Hepatitis B virus-X protein regulates high mobility group box 1 to promote the formation of hepatocellular carcinoma

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Abstract. Hepatitis B virus (HBV) infection is a risk factor for hepatocellular carcinoma (HCC). HBV X protein (HBx) is an important carcinogen for HBV-induced HCC. When the HBx gene is integrated into the host cell genome, it is difficult to eradicate. The identification of an effective target to inhibit the oncogenic function of HBx is therefore critically important. The present study demonstrated that HBx, particularly truncated HBx, was expressed in several HBV-derived cell lines (e.g., Hep3B and SNU423). By analyzing data from The Cancer Genome Atlas, it was revealed that high expression of high mobility group box 1 (HMGB1) was associated with the process and prognosis of HCC. In vitro experiments confirmed that HBx could regulate the expression of HMGB1 and knockdown of HMGB1 could decrease the ability of HBx to promote cellular proliferation. HBx could also upregulate six transcription factors (GATA binding protein 3, Erb-B2 receptor tyrosine kinase 3, heat shock transcription factor 1, nuclear factor κB subunit 1, TATA-box binding protein and Kruppel-like factor 4), which could directly regulate HMGB1. By analyzing genes that are co-expressed with HMGB1, several signaling pathways associated with the development of HCC were identified. HBx and HMGB1 were revealed to be involved in these pathways, which may be the mechanism by which HBx promotes HCC by regulating HMGB1. These findings suggested that HMGB1 may be an effective target for inhibiting HBV-induced HCC.

Introduction

Hepatocellular carcinoma (HCC) is the most common type of liver tumor, and the mortality rate for HCC is the third highest of all types of cancer, following lung and stomach cancer (1). Hepatitis B virus (HBV) chronic infection is a risk factor for HCC (2), particularly in Asia and South Africa (3). HBV X protein (HBx), a 17-kDa multifunctional protein, serves an important role in HBV-associated HCC (4-6).

During chronic HBV infection, HBV DNA can integrate into the host genome (7). The HBx gene can be maintained and transcribed in human HCC cells, although complete HBV replication does not occur (7). Recent studies have demonstrated that HBx influences the entire process of HBV-associated HCC, including inflammation, cirrhosis and HCC development (8-10). HBx regulates a variety of signaling pathways, including phosphoinositide 3-kinase (PI3K)/protein kinase B (Akt)/mechanistic target of rapamycin, Wnt/β-catenin and P53, through modulating the expression and activity of numerous genes (11-13). These events also include epigenetic modification and mutation. However, it is difficult to eliminate HBx once it has been integrated into the host genome. Our previous study detected HBx, particularly truncated HBx, in HBV-derived HCC cell lines (e.g., Hep3B and SNU423) (14). Therefore, the identification of an effective target is critical for inhibiting the oncogenic function of HBx.

High mobility group box 1 (HMGB1) is a non-histone, nuclear DNA-binding protein (15). HMGB1 is also a multifunctional protein, and its function depends on its location in the cell (15). In the nucleus, as a DNA binding protein, HMGB1 has a role in a number of crucial DNA events (16-20). Under certain conditions, including starvation and reactive oxygen species (ROS), HMGB1 can translocate to the cytoplasm and can transit to the extracellular environment (21). Extra-nuclear HMGB1 regulates several signaling pathways affecting cell functions, including proliferation, autophagy and apoptosis (22-24). Additionally, extracellular HMGB1 can affect the microenvironment by inducing inflammation, fibrosis and angiogenesis (25-27). Our previous study revealed that the deletion of HMGB1 worsened liver ischemia/reperfusion...
injury (28). Recently, increasing evidence has indicated that HMGB1 has a significant association with HCC. For example, the HMGB1 serum level is increased in patients with chronic HBV and this is associated with the disease stage (29), and HMGB1 can also translocate outside of the nucleus in response to HBV infection (15). These findings suggested that HBV may regulate HMGB1.

The present study demonstrated that the HBx gene was expressed in HBV-derived cell lines. The expression of HMGB1 was associated with the HCC pathological grade and the overall survival of patients. The non-HBV-derived cell line Huh7 was selected to investigate the association between HBx and HMGB1. It was revealed that HBx could regulate the expression of HMGB1 and knockdown of HMGB1 could decrease the ability of HBx to promote cellular proliferation. Next, several transcription factors that had been reported to regulate HMGB1 were investigated using database analysis (e.g., Genecards and Targetscan). Through analysis of these transcription factors, the mechanism for HBx-mediated regulation of HMGB1 was identified. By analyzing the genes co-expressed with HMGB1 in patients with HBV, several key pathways that could promote the formation of HCC were identified. These findings suggested that HMGB1 is involved in the process of HBV-induced HCC.

Materials and methods

Acquisition of human tissue specimens. The three patients were aged 63, 48 and 46 years old (mean was 52.3 years) and comprised of 2 females and 1 male. All these three tissue were obtained in April 2015. Inclusion criteria for the present study were: Each patient was infected by HBV and HBx and HMGB1 had a higher expression in tumor tissue when compared with normal tissue. Paired HCC and adjacent liver tissues were obtained from patients who underwent hepatectomy at the Department of Hepatopancreatobiliary Surgery of the Second Affiliated Hospital of Harbin Medical University (Harbin, China). All human tissues were acquired in accordance with the protocol approved by the Ethics Committee of Harbin Medical University and written informed consent was obtained from all the patients.

Cell lines. The non-HBV-derived HCC Huh7 cell line was obtained from the Department of Surgery of the University of Pittsburgh Medical Center. The cells were used immediately following receipt and were cultured in Dulbecco's modified Eagle's medium with 10% fetal bovine serum (Hyclone; GE Healthcare Life Sciences, Logan, UT, USA) at 37°C with 5% CO2.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted with TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) according to the manufacturer's protocol. Next, 1 μg total RNA from each sample was reverse transcribed to single-stranded cDNA with RNA to cDNA EcoDry™ Premix (Clontech Laboratories, Inc., Mountainview, CA, USA). cDNA (1 μl) was diluted 50-fold with nuclease-free water and was used as a template for RT-qPCR. All the primers were purchased from Sangon Biotech Co., Ltd. (Shanghai, China). HBx forward, 5'-TTTTTCTCCTCGAGTCG-3' and reverse, 5'-TCAGATTCGACTTGGC-3'; HMGB1 forward, 5'-CAGTTGACACCTGGCT-3' and reverse, 5'-CTTGTTCTGAGGACGCT-3'; GAPDH forward, 5'-GGACGCGATGATCCTCGAG-3' and reverse, 5'-GGTTTCTATGCTGTGCT-3'; TBP forward, 5'-ACTCCACCTGTTATCCCTC-3' and reverse, 5'-TATATGGCCTGCGTTTCTGGCA-3'; HSF1 forward, 5'-CCATGAAAGCATGAGTATGGAGG-3' and reverse, 5'-CTTTGTTGACGACTTGGCTT -3'; ERBB3 forward, 5'-GGTTGATGGGGAACCTGAGAT-3' and reverse, 5'-CTGTCATCTCTGCAATCCACTG-3'; KLFR forward, 5'-CCCCATGAAGGGCAGTCC-3' and reverse, 5'-CAGGTTCCAGGAGATCTGGA-3'; NFKB1 forward, 5'-AACAGAGAGAGTTTCGTTTCG-3' and reverse, 5'-TTGAGCTCAGGGAAGACTCTT-3'.

The thermocycling conditions were as follows: 94°C 20 sec, 60°C 20 sec, 72°C 2 min, 30 cycles. Analysis of relative gene expression data was used real-time quantitative PCR and the 2ΔΔCq method (30). Gene expression of HBx and HMGB1 were normalized to GAPDH. Gene expression of TBP, HSF1, ERBB3, KLFR and NFKB1 were normalized to the control group. The reverse transcription reactions were prepared using the TaqMan Reverse Transcription Kit (Applied Biosystems; Thermo Fisher Scientific, Inc.). Each 15-μl multiplex reaction contained 10 ng total RNA as a template.

Western blots analysis. Whole protein was extracted using cell lysis buffer (Cell Signaling Technology, Inc., Danvers, MA, USA). The nuclear protein was extracted as previously described (23). BCA was used as the protein determination method. A total of 20 μg nuclear protein was electrophoresed on 10% SDS-polyacrylamide gels and transferred onto polyvinylidene difluoride membranes. Following blocking with 5% skimmed milk at room temperature for 1 h, the membranes were incubated with a 1:5,000 dilution of the primary antibodies with bovine serum albumin (Thermo Fisher Scientific, Inc.) for HMGB1, HBx and GAPDH (cat. nos. ab79823, ab39716 and ab181602, respectively) at 4°C overnight. Next, the membranes were washed with Tris-buffered saline and Tween-20 (TBST) three times, incubated with a 1:10,000 dilution of the secondary antibody (goat anti-rabbit; HRP; cat. no. ab19866) at room temperature for 1 h, and developed onto X-ray film using a chemiluminescent reagent (cat. no. c510043; Sangon Biotech, Co., Ltd.). All of the antibodies were purchased from Abcam (Cambridge, UK).

Plasmid construct and cell transfection. The pCDNA-3.1-HBx plasmid was purchased from Sangon Biotech Co., Ltd. The control shRNA and HMGB1 shRNA were purchased from Sigma-Aldrich; Merck KGaA (Darmstadt, Germany). The FuGene transfection reagent (Promega Corporation, Madison, WI, USA) was used at a reagent to plasmid ratio of 3:1 and the concentration was 1 μg/ml. Transfection was performed according to the manufacturer's protocol, and cells were harvested after 48 h for the following experiments.

Database analysis and statistical analysis. The Cancer Genome Atlas (TCGA) data were obtained from Synapse
Gene expression data and patient data from 377 patients were analyzed, however 6 patients did not have HBV information. The gene expression association analysis was completed using R2 (http://r2.amc.nl/) and Prism 5. The signaling pathway analysis was performed using Toppcogene (https://toppcogene.cchmc.org/) and R2. The statistical analysis was performed using Prism 5, and gene information was collected from Genecards (http://www.genecards.org/). Data are presented as the mean ± standard deviation. Two-way analysis of variance with Tukey's multiple comparison test was used.

Figure 1. HBx is expressed at a higher level in tumor tissue than in adjacent non-cancerous tissue, and truncated HBx can be detected in hepatitis B virus-derived HCC cell lines. (A) Western blot analysis and (B) relative protein expression of HBx, which was higher in tumor tissues than in adjacent non-cancerous tissues (**P<0.0001). (C) In the HBV-derived cell lines, truncated HBx was detected. HCC, hepatocellular carcinoma; HBx, hepatitis B virus-X protein.

Figure 2. HMGB1 is highly expressed in tumor tissue and high HMGB1 expression is associated with patient prognosis. (A) Western blot analysis and (B) relative protein expression of HMGB1, which was higher in HCC tumor tissue than in the adjacent non-cancerous tissue (**P<0.0001). (C) Patients overexpressing HMGB1 exhibited a poor prognosis. (D) High expression of HMGB1 was associated with tumor grade. Gene expression data were retrieved from The Cancer Genome Atlas. HCC, Hepatocellular carcinoma; HMGB1, high mobility group box 1.

TCGA (https://www.synapse.org/).
A comparison test was used for raw data analysis and P<0.05 was considered to indicate a statistically significant difference.

**Results**

*HBx expression is higher in tumor tissue than in adjacent noncancerous tissue, and truncated HBx can be detected in HBV-derived cell lines.* The results of in vivo studies revealed that HBx protein expression was higher in HCC tumor tissue than in the adjacent non-cancerous tissue (Fig. 1A and B). Once the HBx gene is integrated into the host genome, it is difficult to eliminate. Accordingly, in *in vitro* studies, truncated HBx was detected (Fig. 1C), which is the form of HBx with a greater effect (14). These results suggested that HBx is expressed in liver cells for a long time and contributes to the process of HCC.

*HMGB1 expression is higher in tumor tissue than in adjacent non-cancerous tissue, and a high level of HMGB1 is associated with the pathological grade of HCC and the survival of patients.* The protein level of HMGB1 was higher in the tumor tissue than in the adjacent non-cancerous tissue (Fig. 2A and B). By analyzing TCGA data, the present study revealed that the prognosis of patients with high-level HMGB1 expression was poor (Fig. 2C). These findings suggested that abnormal expression of HMGB1 can induce HCC.

*HBx can regulate the expression of HMGB1.* The gene expression level of HMGB1 and HBx were also measured. HBx, hepatitis B virus-X protein; HMGB1, high mobility group box 1; HBV, hepatitis B virus.

*HMGB1 is required for HBx to promote the cellular proliferation.* To investigate the role of HMGB1 in the carcinogenic process of HBx, a HBx stable expression cell
Figure 4. HMGB1 is required for HBx to promote cellular proliferation. (A) Reverse transcription-quantitative polymerase chain reaction for HMGB1 gene expression in the HBx stable expression cells following transfection for 48 h with control shRNA or HMGB1 shRNA. (B) Knockdown of HMGB1 decreased cellular proliferation in HBx stable expression cells. (C) Western blot analysis for HMGB1 protein expression in the indicated cells following transfection for 72 h with control shRNA or HMGB1 shRNA. HMGB1, high mobility group box 1; HBx, hepatitis B virus-X protein; shRNA, short hairpin RNA; OD, optical density.

Figure 5. HBx increases the expression of HMGB1 by regulating transcription factors associated with HMGB1. (A) The gene expression of the six transcription factors in patients with hepatocellular carcinoma. (B) Several of these transcription factors were highly expressed in HBV-infected patients. (C) HBx could upregulate the expression of these transcription factors. Gene data were obtained from The Cancer Genome Atlas. R2 and Prism5 were used to analyze these data. HBx, hepatitis B virus-X protein; HMGB1, high mobility group box 1; HBV, hepatitis B virus; GATA3, GATA binding protein 3; ERBB3, Erb-B2 receptor tyrosine kinase 3; HSF1, heat shock transcription factor 1; NFKB1, nuclear factor κB subunit 1; TBP, TATA-box binding protein; KLF4, Kruppel-like factor 4.
line was created (Fig. 4A and B). HMGB1 was knocked down in the HBx stable expression cell line and it was revealed that the ability of HBx to promote proliferation was decreased (Fig. 4C).

**HBx increases the expression of HMGB1 by regulating transcription factors associated with HMGB1.** To clarify the molecular mechanism of HBx-mediated regulation of HMGB1, several transcription factors associated with HMGB1 that could be expressed in HCC were investigated. Eventually, six possible transcription factors: GATA3, ERBB3, HSF1, NFKB1, TBP and KLF4 were identified (Fig. 5A). Several of these transcription factors were highly expressed in patients with HBV (Fig. 5B).

**HBx affects a variety of cellular functions through regulation of HMGB1.** A number of genes that are co-expressed with HMGB1 were abnormally expressed in HBV-induced HCC. Therefore, several genes that exhibited a significant associated with HMGB1 were further examined. Next, the top 19 genes were selected to analyze the functions associated with HMGB1 (Table I). By analyzing these genes using Toppgene, the top 16 signaling pathways enriched for genes that are co-expressed with HMGB1 were obtained (Table II).

**Discussion**

HBx serves an important role in the process of HBV-induced HCC (Fig. 1A and B). The carcinogenic mechanisms of HBx have been reported in numerous previous studies (7-13). The HBx gene integrates into the host cell genome during HBV infection (Fig. 1C) (14) and is very difficult to completely remove. Therefore, the best method for inhibiting the function of HBx is to identify an effective target to prevent integration.

HMGB1 is a multifunctional protein, and its function depends on its location. Abnormal expression of HMGB1 in different organs can induce different diseases (15). High expression of HMGB1 can influence several cell functions and the microenvironment of the liver (15‑27); accordingly, HMGB1 has been associated with the development and progression of HCC (Fig. 2) and has also been associated with HBV infection (29). In the present study, it was confirmed that HBx could increase the expression of HMGB1 through the regulation of transcription factors (Figs. 3 and 5). Therefore, HMGB1 may be an ideal target for inhibiting the carcinogenic function of HBx.

To study the molecular mechanisms of HBx-induced HCC through the regulation of HMGB1, genes significantly associated with HMGB1 in patients with HBV were investigated (Table I). Next, these genes were sorted into signaling pathways, as presented in Table II. Abnormalities in some of these pathways have been reported to be associated with HBx and HMGB1. For example, the P53 signaling pathway is a classical tumor suppressor pathway, and HBx and HMGB1 can regulate cell proliferation and apoptosis by suppressing P53 (2,31). PI3K/Akt is an important signaling pathway through which HBx can induce autophagy (32), and HMGB1 is involved in the same pathway when responding to oxidative stress (33). Furthermore, the Toll-like receptor (TLR) signaling pathway also contributes to HBx-induced carcinogenesis (34), and a previous study demonstrated that HMGB1 could increase the expression of mitochondria in HCC by binding to TLRs (35).

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**Table I. Top 19 genes co-expressed with HMGB1 in patients with HBV.**

| Gene     | Function                                                                 | R²     | P-value            |
|----------|---------------------------------------------------------------------------|--------|--------------------|
| CDKN1B   | Cell cycle, development                                                  | 0.303  | 2.5x10⁻⁹           |
| PCNA     | DNA repair, signaling transduction                                        | 0.289  | 1.5x10⁻⁸           |
| CDK2     | Cell cycle, kinase                                                       | 0.204  | 7.6x10⁻⁵           |
| CREB1    | TF, drug target, signal transduction, transcription regulator activator  | 0.199  | 1.2x10⁻⁴           |
| CCNE2    | Cell cycle                                                               | 0.193  | 1.9x10⁻⁴           |
| BIRC5    | Apoptosis, cell cycle                                                    | 0.188  | 2.7x10⁻⁴           |
| NFKB1A   | Apoptosis, signal transduction                                            | 0.186  | 3.1x10⁻⁴           |
| CCNA2    | Cell cycle                                                               | 0.171  | 9.8x10⁻⁴           |
| YWHAQ    | Signal transduction                                                      | 0.169  | 1.1x10⁻³           |
| E2F1     | TF, apoptosis, cell cycle, development, transcription regulator activator | 0.168  | 1.2x10⁻³           |
| CREB3L4  | TF, transcription regulator activator                                     | 0.144  | 5.5x10⁻³           |
| CDK4     | Cell cycle, kinase                                                       | 0.144  | 5.5x10⁻³           |
| RB1      | TF, cell cycle, drug target, signal transduction, transcription regulator activator | 0.133  | 1.0x10⁻²           |
| TGFBR1   | Kinase, membrane, signal transduction                                     | 0.123  | 2.0x10⁻²           |
| E2F2     | TF, cell cycle, transcription regulator activator                         | 0.117  | 2.0x10⁻²           |
| SMAD4    | TF, development, signal transduction, transcription regulator activator  | 0.113  | 3.0x10⁻²           |
| STAT4    | TF, signal transduction, transcription regulator activator               | 0.109  | 4.0x10⁻²           |
| APAF1    | Apoptosis, development, drug target                                      | 0.105  | 4.0x10⁻²           |
| BAX      | Apoptosis, cell cycle, development, differentiation, membrane           | 0.102  | 5.0x10⁻²           |

HBV, hepatitis B virus; HMGB1, high expression of high mobility group box 1.
Table II. Top 16 signaling pathways enriched for genes that were co-expressed with high mobility group box 1.

| Name                                           | Source                           | P-value    | FDR B&H   | FDR B&Y   | Bonferroni | Gene input |
|------------------------------------------------|----------------------------------|------------|-----------|-----------|------------|------------|
| Cell cycle                                     | BioSystems: WikiPathways         | 6.16x10^-17| 1.57x10^-14| 1.09x10^-13| 3.6x10^-14| 11         |
| p53 signaling pathway                          | MSigDB C2 BIOCARTA (v5.1)       | 1.07x10^-15| 1.04x10^-13| 7.23x10^-13| 6.25x10^-13| 7          |
| DNA damage response                             | BioSystems: WikiPathways         | 8.61x10^-15| 6.28x10^-13| 4.37x10^-12| 5.03x10^-12|            |
| Viral carcinogenesis                            | BioSystems: KEGG                 | 1.67x10^-13| 7.49x10^-12| 5.20x10^-11| 9.74x10^-11| 11         |
| Pathways in cancer                              | BioSystems: KEGG                 | 8.38x10^-13| 3.49x10^-11| 2.43x10^-9 | 4.89x10^-10| 12         |
| PI3K-Akt signaling pathway                      | BioSystems: KEGG                 | 1.64x10^-10| 6.26x10^-11| 4.35x10^-9 | 9.55x10^-10| 12         |
| E2F transcription factor network                | BioSystems: Pathway Interaction Database | 1.82x10^-12| 6.26x10^-11| 4.35x10^-9 | 1.06x10^-9 | 8          |
| DNA replication                                 | BioSystems: REACTOME             | 2.01x10^-9 | 4.71x10^-8 | 3.27x10^-7 | 1.18x10^-6 | 7          |
| Regulation of IFNA signaling                    | BioSystems: REACTOME             | 2.10x10^-9 | 4.72x10^-8 | 3.28x10^-7 | 1.23x10^-6 | 5          |
| TRAF6-mediated IRF7 activation                  | BioSystems: REACTOME             | 6.38x10^-9 | 1.1x10^-7 | 7.61x10^-7 | 3.73x10^-6 | 5          |
| Regulation of autophagy                         | BioSystems: KEGG                 | 8.79x10^-9 | 1.39x10^-7 | 9.64x10^-7 | 5.13x10^-6 | 5          |
| Integrated cancer pathway                       | BioSystems: WikiPathways         | 1.02x10^-8 | 1.57x10^-7 | 1.09x10^-6 | 5.98x10^-6 | 5          |
| Regulation of p27 phosphorylation during cell cycle progression | MSigDB C2 BIOCARTA (v5.1) | 1.27x10^-8 | 1.85x10^-7 | 1.29x10^-6 | 7.41x10^-6 | 4          |
| RIG-I/MDA5-mediated induction of IFN-α/β pathways | BioSystems: REACTOME             | 1.55x10^-8 | 2.21x10^-7 | 1.54x10^-6 | 9.06x10^-6 | 6          |
| IL-7 signaling pathway                          | BioSystems: WikiPathways         | 3.38x10^-8 | 4.45x10^-7 | 3.09x10^-6 | 1.97x10^-4 | 5          |
| Regulation of TLR signaling pathway             | BioSystems: WikiPathways         | 6.74x10^-8 | 8.2x10^-7  | 5.7x10^-6  | 3.94x10^-5 | 6          |

PI3K-Akt, phosphoinositide 3-kinase-protein kinase B; IFNA, interferon α; TRAF6, tumor necrosis factor receptor associated factor 6; IRF7, interferon regulatory factor 7; RIG-I/MDA5, retinoic acid-inducible gene 1/melanoma differentiation-associated protein 5; IL-7, interleukin 7; TLR, toll-like receptor.
In conclusion, HBx and HMGB1 have a synergistic effect in promoting HCC. The present study confirmed that HBx could regulate HMGB1. Additionally, several signaling pathways associated with HBx and HMGB1 that may serve as the mechanism whereby HBx promotes HCC through the regulation of HMGB1 were identified. Nevertheless, further investigation is required to identify the precise mechanism through which HBx induces HCC through HMGB1 regulation.

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Availability of data and materials

HCC TCGA data were obtained from http://www.synapse.org/. All other data analyzed during the current study are included in the published article.

Authors' contributions

DW and HL contributed to experiment design, drafting the manuscript and revising. HW, CD, HY and IJZ conducted the western blotting and PCR experiments, and data collection. YP, BS, ZS, JW and TC contributed to data analysis. ST contributed to experiment design and drafting the manuscript.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of Harbin Medical University and written informed consent was obtained from all the patients.

Consent for publication

All patients provided consent for the publication of the paper and any associated images.

Competing interests

The authors declare that they have no competing interests.

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