Hepatitis B virus X protein promotes epithelial-mesenchymal transition and metastasis in hepatocellular carcinoma cell line HCCLM3 by targeting HMGA2

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Abstract. Chronic hepatitis B virus (HBV) infection is a major risk factor for hepatocellular carcinoma (HCC), and HBV X protein (HBx) serves an essential role in the development of HCC. However, its mechanism remains to be elucidated. The aim of the present study was to investigate the role and mechanism of the HBx protein in the epithelial-mesenchymal transition (EMT) and metastasis of HCC. The HCCLM3 cell line was transfected with a HBx-expressing vector. The effects of HBx overexpression on proliferation, migration, invasion and EMT capacities of the HCCLM3 cell line were evaluated using MTT, migration and invasion assays, and western blotting, respectively. Furthermore, the impact of High mobility group AT-hook 2 (HMGA2) knockdown on HBx-mediated metastasis was investigated in the HCC cell line HCCLM3. The results demonstrated that HBx significantly upregulated HMGA2 expression, and enhanced the proliferation, EMT, invasion and migration in HCC cells. Furthermore, HMGA2 knockdown almost abolished HBx-induced EMT and metastasis in HCC. The results of the present study suggest that HBx promotes the proliferation, EMT, invasion and migration of HCC cells by targeting HMGA2. HMGB2 may serve as a potential therapeutic target for HBV-associated HCC.

Introduction

Hepatocellular carcinoma (HCC) is among the most lethal types of cancer, characterized by diagnosis at an advanced stage with regional vascular involvement, and distant metastasis (1).

Hepatitis B virus (HBV) infection is one of the most common risk factors for HCC, particularly in Asia (2).

Hepatitis B virus x (HBx), a protein encoded by HBV virus, is essential for viral replication. Previous studies have demonstrated that HBx contributes to the progression of HCC by interacting with certain signal pathways, and promoting proliferation and invasive potential of HCC cells (3-6). HBx activates phosphatidylinositol 3-kinase (PI3K)/protein kinase B (AKT), the Notch signal pathway, and various transcription factors, including nuclear factor-kB (NF-kB) and activator protein 1 (AP-1) (3-5). However, the precise mechanisms by which HBx promotes the epithelial-to-mesenchymal transition (EMT) and metastasis of HCC remain unclear.

High mobility group AT-hook 2 (HMGA2), an architectural transcription factor, is overexpressed during embryogenesis, but not expressed in normal adult tissues (7,8). Previous studies have reported HMGA2 is overexpressed in a variety of tumors, including HCC, breast cancer, non-small-cell lung cancer and gastric cancer (9-12). HMGA2 is involved in various essential biological processes, including DNA repair, apoptosis, cell proliferation, EMT and telomere restoration by regulating a wide range of gene expression levels in HCC (13-16). Given that HBx and HMGA2 serve important roles in HCC metastasis, the effects of HBx overexpression on EMT markers, invasion and metastasis of HCC cells were investigated in the present study. Furthermore, the role of HMGA2 in HBx-mediated HCC metastasis was explored.

Materials and methods

Cell culture. The HCCLM3 cell line was obtained from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). The cells were cultured in a humidified atmosphere of 5% CO₂ at 37°C in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) (both from Hyclone; GE Healthcare Life Sciences, Logan, UT, USA).

HBx-expression vector construct and transfection. The construction of the HBx-expressing vector (pcDNA3.1-HBx) and control vector (pCDNA3.1-EGFP Vector) has been described in a previous study (5,17). A full-length HBx gene was inserted into a pcDNA3.1 vector, and was identified using.
polymerase chain reaction (PCR), restriction endonuclease digestion and DNA sequencing methods. The PCR primers were as follows: HBx forward, 5'-CGGCGTCTGAGTCTGCTG-3' and reverse, 5'-CGGAATTCTT��GCGAGGTCGAGTGGTGAAT-3'. The PCR product was digested with XhoI and BglII. The EGFP was inserted into a pCDNA3.1 vector between EcoRI and NotI as a control. The PCR primers for the control vector were as follows: CMV-f 5'-CGC AAA TGG GCG GTA GGC GTG-3' and BGH-r 5'-TAG AAG GCA CAG TCG AGG-3'. All ligated vectors were confirmed by DNA sequence analysis. HCCLM3 cells were transfected with the HBx-expressing vector, and then screened with puromycin to obtain stable cell clones. The stable HBx-expressing cells were termed HCCLM3-HBx cells.

RNA interference. The stable HBx-expressing HCCLM3 cell line was further assigned into two subgroups: HBx group and HMGA2 knockdown group [HBx + HMGA2 small interfering RNA (siRNA)]. The HMGA2 siRNA and corresponding control siRNA were designed and synthesized by Shanghai GenePharma Co., Ltd. (Shanghai, China). The HMGA2 siRNA target sequences was as follows: 5'-GGA AAT GGC CAC AAC AAG TTG-3'. The cells were transfected using Lipofectamine 2000 (cat. no. 11668019; Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) according to the manufacturer's protocol. Briefly, the cells were seeded on 6-well plates at a density of 5x10^5 cells/well, and cultured for 48 h to reach 80% confluence. The cells were washed twice with PBS. A mixture of individual HMGA2 siRNA (1 µg/ml), transfection medium and Lipofectamine 2000 was added. After 30 min, 500 µl of Opti-MEM (cat. no. 31985062; Gibco; Thermo Fisher Scientific, Inc.) was added. After 6 h, the transfection medium was removed, and the cells were cultured in RPMI-1640 medium with 10% fetal bovine serum. The gene silencing efficiency was confirmed by western blotting and immunocytochemistry 48 h after the transfection.

Proliferation assays. The cells were seeded at a density of 2x10^3 cells/well in 96-well plates and incubated with RPMI-1640 medium at 37°C for 1-7 days. PBS (cat. no. SH30256.01; Hyclone, Logan, UT, USA) was used to dissolve the purple formazan. MTT was added to each well and incubated at 37°C for 4 h. The absorbance was measured using a microplate reader with a wavelength of 490 nm. Each experiment was repeated three times.

Transwell migration and invasion assays. The invasion assay was performed as follows. At 48 h after the transfection, 5x10^5 cells were seeded into the upper compartments of a 24-well Transwell chamber, which was coated with 100 µl Matrigel (200 µg/ml; cat. no. 356234; BD Biosciences, Franklin Lakes, NJ, USA). The medium contained with 20% FBS was added into the lower compartment. After 24 h incubation, the filter membrane was washed with PBS. The cells were stained with 0.05% crystal violet and fixed with 10% formaldehyde. The cells in five random fields of the membrane were counted with microscope. Cell migration assays were performed according to the same protocol, except without Matrigel-coating.

Scratch wound-healing assays. A total of 1x10^6 cells were seeded into 6-well tissue culture plates and grown to reach 80% confluence. A linear wound was created in the cell surface using a 10-µl sterilized pipette tip. The wounded cell layers were washed twice with PBS. The migrated distance at the wound space was measured at 0, 24 and 48 h in five random microscopic fields at magnification, x40 under an Olympus BX53 microscope (Olympus Corporation, Tokyo, Japan). Each independent experiment was repeated three times.

Immunofluorescence. A total of 2x10^5 cells were seeded and cultured onto glass cover slips in 24-well plates.
were fixed in 4% paraformaldehyde for 20 min at 37˚C, and incubated with a primary antibody against HMGA2 (rabbit anti-human; 1:100; cat. no. ab97276; Abcam, Cambridge, UK) overnight at 4˚C. Next, the slides were incubated with an Alexa Fluor 594-conjugated secondary antibody (anti-rabbit immunoglobulin G; 1:500; cat. no. 7074; CST, Boston, USA) for 1 h at room temperature. Lastly, the cells were stained with DAPI at 37˚C for 5 min and observed using fluorescence microscopy at x200 magnification.

Western blot analysis. The protein was extracted from the cells with 10% MSDS (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) and the protein quantity was determined using a bicinchoninic acid assay. The mass of the proteins loaded in per lane was 80 µg. The proteins were separated using 10% SDS-PAGE, and then transferred onto polyvinylidene fluoride membranes. The membranes were blocked in 5% bovine serum albumin (cat. no. 10099141; Gibco; Thermo Fisher Scientific, Inc.) at 37˚C for 1 h, and washed with TBST buffer three times. The membranes were exposed to primary rabbit anti-human HMGA2 antibodies (cat. no. ab97276; Abcam, San Francisco, USA), HBx (cat. no. ab2741; Abcam, Cambridge, UK), E-cadherin, Vimentin, N-cadherin (all 1:500; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) and GAPDH (1:3,000, ProteinTech Group, Inc., Chicago, IL, USA) overnight at 4˚C. The membranes were washed with TBST three times, and incubated with secondary antibody (anti-rabbit Immunoglobulin G; 1:2,000; cat. no. 7074; CST, Boston, USA) for 2 h at room temperature, and washed with TBST three times. The bands were visualized with the WEST ZOL Plus system and quantified using ImageJ software (version 1.44P; National Institutes of Health, Bethesda, MA, USA).

Statistical analysis. Statistical analysis was performed using the SPSS version 18.0 (SPSS, Inc., Chicago, IL, USA). Data are expressed as the mean ± standard deviation of three independent experiments, and were analyzed using the Student’s t-test or one-way analysis of variance with the Least Significant Difference (LSD) post hoc test to determine the different between two groups. P<0.05 was considered to indicate a statistically significant difference.

Results

HBx promotes proliferation, migration, invasion, and EMT of HCC cells. In order to investigate the effect of HBx on the proliferation of HCC cells, HCCLM3 cells were transfected with the HBx-expressing (pcDNA3.1-HBx) and control vectors (NC). The transfection efficiency was confirmed with western blotting (Fig. 1). MTT analysis demonstrated that the proliferation of HCCLM3 cells transfected with pcDNA3.1-HBx vectors was significantly promoted when compared with the control cells (Fig. 2).

Then, Transwell migration and Matrigel invasion assays were performed to determine the roles of HBx in HCC metastasis. The results revealed that HCCLM3 cells transfected with HBx-expressed vectors significantly increased the migratory and invasive capacities compared with empty vector-transfected control cells (Figs. 3 and 4). In addition, Western blot analysis demonstrated that the upregulation of HBx resulted in a significant increase in the epithelial cell marker E-cadherin expression and a decrease in the mesenchymal cell marker Vimentin protein expression in HCCLM3 cells (Fig. 5).

HBx promotes the expression of HMGA2 in HCC cells. To explore the mechanism of HBx on HCC metastasis, the influence of HBx on expression of HMGA2 in HCCLM3 cells was evaluated. Western blotting analyzed indicated that HMGA2 expression was significantly upregulated in HCCLM3 cells transfected with pcDNA3.1-HBx vectors at 48 h (Fig. 5). Fluorescent microscopy revealed that HBx-transfected HCCLM3 cells had markedly increased HMGA2 expression in the nucleus (Fig. 6). These results suggest that HBx may enhance the metastasis of HCCLM3 cells through HMGA2.
**HMGA2 silencing restrains EMT and metastasis of HCCLM3 cells induced by HBx.** To further confirm that HMGA2 was a target of HBx, the effect of HMGA2 knockdown on HBx-induced HCC metastasis was examined. Transwell invasion and scratch wound-healing assays showed that the migratory and invasive capacities of HBx-expressed HCCLM3 cells were significantly inhibited by HMGA2 silencing (Figs. 3 and 4). Furthermore, western blotting analysis demonstrated that HMGA2-siRNA significantly inhibited the expression of E-cadherin and increased in Vimentin protein expression, reversing the EMT in HBx-expressed HCCLM3 cells (Fig. 5). Collectively, these data indicated that HBx enhanced the invasiveness and migration capacities of HCC cells via HMGB2, HMGA2 knockdown was an effective strategy to prevent HBx-induced HCC progression.

**Discussion**

HBx is a risk factor for hepatocarcinogenesis and has been implicated in HCC progression. Previous studies reported HBx promoted the proliferation of HCC cells (5,13). HBx also disrupts intercellular adhesion, and induces EMT, invasion, migration and metastasis in HCC (13,14). In the present study, it was demonstrated that HBx markedly upregulated the expression of the epithelial cell marker E-cadherin and downregulated the expression of the mesenchymal cell marker Vimentin in HCCLM3 cells. In addition, the overexpression of HBx in HCCLM3 cells resulted in significantly increased migratory and invasive capacities compared with the control cells. These data indicated that HBx promoted the EMT, migration and invasion capabilities in HCC.

![Figure 4](image4.png)

Figure 4. The effect of HBx overexpression and HMGA2 suppression on HCCLM3 cell invasive potential was assessed by the Matrigel invasion assay. (A) Representative image of Matrigel invasion assay and (B) quantification. The number of cells that invaded the basal side of the Matrigel-coated Transwell inserts were counted at 24 h (magnification, x100). *P<0.05. HBX, HBV X protein; siRNA, small interfering RNA; HMGA2, high mobility group AT-hook 2; NC, negative control.

![Figure 5](image5.png)

Figure 5. Western blot analysis of HMGA2, E-cadherin, Vimentin and N-cadherin in HCCLM3 cells. (A) Representative blot and (B) quantification. GAPDH was used as a loading control, vs. control and NC groups. *P<0.05. HBX, HBV X protein; siRNA, small interfering RNA; HMGA2, high mobility group AT-hook 2; NC, negative control.
Previous studies have reported that HBx promotes HCC metastasis through differing mechanisms. Jin et al. (18) reported that HBx promoted HCC cell metastasis and induced EMT by mediating long noncoding RNA, ZEB2 antisense RNA 1. Hou et al. (19) reported that HBx promoted HCC cell invasion and migration through the HBx-metastasis associated lung adenocarcinoma transcript 1 (non-protein coding)/latent transforming growth factor β binding protein 3 signaling axis. Chen et al. (20) demonstrated that HBx overexpression induced the secretion of high-mobility group box 1 to promote the invasion and metastasis of HCC in an autocrine/paracrine manner. In current study, it was revealed that HMGA2 knockdown inhibited HBx-induced EMT and metastasis in HCCLM3 cells. The HMGA2 protein overexpression has been reported to be associated with metastasis in HCC (20-22). Ou et al. (20) demonstrated that HMGA2 promoted HCC cell proliferation and invasion via the Wnt/β-catenin signaling pathway. Luo et al. (21) reported that HMGA2 expression was significantly associated with the expression of EMT markers, whereby HMGA2 induced EMT by upregulating the expression of Twist and Snail in HCC cell lines. The results suggest that HBx promotes HCC progression at least in part through targeting HMGA2, HMGA2 is an effective target to prevent HBx-induced HCC metastasis.

Previously, certain studies have reported that HBx upregulated transforming growth factor-β1 (TGFβ1) expression (23,24). TGF-β was also a vital cytokine to activate HMGA2 and induce EMT in HCC (25). Increased TGF-β levels may upregulate HMGA2 expression, which in turn promotes the EMT, invasion and migration capabilities HCC. The data suggested that HBx activated HMGA2 through TGF-β. However, further studies are required to confirm this hypothesis.

There are certain limitations to the present study. Firstly, the use of different experimental systems and HBx expression levels may result in differences in the effects of HBx on cellular signal transduction pathways and influence HCC metastasis. Secondly, since HBx has been identified to serve a vital function in the regulation of chronic liver inflammation and the liver tumor microenvironment (26), further study is required to clarify the interaction between HBx and HMGA2 under the specific liver tumor microenvironment. Finally, it has been reported that overexpression of HBx in hepatocellular carcinoma cells may induce the secretion of high-mobility group box 1 (HMGB1) to promote invasion and metastasis of HCC in an autocrine/paracrine manner (6). However, the interactions between HMGB1 and HMGB2 in HBx-induced HCC metastasis remain largely unclear.
In conclusion, in spite of the aforementioned limitations in the current study, an important role of the HBx-induced HMGA2 signaling pathway in regulating EMT was identified, which subsequently promotes the invasive and migratory invasive capacities of HCC. The present study suggests that HMGA2 may serve as a potential therapeutic target for the treatment of patients with HBV-associated HCC.

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Availability of data and materials

All analyzed data and materials in this study are included in this published article.

Authors' contributions

YZ designed the study. QY, JSL, YYW and WMS completed the experiment. YZ drafted the manuscript. All authors have approved the final version.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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