

HBx promotes the proliferative ability of HL-7702 cells via the COX-2/Wnt/ β -catenin pathway

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Abstract. Hepatitis B virus X protein (HBx) has been termed a viral oncoprotein, and is involved in the initiation and progression of hepatocellular carcinoma (HCC). Cyclooxygenase-2 (COX-2) and β -catenin have been attributed to the oncogenic activity of HBx in HBV-associated HCC. The present study aimed to determine whether there is crosstalk between COX-2 and the Wnt/ β -catenin signaling pathway during HL-7702-HBx cell proliferation, and to investigate the associated underlying molecular mechanism. In the present study, cell proliferation assay, colony formation assay and flow cytometric analysis were used to detect the proliferative ability of cells. Reverse transcription-quantitative polymerase chain reaction and western blotting were performed to examine the mRNA and protein expression of COX-2, β -catenin, cyclin-D1 and c-myc. The results demonstrated that HL-7702-HBx exhibited increased cell proliferation, higher colony formation efficiency and a shortened G1 period of the cell cycle. In addition, the mRNA and protein expression levels of COX-2 were increased, and this was associated with HL-7702-HBx cell growth. Furthermore, the expression of β -catenin and its target genes, cyclin-D1 and c-myc proto-oncogene protein, was upregulated by HBx via COX-2. Finally, HBx promoted HL-7702 cell proliferation through the Wnt/ β -catenin signaling pathway. In conclusion, the primary finding of the present study was that HBx may promote HL-7702 cell proliferation via the COX-2/Wnt/ β -catenin pathway. Thus, it may be helpful to further investigate the molecular mechanism of HBV-associated hepatocellular carcinoma.

Introduction

Hepatitis B virus (HBV) is a widespread human pathogen associated with liver inflammation, cirrhosis and hepatocellular carcinoma (HCC). Among HBV proteins, HBx has been termed the viral oncoprotein and is involved in the initiation and progression of HCC (1). However, the underlying mechanism by which HBx contributes to the development of HCC remains unclear. Various studies have provided considerable evidence that HBx is a multifunctional protein that acts on cell cycle regulation, signaling pathways, DNA repair, cell proliferation, autophagy and apoptosis (2).

The different subcellular localizations of HBx indicate its different functions. It is primarily localized in the cytoplasm, with a fraction in the mitochondria and a small amount in the nucleus (3). A previous study demonstrated the colocalization of HBx with the inner mitochondrial membrane protein cytochrome c oxidase III (COXIII) and, reported an alteration of mitochondrial function and an upregulation of reactive oxygen species (ROS) generation in HL7702 and HepG2 cells. Subsequently, the key region in HBx for binding with COXIII was identified to be aa72-117, and ROS from mitochondria stimulated COX-2 expression (4-7).

COX-2 is an isoform of cyclooxygenase that mediates the oncogenic actions of HBx. COX-2 activity upregulation results in induced proliferation, angiogenesis and invasiveness in HCC (8,9). The Wnt/ β -catenin signaling pathway is involved in cell proliferation, differentiation and oncogenesis. Previously, studies have documented that HBx serves an important role in the modulation and induction of the canonical Wnt signaling pathway. β -catenin, upregulated by HBx, is associated with the oncogenic activity of HBx in HBV-associated HCC. Stabilization of β -catenin in the cytoplasm and its translocation to the nucleus are the two features of the activation of the canonical Wnt pathway. The expression of β -catenin targeted genes, including cyclin-D1 and c-myc proto-oncogene protein (c-myc) are activated following its translocation to the nucleus (10-12). A previously study demonstrated that β -catenin is associated with COX-2 overexpression (13). COX-2 activates the Wnt/ β -catenin pathway in gastric cancer (14). However, it remains unclear whether the COX-2 and β -catenin signaling pathways converge during HCC. A study on the underlying antitumor mechanism showed that the Wnt/ β -catenin signaling pathway mediated by COX-2 is

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involved in HCC (15). Therefore, the aim of the present study was to clarify the role of the COX-2/Wnt/ β -catenin pathway in HL-7702-HBx cells.

In the present study, the role of HBx in the oncogenesis of HBV associated with HCC was investigated by stably expressing HBx in HL-7702 cells. It was concluded that HBx promoted HL-7702 cell proliferation, and was dependent on the COX-2/Wnt/ β -catenin pathway.

Materials and methods

Antibodies and reagents. Anti-COX-2 antibody was purchased from Abcam (Cambridge, UK; cat no. ab151571). Anti- β -catenin antibody was purchased from OriGene Technologies, Inc. (Rockville, MD, USA; cat no. sc-7963). Anti-c-myc antibody was purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA; cat no. SC-40). Anti-cyclin D1 antibody was purchased from Santa Cruz Biotechnology, Inc. (cat no. sc-70899). Anti- β -actin antibody was purchased from OriGene Technologies, Inc. (cat no. TA-09). Peroxidase-conjugated goat anti-rabbit immunoglobulin (Ig)G (H+L) was purchased from OriGene Technologies, Inc. (cat no. ZB-2301). Peroxidase-conjugated goat anti-mouse IgG (H+L) was purchased from OriGene Technologies, Inc. (cat no. ZB-2305). XAV939 was purchased from Selleck Chemicals (Houston, TX, USA). Cells were treated with 20 μ mol/l XAV939 (a β -catenin inhibitor) and DMSO (as a control) for 24 h in cell proliferation assay. NS-398 was purchased from Beyotime Institute of Biotechnology (Haimen, China). Cells were treated with 50 μ mol/l NS-398 (a selective COX-2 inhibitor) and DMSO (as a control) for 72 h in western blot analysis and colony formation assay. Primers were synthesized by Biosune Biotechnology Co., Ltd (Shanghai, China; www.biosune.com). The enhanced chemiluminescence kit was purchased from OriGene Technologies, Inc. TRIzol reagent was purchased from Life Technologies (Thermo Fisher Scientific, Inc., Waltham, MA, USA). Moloney murine leukemia virus (MMLV) transcriptase was purchased from New England BioLabs., Inc. (Ipswich, MA, USA). Dulbecco's Modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from HyClone Company; GE Healthcare (Chicago, IL, USA).

Cell cultures. The human hepatocyte HL-7702 cell line was purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). HL-7702-HBx cells (HL-7702 cells transfected with lentiviral vector pLOV.flag-HBx to stably express the HBx gene: HBV subtype ayw) and HL-7702-mock cells (HL-7702 cells transfected with lentiviral vector pLOV.flag) were constructed previously by the authors of the present study (5). HBx, mock and control represent HL-7702-HBx, HL-7702-mock and HL-7702 cells, respectively. Cells were maintained in DMEM supplemented with 10% FBS and 1% penicillin-streptomycin at 37°C in a humidified atmosphere of 5% CO₂.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was isolated using TRIzol reagent. The primer sequences of each gene are listed in

Table I. First-strand cDNA was generated using MMLV transcriptase (2 μ g total RNA, 1 μ g primer, 5 μ l dNTP, 5 μ l M-MLV 5X Reaction Buffer and final volume 25 μ l), incubated for 60 min at 37°C. The extension temperature is 37°C, and qPCR was performed using FastStar universal SYBR Green master mix (Roche Applied Science, Penzberg, Germany) in triplicate using an Applied Biosystems Step one plus Real time PCR system (Life Technologies; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. Thermocycling conditions of the qPCR reaction are as follows: 95°C for 3 min, then 40 cycles of 95°C for 15 sec followed by 60°C for 30 sec. Specific primers were used to detect the relative mRNA expression by the 2^{- $\Delta\Delta$ C_q} method. The expression level was normalized against endogenous β -actin (16).

Western blot analysis. Cells were harvested and lysed by RIPA buffer from Beyotime Institute of Biotechnology. The protein concentration of each sample was determined using a bicinchoninic protein assay kit. A total of 20 μ g protein per lane was loaded on a 10% SDS/PAGE gel, and subsequently transferred to a nitrocellulose membrane. The membrane was blocked in 5% milk in TBS-Tween 20 [0.1% Tween 20, 20 mM Tris (pH 7.4) and 150 mM NaCl] for 2 h at room temperature, followed by overnight incubation with the primary antibody at 4°C. Primary antibodies were used at 1:1,000 (Anti-COX-2 antibody, Anti- β -catenin antibody, Anti-c-myc antibody and Anti- β -actin) or 1:500 (Anti-cyclin D1 antibody), and secondary antibodies conjugated with horseradish peroxidase were used at a 1:2,000 dilution in 5% nonfat dry milk at room temperature for 1 h. Following a final wash, proteins were visualized using the enhanced chemiluminescence kit. Images of the blots were captured using an Image Scanner (Epson, Nagano, Japan). Expression levels of protein were semi-quantitatively analyzed by using Image J Launcher (Broken Symmetry Software 1.4.3.67), normalized to β -actin density.

Cell proliferation assay. A Cell Counting Kit-8 (CCK-8) assay (Dojindo Molecular Laboratories, Inc., Kumamoto, Japan) was used to analyze cell proliferation. Cells were seeded into 96-well plates at 3x10³ cells/well in 100 μ l complete DMEM and incubated at 37°C and 5% CO₂. The plates were incubated for 24, 48 and 72 h, the medium was removed and 100 μ l fresh complete DMEM was added, containing 10 μ l CCK-8, to each well. The plates were incubated for 3 h at 37°C. The absorbance was measured at a wavelength of 450 nm using an ELISA plate reader. Proliferation inhibition rate was calculated according to the following formula: Proliferation inhibition rate (%)=(1-OD₄₅₀/OD_{450 with XAV939}) x 100%.

Colony formation assay. Cells were seeded into 6-well plates at 500 cells/well in triplicate and incubated at 37°C and 5% CO₂ for 14 days. Clones were observed in the dish. Each well was washed with PBS twice, fixed with 1 ml 100% methanol for 15 min, then washed with PBS for three times and stained with crystal violet staining solution (Beyotime Institute of Biotechnology) for 10 min at room temperature. Directly counted by naked eyes based on experience, in case of doubting whether 50 or more cells formed the suspected colony, counted

Table I. Primer sequences for the genes used in the RT-qPCR.

Gene	Primer sequences (5'→3')
COX-2	F: TGAAACCCACTCCAAACACA R: GAGAAGGCTTCCCAGCTTTT
β-catenin	F: AAATGGTTGCCTTGCTCAAC R: TCAGCACTCTGCTTGTGGTC
c-myc	F: CCTACCCTCTCAACGACAGC R: CTCTGACCTTTTGCCAGGAG
Cyclin D1	F: CCCTCGGTGTCCTACTTCAA R: GGGGATGGTCTCCTTCATCT
β-actin	F: CTCCATCCTGGCCTCGCTGT R: GCTGTCACCTTCACCGTTCC

F, forward; R, reverse; COX-2, cyclooxygenase-2.

the colony by stereomicroscope under lower magnification (x40). Clone formation rate and Clone formation inhibition rate were calculated according to the following formula: Clone formation rate=number of formed colonies/number of seeded cells x100. Clone formation inhibition rate=(1-number of formed colonies/number of formed colonies with NS398) x100%.

Flow cytometric analysis. Cells were harvested and resuspended in PBS (1x10⁶ cells/ml). For analysis of the cell cycle, cells were fixed in 70% ethanol overnight at 4°C. Subsequently, cells were digested with 50 μl RNase (TakaRa Biotechnology Co. Ltd. Dalian, China; 10 mg/ml) at 37°C for 30 min and stained with 20 μl 50 μg/ml propidium iodide (Beyotime Institute of Biotechnology) at 37°C for 10 min in the dark. The DNA histograms were determined by flow cytometry (C6; Becton-Dickinson; BD Biosciences, San Jose, CA).

Statistical analysis. Experiments were performed in triplicate. All statistical analyses were performed using the SPSS version 16.0 statistical software package (SPSS, Inc., Chicago, IL, USA). Continuous variables are expressed as the mean ± standard deviation. One-way analysis of variance was used for the statistical analyses, followed by the Fisher's Least Significant Difference. P≤0.05 was considered to indicate a statistically significant difference.

Results

HBx promotes the proliferative ability of HL-7702 cells. Considerable efforts have been made to detect the role of HBx in cell proliferation (17). A cell viability assay and plate colony formation assay were performed in the present study to observe the role of HBx in HL-7702 cell proliferation. As demonstrated in Fig. 1A, HL-7702-HBx cells grew faster compared with the mock and control groups on days 1, 2 and 3 (P<0.05). As presented in Fig. 1B, following incubation for 2 weeks, HL-7702-HBx cells formed more colonies and had higher colony formation efficiency compared with the mock and control groups. In addition, HL-7702-HBx cells

exhibited a shortened G1 phase of the cell cycle compared with the HL-7702-mock and HL-7702-control cells (Fig. 1C). These results suggested that HBx promoted the proliferation of HL-7702 cells.

Upregulation of COX-2 promotes the proliferation ability of HL-7702 cells. Previous studies have demonstrated that HBx promoted the levels of mitochondrial ROS in HL-7702 cells (4,5). ROS from mitochondria lead to COX-2 induction (18). As demonstrated in Fig. 2A and B, HBx enhanced the mRNA and protein expression levels of COX-2 in HL-7702 cells. COX-2 increases proliferation in various types of tumor and its expression appears to be associated with early HCC events (19). The present study investigated whether upregulation of COX-2 promoted the proliferative ability of HL-7702 cells and treated cells with NS-398. COX-2 protein expression levels and colony formation efficiency were analyzed. As presented in Fig. 2C and D, NS-398 suppressed the colony formation efficiency in the three groups investigated, the colony formation inhibition rate of HL-7702-HBx cells was significantly higher than that of other two groups after down-regulation of COX-2 at the protein level (Fig. 2E). Therefore, HBx may promote cell proliferation through upregulation of COX-2.

HBx activates the Wnt/β-catenin signaling pathway in HL-7702 cells. The Wnt/β-catenin signaling pathway is involved in cell proliferation, differentiation and oncogenesis (10,12). The present study questioned whether aberrant activation of the Wnt/β-catenin signaling pathway occurs in HBx-transfected cells. The mRNA expression level of β-catenin was increased in HL-7702-HBx cells compared with the control and mock groups (Fig. 3A). In support of this upregulation at the mRNA level, the protein expression of β-catenin was increased in HL-7702-HBx cells compared with the other two cell groups (Fig. 3B). The results demonstrated that the mRNA and protein expression levels of β-catenin responsive genes, c-myc and cyclin-D1, were activated compared with the mock and control groups (Fig. 3C-E). These data suggested that HBx may activate Wnt/β-catenin signaling in HL7702 cells.

HBx promotes the proliferative ability of HL-7702 cells via the COX-2/Wnt/β-catenin signaling pathway. To further examine the association between β-catenin and COX-2 in HL-7702-HBx cell proliferation, cells were treated with NS-398 (50 μmol/l) for 72 h, and as presented in Fig. 3B and Fig. 4A, NS398 attenuated the effects of HBx on β-catenin expression in HL7702-HBx cells. The present study considered whether the upregulation of β-catenin resulted in increased proliferation of HL-7702-HBx cells. To support this hypothesis, cell viability assays were performed following treatment of the cells with a β-catenin inhibitor, XAV939 (20 μmol/l), for 24 h. As indicated in Fig. 4B, treatment with XAV939 suppressed cell proliferation in the three groups. HL7702-HBx cells treated with XAV939 demonstrated higher proliferative inhibition rate than the mock and control groups (Fig. 4C). Therefore, it was concluded that upregulation of COX-2 may promote the proliferation of HL-7702-HBx cells via activation of the Wnt/β-catenin signaling pathway.

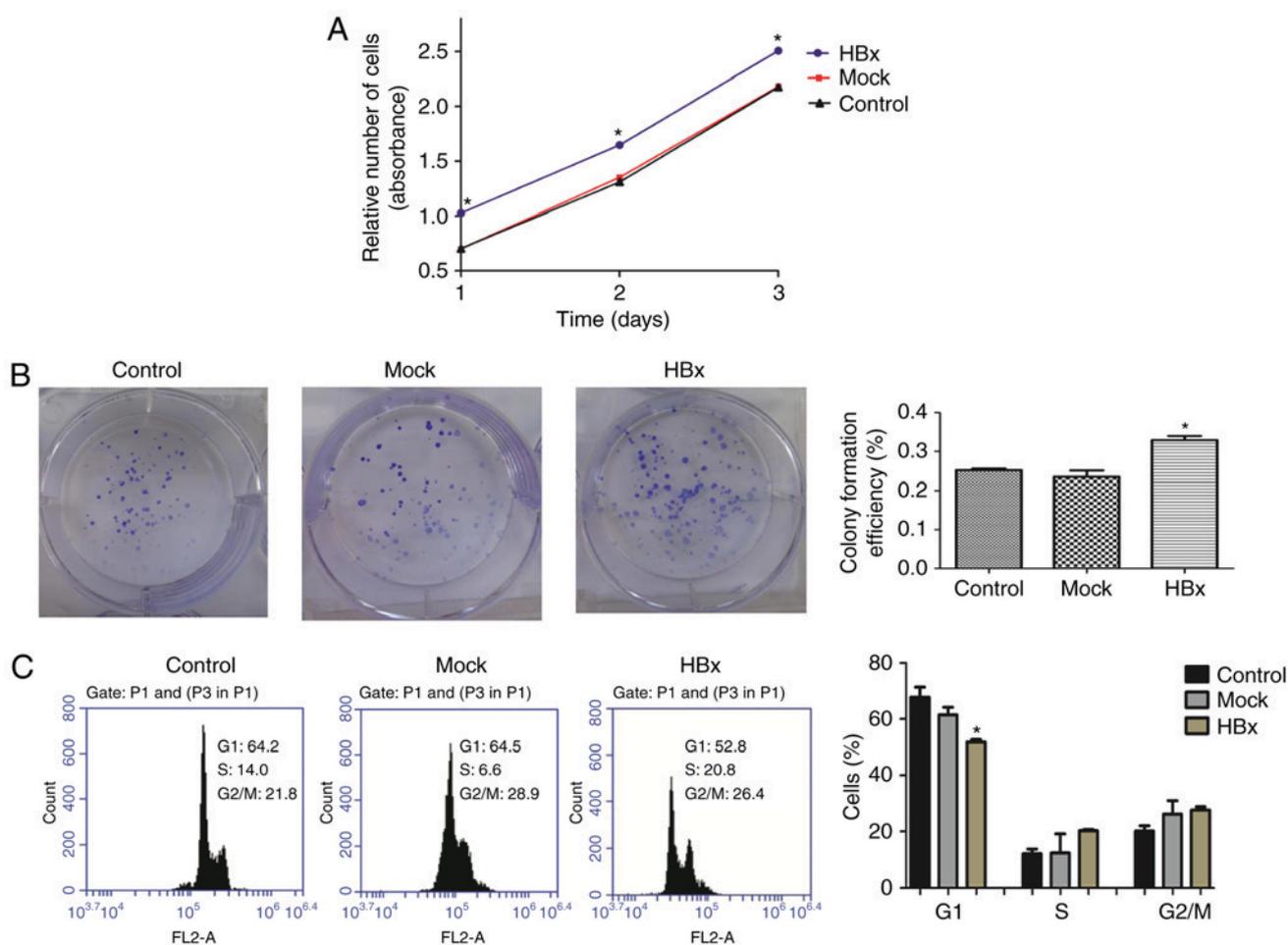


Figure 1. HBx promotes the proliferative ability of HL-7702 cells. (A) Following incubation for 24, 48 and 72 h, a cell viability assay using a Cell Counting Kit-8 was used to examine cell proliferation. (B) A plate clone formation assay was performed to detect the colony formation efficiency. Following incubation for 14 days, cells were stained with crystal violet staining solution and counted by naked eyes. (C) The cell cycle distribution was assessed with the fluorescent dye propidium iodide, followed by flow cytometric analysis. Values represent the mean \pm standard deviation. * $P < 0.05$ vs. HL-7702-mock cells and * $P < 0.05$ vs. HL-7702 cells. HBx, hepatitis B virus X protein.

Discussion

HBx, a key regulatory HBV protein that is important for HBV replication, is involved in the initiation and progression of HCC (20). Research has demonstrated that HBx leads to cell proliferation and is involved in the initiation of HCC (20,21). A previous study confirmed the colocalization of HBx with COXIII and the upregulation of ROS generation in HL7702 and HepG2 cells, and ROS from mitochondria induced COX-2 expression that promoted HepG2 cell growth (5,6). However, the role of HBx in HL-7702 cells, which are human normal hepatocytes and serve as a biologically relevant system for examining the role of HBx, remains unclear. The results of the present study demonstrated that HBx increased the proliferation rate, led to the formation of more colonies and shortened the G1 phase in HL-7702 cells. Therefore, it was concluded that HBx may promote HL-7702 cell proliferation.

Increased COX-2 activity induces proliferation and mediates the oncogenic action of HBx (8). The present study demonstrated that COX-2 mRNA and protein expression levels were increased by HBx, and when cells were treated with NS-398, the colony formation efficiency in HL-7702-HBx cells was significantly suppressed following

reduced expression of the COX-2 protein. Therefore, it was concluded that the upregulation of COX-2 may promote HL-7702-HBx cell proliferation. However, the underlying molecular mechanism remains unknown. COX-2 was observed to promote cell proliferation by mediating the activation of downstream oncogenic pathways (22). Thus, it is important to investigate the downstream pathways of COX-2 and the role of HBx in HCC.

The Wnt/ β -catenin signaling pathway has long been considered to be an important developmental pathway in human HCC (23). It has been reported that HBx upregulates β -catenin expression during the oncogenesis of HBV-associated HCC (24). The present study examined the mRNA and protein expression levels of β -catenin, which were increased in HL-7702-HBx cells. When activated, β -catenin is translocated to the nucleus leading to increased expression of β -catenin targeted genes, including cyclin-D1 and c-myc (10), which serve important roles in oncogenesis (12). The present study detected that the mRNA and protein expression levels of cyclin-D1 and c-myc were increased. Experimental data has demonstrated that β -catenin is associated with COX-2 overexpression (13). The Wnt/ β -catenin pathway is activated by COX-2 in gastric

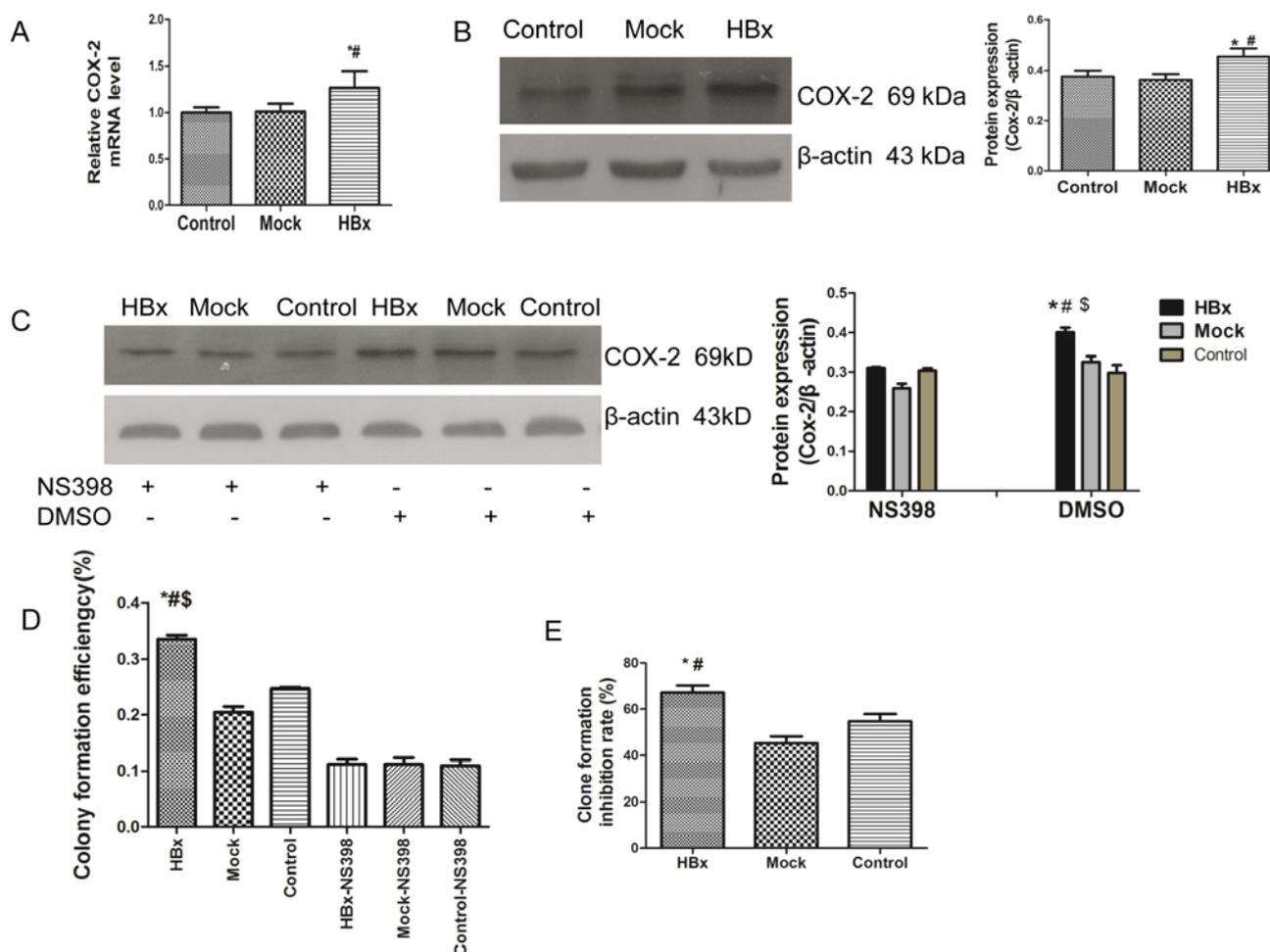


Figure 2. Upregulation of COX-2 promotes the proliferative ability of HL-7702 cells. (A) The mRNA expression levels of COX-2 in cells were examined using the reverse transcription-quantitative polymerase chain reaction. (B) The expression levels of the COX-2 protein in cells were detected by western blotting. (C) Western blot analysis of COX-2 protein expression levels. A total of 50 μ mol/l COX-2 inhibitor, NS-398, was added to cells in the HBx, mock and control groups, or DMSO was added instead, for 72 h. (D) A total of 50 μ mol/l NS-398 was added to cells, or DMSO as a control, for 72 h. A plate clone formation assay was performed to detect the colony formation efficiency. (E) Clone formation inhibition rate was calculated. Clone formation inhibition rate=(1-number of formed colonies/number of formed colonies with NS398) \times 100% ^{*}P<0.05 vs. HL-7702-mock cells and [#]P<0.05 vs. HL-7702 cells. ^{\$}P<0.05 vs. HL-7702-HBx cells with NS398. COX-2, cyclooxygenase-2; DMSO, dimethyl sulfoxide; HBx, hepatitis B virus X protein.

cancer (14). The COX-2-mediated Wnt/ β -catenin signaling pathway is involved in HCC (15). These previous results question the effect of COX-2 in the regulation of β -catenin in HL-7702-HBx cells. The present study treated cells with NS-398, and the expression of β -catenin protein was decreased with the downregulated expression of COX-2. Therefore, the results of the present study may provide evidence that the upregulation of β -catenin is activated by COX-2. In addition, the present study demonstrated that the upregulation of β -catenin resulted in increased proliferation of HL-7702-HBx cells by adding the β -catenin inhibitor, XAV939, to the cells. Treatment with XAV939 significantly suppressed cell proliferation. Thus, the results of the present study demonstrated that that activation of COX-2 may lead to the upregulation of β -catenin, resulting in HL-7702 cell proliferation.

In conclusion, the present study demonstrated that HBx activated the expression of COX-2. In addition, it upregulated the expression levels of β -catenin and ultimately promoted HL-7702-HBx cell proliferation. The authors of the present study hypothesize that therapies aimed at simultaneous

disruption of the COX-2/Wnt/ β -catenin pathways may produce effective chemopreventive and antitumorigenic effects. The present study may provide a novel insight into the underlying molecular mechanism of HBV-associated HCC. In future studies, small interfering RNA knockdown may be used to specifically silence COX-2 and β -catenin, in order to further examine the role of the COX-2/Wnt/ β -catenin pathways in the oncogenesis of HBV-associated HCC.

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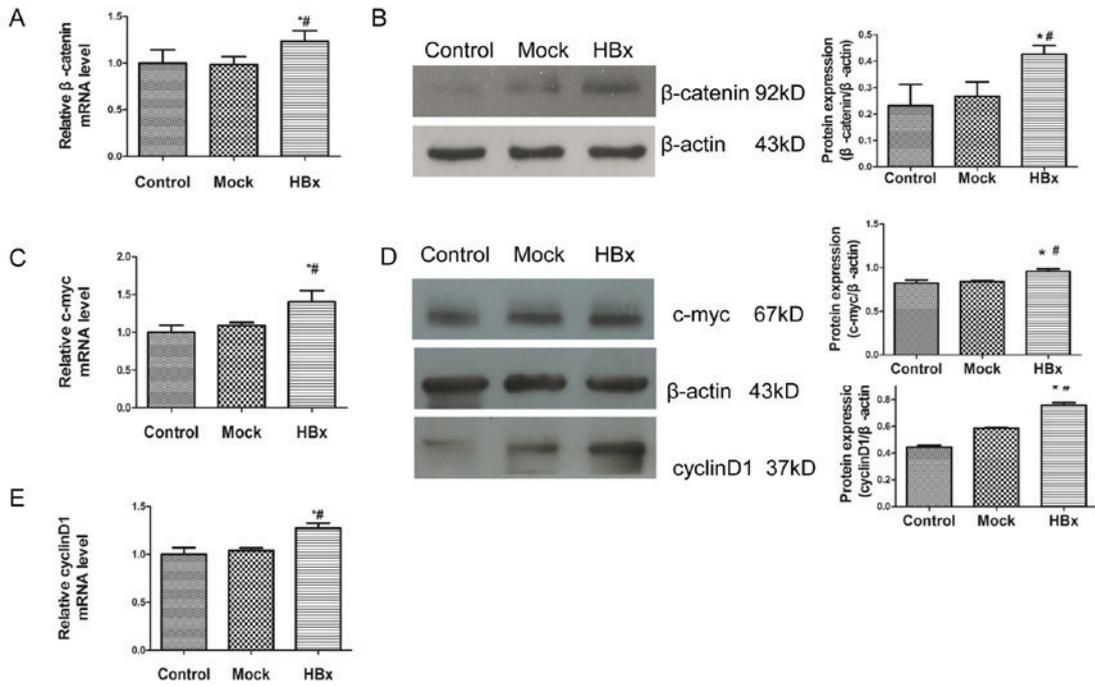


Figure 3. HBx activates the Wnt/ β -catenin signaling pathway in HL7702 cells. (A) The mRNA expression levels of β -catenin in cells were detected using RT-qPCR. (B) The expression levels of the β -catenin protein were detected in the lysates of cells by western blotting using specific antibodies against β -catenin protein and β -actin. (C) The expression of c-myc mRNA in cells was detected using RT-qPCR. (D) The expression levels of c-myc and cyclinD1 protein were detected by western blotting using specific antibodies against c-myc, cyclinD1 and β -actin. (E) The expression of cyclinD1 mRNA was detected using RT-qPCR. * $P < 0.05$ vs. HL-7702-mock cells and # $P < 0.05$ vs. HL-7702 cells. HBx, hepatitis B virus X protein; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; c-myc, c-myc proto-oncogene protein.

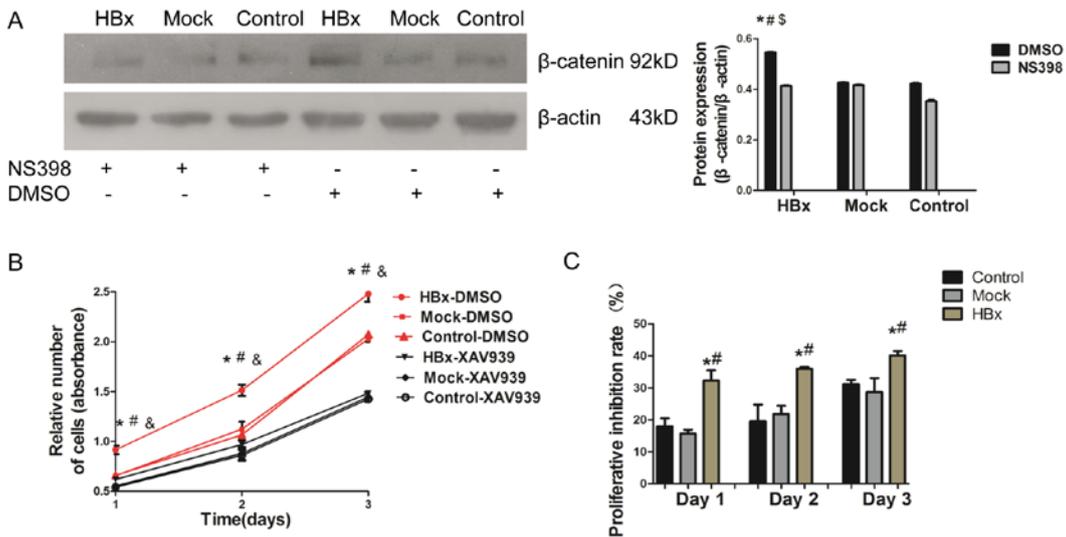


Figure 4. HBx promotes the proliferative ability of HL-7702 cells via the COX-2/Wnt/ β -catenin pathway. (A) Following the addition of 50 $\mu\text{mol/l}$ NS-398 and DMSO as a control, to cells for 72 h, the expression of the β -catenin protein was examined in the lysates of HL-7702, HL-7702-mock and HL-7702-HBx cells by western blotting. (B) Cells were treated with a β -catenin inhibitor, XAV939 (20 $\mu\text{mol/l}$), or DMSO as a control. Following incubation for 24, 48 and 72 h, cell proliferation was determined by a Cell Counting Kit-8 assay. (C) Proliferation inhibition rate was calculated. Proliferation inhibition rate (%) = $(1 - \text{OD}_{450} / \text{OD}_{450}^{\text{with XAV939}}) \times 100\%$. * $P < 0.05$ vs. HL-7702-mock cells and # $P < 0.05$ vs. HL-7702 cells. $^{\$}P < 0.05$ vs. HL-7702-HBx cells with NS398. $^{\&}P < 0.05$ vs. HL-7702-HBx cells with XAV939. DMSO, dimethyl sulfoxide; HBx, hepatitis B virus X protein.

Availability of data and materials

The materials described in the manuscript, including all relevant raw data, will be freely available to any scientist wishing to use them for non-commercial purposes, without breaching participant confidentiality.

Author contributions

BYZ and XZW conceived and designed the experiments. BYZ and WYG performed the experiments; XYH, LYL, XFF and ZXC analyzed the data; BYZ wrote the paper.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare they have no competing interests.

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