globally, ~2 billion people are infected with HBV, and HBV infection is particularly prevalent in China (3). The gross population of HBV infection is estimated to be ~100 million (3). HBV infection leads to repeated damage and repair of liver tissues and may thus lead to chronic hepatitis B, cirrhosis and HCC (16). As a malignant tumor with extremely high rates of morbidity and mortality, HCC is an important manifestation of poor prognosis in patients with HBV infection (17). The majority of patients with HCC are middle-aged males; therefore, HBV infection and associated HCC can cause significant

economic pressure and mental stress for patients, their families and society (18). Early diagnosis and early treatment are the most fundamental measures to improve the prognosis of patients with HCC. However, the early diagnosis of HCC remains challenging (19).

The present study assessed HBV-induced miR-15a expression in patients with HCC. miRNAs have important regulatory effects on various activities of the body (9), and specific miRNA expression profiles are observed in different types of cancer. Therefore, miRNAs are regarded as potential markers for cancer classification and have important clinical significance (12). Research has demonstrated that miRNA expression is stable in circulating blood, and may be useful as prognostic and diagnostic markers for diseases, particularly tumors (20). In the present study, anti-miR-15a was demonstrated to significantly decrease HepG2 cell viability.

Smad-7, an inhibitory Smad, has important effects. Smad-7 competitively associates with the T β R1-type receptor and suppresses Smad phosphorylation; thus, Smad-7 regulates TGF- β /Smad signaling via negative feedback and maintains the balance of the pathway (13). Dysregulated Smad-7 expression may affect cell responses to TGF- β and promote the malignant progression of cells (21). TGF- β strongly induces Smad-7 expression. In the TGF- β signaling pathway, Smad-7 is a self-regulating negative feedback signal, and TGF- β signal strength and duration is determined by Smad-7 levels in cells (22). Endogenous Smad-7 can inhibit TGF- β signaling in a number of ways. It can inhibit TGF- β signal transduction via combining with T β R1 competitively and inhibiting receptor-regulated Smad phosphorylation (23). Research has also shown that, when Smad-7 accumulates in the cytoplasm, it combines with activated T β R1 via its MH2 domain to form a stable complex, blocking the transduction of the TGF- β signal (24). Smad-7 can thus decrease cell reactivity to TGF- β . The aforementioned processes occur in the cytoplasm (24). Smad-7 can also decrease histone acetylation to inhibit transcription. Smad-7 combines with DNA competitively via its MH2 structural domain, thereby restricting the formation of functional Smad-DNA complexes induced by TGF- β signaling (15). This competitive mechanism is widespread in the negative feedback Smad-7/TGF- β signaling pathway (15). In the present study, the following two aspects were validated experimentally. Firstly, increased Smad-7 protein expression was detected in HCC patients with HBV infection compared with normal tissue samples. Secondly, transfection with anti-microRNA-15a led to significant activation of Smad-7 protein expression in HepG2 cells infected with HBV.

A previous study, demonstrated that the expression of Smad-7 protein in HCC tissue was markedly higher than that in carcinoma-adjacent and normal tissues, the anticancer effect of TGF- β /Smad signaling was suppressed and the development of liver cancer was promoted (25). In another study, following the transfection of mouse hepatic cells with a Smad-7-containing adenovirus, Smad-7 was observed to decrease the intranuclear accumulation of Smads, including Smad3 induced by activin A, promote DNA synthesis stimulated by epithelial growth factor and alleviate the growth-inhibitory effect of activin A on hepatic cells (26). Furthermore, a study by Liu *et al* (27) revealed that, after an exogenous Smad-7 gene was transferred into L-02 hepatic cells, Smad-7 rescued the TGF- β -induced inhibition of L-02 hepatocellular proliferation and its apoptosis-inducing effect (27). The present study, reported that HBV induced apoptosis via the miR-15a/Smad-7/TGF- β pathway (27). The findings of the present study indicated that miR-15a/Smad-7/TGF- β signaling suppressed the cell growth of HBV-associated liver cancer via Smad-2/-4/TGF- β /FSP1 in HepG2 cells. In summary, the results indicate an association between HBV-associated liver cancer and miR-15a, which is a critical regulator of Smad-7/TGF- β /Smad-2/-4/FSP1/caspase-3/-7 signaling (Fig. 15).

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