The Effects and Underlying Mechanisms of Hepatitis B Virus X Gene Mutants on the Development of Hepatocellular Carcinoma

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Research

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Abstract

Background: We aimed to elucidate the mechanism by which hepatitis B virus X (HBx) gene mutations increase the risk of hepatocellular carcinoma (HCC) and identify novel therapeutic targets.

Methods: Wild-type and four HBx mutants (M1, A1762T/G1764A; M2, T1674G+T1753C+A1762T/G1764A; M3, C1653T+T1674G+A1762T/G1764A; Ct-HBx, carboxylic acid-terminal truncated HBx) were delivered into the livers of fumarylacetoacetate hydrolase-deficient mice by using the *Sleeping Beauty* (SB) transposon system, respectively. Seven liver tissues and seven tumor tissues of the SB mouse models were subjected to HBV-capture sequencing. Three liver tissues from WT-HBx mice, three tumor tissues from M3-HBx mice, and three tumor tissues from Ct-HBx mice were subjected to cDNA microarray analysis. HeLa cells stably expressing WT-HBx and the four HBx mutants were also subjected to cDNA microarray assay.

Results: The incidence of HCC was higher in the mice injected with M3-HBx or Ct-HBx. M3-HBx had a stronger capacity of upregulating inflammatory cytokines than other HBx variants. HBV-capture sequencing showed that the HBx fragments were mainly integrated into intergenic and intron regions. No significant difference was observed in the number of insertion sites between tumors and liver tissues. Ectopic expression of the HBx mutants, especially M3-HBx and Ct-HBx, significantly increased cell proliferation and the S phase proportion of HepG2 and HeLa cells, compared to WT-HBx. Liver tissues of the SB mice and the transfected cells were subjected to cDNA microarray analysis. Plasminogen activator inhibitor-1 (PAI1) and cell division cycle 20 (CDC20) were identified as novel effectors. M3-HBx and Ct-HBx significantly upregulated the expression of PAI1 and CDC20 in HepG2 and HeLa cells as well as the livers of the SB mice. PAI1 silencing attenuated the effect of M3-HBx and Ct-HBx on the growth of HepG2 cells and greatly decreased the growth of HeLa cells with Ct-HBx.

Conclusion: HBx C1653T+T1674G+A1762T/G1764A mutant and Ct-HBx promote carcinogenesis via upregulating PAI1 and CDC20. PAI1, an important player bridging the HBx mutants and HCC, should be a promising candidate as a predictive and prognostic biomarker and therapeutic target in HBV-related HCC.

Background

Primary liver cancer (PLC) was the third leading cause of cancer death worldwide in 2020 and hepatocellular carcinoma (HCC) accounts for 75–85% of PLC cases [1]. Chronic infection with hepatitis B virus (HBV) is the leading cause, contributing to 44–55% of global HCC [2]. Different countries have different rates of the seropositivity for HBV surface antigen (HBsAg) in HCC. Around 50–80% HCC cases in China are seropositive for HBsAg [3]. HBV-related HCC (HBV-HCC) is associated with 10-year earlier onset, higher α -fetoprotein (AFP), and more microvascular invasion than HCC caused by other causes [4], indicating that HBV is more powerful in promoting HCC development than other etiological factors.

During HBV-induced hepatocarcinogenesis, HBV often evolves via accumulating its mutations adapted to the inflammatory microenvironment and integrating into the human genome [5, 6]. HBV mutations,

including A1762T/G1764A, C1653T, T1753V, and T1674G/C, in the core promoter (CP) region of the viral genome are typically the ones that increase the risk of HCC [7–9]. It has been demonstrated that the combination (combo) mutant, rather than HBx with single or double CP mutations, accelerates cell cycle progression and p21 degradation, possibly via increasing expression of S-phase kinase-associated protein 2 (SKP2) [10]. Gene expression profiling of human hepatocytes infected with HBV genotype F1b, particularly the CP mutant, indicates more cancer-related signaling pathways compared with other genotypes [8]. HBV integration into the host genome often leads to the truncation of the HBV genome, particularly at the C terminus of HBx, resulting in the generation of carboxylic acid terminal-truncated HBV X protein (Ct-HBx) [11]. The HBV X genes encoding Ct-HBx, which are most frequently detected in HBV-HCC samples, significantly increase the aggressiveness of HCC compared to the full-length X gene, possibly via downregulating thioredoxin-interacting protein, a well-established regulator of glucose metabolism [12]. However, the gene expression profiles of the HBV mutant-integrated liver of animal models with intact immune system, which represents the key molecular event in humans, are not reported. Here, we investigated tumorigenic effects of combo HBx mutations (A1762T/G1764A, C1653T, T1753C, and T1674G), Ct-HBx, and wild-type (WT) HBx on HCC using the *Sleeping Beauty* (SB) transposon system to deliver WT-HBx and HBx mutants into the livers of fumarylacetoacetate hydrolase (*Fah*)-deficient mice. The functional genes with similar profiles in the animal model and cell lines were functionally investigated. This study not only helps elucidate the mechanisms by which HBx mutants promote HBV-induced carcinogenesis but also reveals effective therapeutic options for HBV-HCC.

Methods

Plasmid construction

HBx fragments (nt. 1374–1838) were amplified from the sera of 10 HBV-infected patients included in a previous study [9]. The details for viral DNA extraction, HBV DNA sequencing, and mutation analysis are provided in the Supplementary Materials. We selected four HBx mutants carrying the HCC-risk mutations: M1, A1762T/G1764A alone; M2, A1762T/G1764A + T1674G + T1753C; M3, A1762T/G1764A + C1653T + T1674G, and Ct-HBx (nt. 1374–1733) (Figure S1). WT-HBx and the HBx mutants were linked with flag tags and inserted into *EcoRI* restriction sites of the pKT2-FAH-mCa-SB plasmid, a SB transposon vector containing the cDNA of *Fah* gene, respectively [13]. The constructs were verified by Sanger sequencing. Primers for the amplification and verification are listed in Table S1. The construction of recombinant lentiviruses expressing WT-HBx and HBx mutants is described in the Supplementary Materials.

Cell experiments

HepG2 and HeLa cell lines were purchased from the Chinese Academy of Sciences (Shanghai, China). Before the experiments, all cell lines were authenticated using the genotyping analysis of short tandem repeat (STR) by Biowing Biotechnology (Shanghai, China). All cell cultures were tested for mycoplasma contamination every three months. The cells with the stable overexpression of HBx variants were constructed with lentivirus. Details are available in the Supplementary Materials. Small interfering RNAs

(siRNAs) against cell division cycle 20 (CDC20), plasminogen activator inhibitor-1 (PAI1), and cyclin dependent kinase inhibitor 1A (P21) were synthesized by GenePharma (Shanghai, China) (Table S2). Lipofectamine 3000 kit (Invitrogen, Carlsbad, CA) was applied for siRNA transfection. Gene expression was measured by quantitative reverse transcription PCR (qRT-PCR) and Western blot (Figure S2). Cell proliferation, migration, invasion, cell cycle assay, qRT-PCR, and Western blot are detailed in the Supplementary Materials.

Mouse models

The mouse model of HBx protein-induced HCC was constructed based on *Fah*-deficient mice [13]. The *Fah*^{-/-} mice were maintained with 7.5 µg/mL 2-(2-nitro-4-trifluoromethylbenzoyl)-1, 3-cyclohexanedione (NTBC). At the age of 5–7 weeks, mice were injected with 15-µg constructs containing *Fah* cDNA and the HBx mutants or WT by hydrodynamic injection via tail vein. After the injection, NTBC was removed from the drinking water. Mice that died within 30 days after the injection were excluded from the following analyses due to the failure in gene delivery. All mice were sacrificed on Day 150 after the infection. The livers and tumors were pathologically examined. Hematoxylin–eosin (H&E) and immunohistochemistry (IHC) are detailed in the Supplementary Materials. The antibodies used for IHC are detailed in Table S3.

Five-week-old Nod-SCID mice were purchased from Jihui Laboratory Animal Care Cooperation (Shanghai, China). Mice were subcutaneously injected with 1.5×10^6 HeLa cells stably expressing wild-type or mutant HBx protein. Five weeks later, tumors were harvested. All animal studies were conducted under the animal welfare protocol approved by the ethics committee of Second Military Medical University.

HBV capture sequencing

Seven liver tissues and seven tumor tissues of the *SB* mouse models were subjected to HBV-capture sequencing. Liver tissues were from the mice without tumors, including three WT-HBx mice, one M3-HBx mouse, and three Ct-HBx mice. Tumor tissues were from one WT-HBx mouse, three M3-HBx mice, and three Ct-HBx mice. DNA was extracted from tissues using Genomic DNA Mini Kit (Invitrogen, Carlsbad, CA). The DNA libraries were constructed using the Fast Library Prep Kit (iGeneTech, Beijing, China). The capture probes of HBV panel were synthesized by iGeneTech. The libraries were captured using the probes of HBV panel and TargetSeq one enrichment kits (iGeneTech). The HiSeq™ 2500 platform (Illumina, San Diego, CA) was applied for the sequencing. The breakpoints of HBV integration were detected using VERSE software [14]. The annotation for the integrated breakpoints was performed with ANNOVAR software [15]. Data of HBV-capture sequencing were uploaded to the Sequence Read Archive (SRA) database under Accession Number PRJNA743432.

Cytokine assessment

The cytokine levels in mice plasma were measured by multiplex immunoassay with ProcartaPlex Kit (ThermoFisher Scientific, MA). In total, 11 cytokines were tested: interleukin-4 (IL-4), IL-5, IL-6, IL-1β, IL-12, interferon-α (IFNα), IFNγ, tumor necrosis factor-α (TNFα), granulocyte macrophage colony stimulating factor (GM-CSF), transforming growth factor β (TGFβ), and vascular endothelial growth factor (VEGF).

Microarray profiling analysis

HeLa cells stably expressing WT-HBx and the four HBx mutants were subjected to cDNA microarray assay by using Human Transcriptome Array 2.0 (Affymetrix, Santa Clara, CA). Differently expressed genes between WT-HBx group and each of the mutant HBx groups were identified using the edgeR package [16]. The significantly expressed genes were subjected to gene set enrichment analysis (GSEA) using GSEA software (<http://software.broadinstitute.org/gsea/>). Based on the significantly enriched genes, protein–protein interaction (PPI) networks were generated using STRING v10 software (<https://string-db.org/>) [17]. Three liver tissues from WT-HBx mice, three tumor tissues from M3-HBx mice, and three tumor tissues from Ct-HBx mice were also subjected to cDNA microarray analysis. The samples were randomly selected from each mouse group. Agilent-074809 SurePrint G3 Mouse GE V2.0 microarray (Agilent Technologies, Santa Clara, CA) was applied for this analysis. Mouse genes were converted to human homologs using the HUGO Gene Nomenclature Committee database. The gene profiling data of the *SB* mouse models and HeLa cells were uploaded to the Gene Expression Omnibus (GEO) database under Accession Numbers GSE179125 and GSE179126, respectively.

Luciferase reporter assay

Promoter region was defined as 2000 bp upstream of the transcription start site of a given gene based on the Ensembl database (GRCh38.p13, https://asia.ensembl.org/Homo_sapiens). The promoter fragments of *CDC20*, *PAI1*, and *P21* were synthesized and sequenced by Obio Technology (Shanghai, China). The correct sequences were inserted into the firefly luciferase reporter vector (pGL4.10-basic vector, Promega, Madison, WI) to evaluate the promoter activity. HepG2 and HeLa cells were co-transfected with the constructed reporter vectors and Renilla luciferase vectors (pRL-TK, Promega). Luciferase activities were tested using a dual-luciferase reporter assay system (Promega). Firefly luciferase activity was normalized to Renilla luciferase activity.

Statistical analysis

Differences in continuous variables were determined by Student's *t*-test. For non-normal data, the Wilcoxon sum rank test was used. The analyses of the data from *in vitro* and *in vivo* experiments were performed with GraphPad Prism version 5.0 (GraphPad Software, San Diego, CA). All statistical tests were two-sided. $P < 0.05$ was considered statistically significant.

Results

Effects of WT-HBx and the HBx mutants on hepatocarcinogenesis in *SB* mice

Empty *SB* vector and those carrying WT-HBx and the HBx mutants were successfully delivered into 42 mice. None of the empty vector-injected mice ($n=9$) developed tumors. Tumors and shrunken and cirrhotic livers were evident in M2-HBx-, M3-HBx-, and Ct-HBx-injected mice (shown in Fig 1). The incidence of tumors was 14.3% (1/7), 50% (2/4), 42.8% (3/7), 87.5% (7/8), and 57.1% (4/7) in the *SB* models whose hepatic genomic DNA was integrated with WT-HBx, M1-HBx, M2-HBx, M3-HBx, and Ct-HBx, respectively.

Compared to WT-HBx-injected mice, all HBx mutants-injected mice displayed a trend toward higher tumor burden, especially the M3-HBx and Ct-HBx groups. No apparent pathological change was observed in the livers of the vector-injected mice. Inflammatory cell infiltration and cancer nests were observed in the HBx mutants-injected mice (shown in Fig 2A), which were similar to the histopathological changes of mouse hepatitis-induced HCC [18]. Histopathologic analyses revealed that inflammatory cell infiltration was more severe in livers injected with HBx mutants than in those with WT-HBx. Our IHC analysis confirmed that the tumors were positive for HBx protein as well as cytokeratin 18 (CK18) and α -fetoprotein (AFP), the classic biomarkers of HCC (shown in Fig 1).

The insertion sites of HBx fragments

The integration sites of WT-HBx and the HBx mutants in the *SB* mice were examined via HBV-capture sequencing. In total, 1750 integrations were identified (Table S4). HBx fragments were mostly integrated in the intergenic and intron regions, with only 3.71% located in the exon regions. There were nine genes with the insertion sites detected in more than three samples (Table S5). The first three most frequently integrated genes were *Fah* (42.8%), catenin alpha 3 (*Ctnna3*) (28.6%), and fragile histidine triad gene (*Fhit*) (28.6%). Gene encoding telomerase reverse transcriptase (TERT) was not an integration site. The nine frequently integrated genes have low ratios of the reads with HBx integration to the normal reads, ranging from 0.09% to 0.25%. The difference in the levels of integration sites was not significant between liver tissues and tumor tissues (shown in Fig 2B). The number of integration sites was not significantly different among WT-HBx-, M3-HBx-, and Ct-HBx-injected *SB* mice (shown in Fig 2C). These data suggest that HCCs developed in the *SB* mice were induced by the HBx proteins, rather than insertional mutations.

Serum levels of proinflammatory cytokine in WT-HBx and the HBx mutants *SB* mouse models

The levels of Th1 cytokines (IFN γ , TNF α , IL-1 β , and IL-12), Th2 cytokines (IL-4 and IL-5), IL-6, IFN α , TGF β , VEGF, and GM-CSF were detected in the sera of the *SB* mice (shown in Fig 3 and Fig S3). Among the Th1 cytokines, IFN γ and TNF α were positive in all groups (shown in Fig 3A, 3B). The level of IFN γ was significantly higher in the M3-HBx-injected mice than in the controls. The positivity rate of IL-1 β was significantly higher in M2-HBx- and M3-HBx-injected mice than in the empty-vector-injected mice (shown in Fig 3C). IL-5 and IL-6 were measurable in all samples. The levels of IL-5 and IL-6 were significantly higher in M3-HBx mice than in the empty vector- and WT-HBx-injected mice (shown in Fig 3E, 3F). The levels of IFN γ , TNF α , IL-5, and IL-6 were significantly higher in the mice with tumors than in the tumor-free mice (shown in Fig 3G). The positive rate of IL-1 β was also significantly higher in the mice with tumors (shown in Fig 3H). IFN α was only positive in two M3-HBx-injected mice and one Ct-HBx-injected mouse (shown in Fig S3). IL-4 was only positive in one Ct-HBx-injected mouse. The HBx mutants did not upregulate the expression of TGF- β , VEGF, and GM-CSF (shown in Fig S3).

Effects of the HBx mutations on malignant phenotypes of cancer cells

To investigate whether the function of the HBx mutation was hepatocyte-specific, we evaluated the oncogenic effects of the HBx mutants in HepG2 and HeLa cells. The expression of WT-HBx and HBx

mutants (M1-HBx, M2-HBx, M3-HBx, and Ct-HBx) in HepG2 and HeLa cells via the lentiviral infection was confirmed by Western blot (shown in Fig S4A). Ectopic expression of M1-HBx, M2-HBx, M3-HBx, and Ct-HBx significantly increased cell proliferation and the S phase proportion of HepG2 and HeLa, compared to WT-HBx (shown in Fig 4A, 4B). No significant difference in migration and invasion was observed between HepG2 cells expressing WT-HBx and those expressing HBx mutants; the same was true for HeLa cells (shown in Fig 4C and shown in Fig S4B–D). The effects of HBx mutants on cell proliferation, cell cycle, and migration were repeated in HeLa cells (shown in Fig 4D–F). As M3-HBx and Ct-HBx displayed the strongest tumorigenic capability in the *SB* mouse models, the effects of M3-HBx and Ct-HBx on the growth of cancer cells were further investigated using the xenograft of HeLa cells in nude mice (HepG2 is not tumorigenic in nude mice). It was found that the tumor weight was significantly higher in HeLa cells with M3-HBx than in those with WT-HBx (shown in Fig 4G). Thus, the HBx mutants, especially M3-HBx and Ct-HBx, promote cancer development, and this effect is not hepatocyte-specific.

Gene expression profiles affected by the HBx mutants

Based on cDNA microarray assay of HeLa cells, we identified the differentially expressed genes in each of the mutant HBx groups relative to WT-HBx group. The PPI network for each HBx mutant group was generated using STRING software. Six hub genes were selected because they had strong PPI function (the highest STRING score) in at least one PPI network (shown in Fig S5A). The six hubs are E3 ubiquitin protein ligase (also named MDM2), replication factor C subunit 3 (RFC3), polo-like kinase 1 (PLK1), cell division cycle 6 (CDC6), early growth response 1 (EGR1), and PAI1. The effects of HBx mutants on the expression of these hub genes were also investigated with the microarray data of the *SB* mouse models with the highest tumor burden, M3-HBx and Ct-HBx. WT-HBx mice served as control. The expression levels of PAI1 were significantly higher in M3-HBx mice (fold change (FC) = 16.93, $P < 0.001$) and Ct-HBx mice (FC = 3.36, $P = 0.001$) than in WT-HBx control (Table S6).

The differentially expressed genes identified in HeLa cells and those identified in M3-HBx- and Ct-HBx-injected mice vs. WT-HBx-injected mice were subjected to GSEA analysis. Four cancer-related gene sets [19–22] were identified in both microarray assays: BERENJENO_TRANSFORMED_BY_RHOA_UP, MARKEY_RB1_CHRONIC_LOF_UP, VERNELL_RETINOBLASTOMA_PATHWAY_UP, and CHIANG_LIVER_CANCER_SUBCLASS_PROLIFERATION_UP. The first three gene sets were significantly enriched by M3-HBx and Ct-HBx in the cell and tissue microarray data. The remaining one was significantly enriched by M3-HBx in both cell and tissue microarray data. The four cancer-related gene sets included PAI1, P21, SKP2, and CDC20, respectively (shown in Fig 5A–D). PAI1 is the hub gene of the PPI network; P21 and CDC20 were also identified as functional molecules in the PPI network. P21 was significantly upregulated in both M3-HBx-injected mice (FC = 3.26, $P = 0.006$) and Ct-HBx-injected mice (FC = 1.77, $P = 0.009$). CDC20 was significantly upregulated in M3-HBx-injected mice (FC = 2.64, $P = 0.005$). SKP2, a molecule previously identified to be involved in HBx-induced carcinogenesis [10], was not identified in the PPI analysis. M3-HBx- and Ct-HBx-injected mice had a trend toward a higher level of SKP2 (M3-HBx, FC = 1.66, $P = 0.08$; Ct-HBx, FC = 1.67, $P = 0.09$), compared to WT-HBx-injected control. We also identified four inflammation-related gene sets [23–27] in the *SB* models. IL-6-related gene set,

CROONQUIST_IL6_DEPRIVATION_DN, was significantly enriched in the M3-HBx- and Ct-HBx-injected mice (shown in Fig S5B, S5C). The IFN- γ receptor 1-related gene set, MATSUDA_NATURAL_KILLER_DIFFERENTIATION, and an inflammatory response-related gene set, NEMETH_INFLAMMATORY_RESPONSE_LPS_DN, were significantly enriched in the M3-HBx-injected mice (shown in Fig S5D, S5E). In total, 317 genes were involved in the four cancer-related gene sets, while 197 genes were involved in the four inflammation-related ones. Overall, 77 genes overlapped in the cancer-related gene sets and the inflammation-related gene sets. In total, 72 genes were involved in the two IL-6-related gene sets enriched in M3-HBx- and Ct-HBx-injected mice. Interestingly, of the 72 genes, 55 (76.3%), including P21, SKP2, and CDC20, were also included in the cancer-related gene sets.

Effects of the HBx mutants on the expression of functional molecules

We then investigated the expression levels of the functional molecules identified in the above bioinformatics in HepG2 and HeLa cells stably expressing WT-HBx or HBx mutants. The transcription levels of *MDM2*, *RCF3*, *PLK1*, *CDC6*, and *EGR1* were not significantly affected by the four HBx mutants (shown in Fig S6). Interestingly, the mRNA and protein levels of PAI1 and CDC20 were significantly upregulated by M3-HBx and Ct-HBx in HepG2 and HeLa cells (shown in Fig 6A–D). HBx combo mutations (M1-HBx, M2-HBx, and M3-HBx) significantly downregulated the mRNA level and protein level of p21 in HepG2 rather than in HeLa cells (shown in Fig 6B, 6D). However, Ct-HBx significantly upregulated the expression of p21 in both HepG2 and HeLa cells. At the protein level, Ct-HBx significantly upregulated SKP2 in HeLa and HepG2 cells. The HBx mutants showed consistent effects on the RNA and protein expression of PAI1, CDC20, and p21, respectively. However, our luciferase assays showed that M3-HBx and Ct-HBx had no direct effects on the promoter activities of the three molecules, compared with WT-HBx in HepG2 and HeLa cell lines (shown in Fig S7). We then investigated the expression of PAI1, CDC20, and p21, the newly identified molecules participating in HBx-induced carcinogenesis, in the tumor tissue of the *SB* mice. The protein levels of CDC20 and PAI1 were significantly higher in the tumors from M3-HBx and Ct-HBx-injected mice, compared to their expression in the tumors from WT-HBx-injected mice (shown in Fig 7A, 7B). The protein level of p21 in the tumors was significantly lower in the M2-HBx group than in the WT-HBx group.

Reversal of HBx mutations' effects by silencing functional molecules

To figure out if the HBx mutations regulate the biological activity of HepG2 and HeLa cells via upregulating PAI1, CDC20, and p21, we knocked-down the three genes and then examined the effect of stably expressed WT-HBx and HBx mutants on the proliferation of HepG2 and HeLa cells. PAI1 silencing significantly neutralized the effects of M3-HBx and Ct-HBx on the proliferation and cell cycle of HepG2 and HeLa cells. CDC20 silencing significantly reversed the effects of M3-HBx on the proliferation and cell cycle in HeLa cells. P21 silencing reversed the Ct-HBx-induced increase in the proportion of S phase cells, rather than cell proliferation in HeLa and HepG2 cells (shown in Fig 8).

Discussion

In this study, we found that the HBx mutants-injected mice displayed a trend toward higher tumor burden, especially the M3-HBx- and Ct-HBx-injected mice, than the WT-HBx counterpart. This result is consistent with a previous study demonstrating that the HBx with A1762T/G1764A (K130M/V131I) mutation (the same as M1-HBx in our study) had a stronger tumorigenic effect than its WT counterpart in a *SB* mouse model [28]. M2-HBx and M3-HBx contain two more HCC-risk mutations [7–9]. M3-HBx- and Ct-HBx-injected mice were more tumorigenic than M1-HBx-injected mice. As WT-HBx and the HBx mutants were integrated into the hepatic genome, HCC development can be caused by the integration site such as TERT [29, 30]. We then applied HBV-capture sequencing to identify the integration sites. TERT was not the target. The introduced WT-HBx or mutant HBx genes express HBx protein, as detected by IHC (shown in Fig. 1). The tumors were confirmed to be HCC as CK-18 and AFP were positive [31]. Thus, HCC developed in the *SB* mice are induced by the HBx proteins, rather than the insertional mutations. To further determine the effects of HBx mutants on the growth, we introduced WT-HBx and the HBx mutants into HepG2 and HeLa cells, respectively. Ectopic expression of HBx mutants significantly increased cell proliferation and the S phase proportion. Thus, the HBx mutants are more carcinogenic than WT-HBx. Of those, M3-HBx and Ct-HBx are the most potent ones.

Our histopathologic analyses revealed more inflammation events in livers of the mice injected with M2-HBx, M3-HBx, and Ct-HBx than their corresponding WT-HBx counterparts and even M1-HBx; furthermore, signs of necrosis, fibrosis, and degenerative changes were also evident in liver sections (shown in Fig. 1). The serum levels of IFN γ , IL-5, and IL-6 were significantly higher in M3-HBx-injected mice than in the empty vector- or WT-HBx-infected controls, while the positive rate of IL-1 β was significantly higher in M2-HBx- and M3-HBx-injected mice than in the vector-injected mice (shown in Fig. 3). IFN γ has both protumor and antitumor activities, although it induces the Th1-recruiting, proinflammatory chemokines in the tumor microenvironment [32]. IL-5 contributes to the differentiation and survival of eosinophils in the tumor microenvironment, which facilitates tumor progression [32, 33]. IL-6 basically promotes cancer immune evasion and facilitates the development of HCC [34]. IL-1 β , a pro-inflammatory cytokine abundantly expressed in the tumor microenvironment, plays a major role in cancer invasiveness, progression, and metastases. Anti-inflammation with canakinumab targeting IL-1 β significantly reduces cancer occurrence and mortality [35]. The higher circulating levels of IFN- γ , IL-5, and IL-6 and higher positivity rate of IL-1 β are quite consistent with the higher occurrence of HCC in M3-HBx-injected mice, indicating M3-HBx-caused inflammation is closely related to HCC development. The three inflammatory related gene sets were significantly enriched in M3-HBx-injected mice, while the IL-6-related gene set was significantly enriched in Ct-HBx-injected mice. We found that 77 genes overlapped in the cancer-related gene sets and the inflammation-related gene sets. Of the 72 genes in the IL-6-related gene sets, 55 (76.3%), including CDC20, P21, and SKP2, were included in the cancer-related gene sets. These data indicate that the inflammatory (especially IL-6-related) signaling pathways are closely associated with the development of HBx mutants-caused HCC.

Ectopic expression of the HBx mutants significantly increased cell proliferation and the S phase proportion of HepG2. These effects were exactly repeated in HeLa cells. Thus, the effects of the HBx mutants on cell biology are not hepatic-specific. HBV hepatotropism is mediated by specific receptor

recognition in the liver [36]. After being introduced, the HBx mutants exhibit pro-carcinogenic effects in non-hepatic cells such as HeLa cells [37]. Due to the ease of cell culture and gene transfer, stable genome, and tumorigenicity in nude mice, HeLa is the most widely used human cancer cell line in elucidating the biochemical pathways of normal and diseased tissue in human cells [38]. Thus, HeLa cells were applied for cDNA microarray assay in this study. Using the PPI network, GSEA assay, and published data, we identified four key molecules (PAI1, CDC20, P21, and SKP2) in the four cancer-related gene sets that were quite consistent in GSEA between cell cDNA microarray assay in humans and tissue cDNA microarray assay in mice. The expression levels of SKP2 and p21 in HepG2 cells with M3-HBx are quite consistent with a previous study [10], indicating that our experiments are reliable. No study reports the effect of Ct-HBx on p21 expression. Here, we found that the expression level of p21 was higher in Ct-HBx-transfected HepG2 and HeLa cells, possibly because the 3'-terminal of HBx inhibits the expression of p21 in cancer cells. The effects of the HBx mutants on CDC20 expression are also not reported. CDC20 is an activator of the anaphase-promoting complex/cyclosome. Recent studies have demonstrated that HBx induces mitotic checkpoint dysfunction via targeting the human serine/threonine kinase BubR1, while HBx binding to BubR1 attenuates the association between BubR1 and CDC20 [37, 39]. Ct-HBx and HBx mutations at the Kunitz domains may fail to bind BubR1, thus facilitating the binding of hBubR1 to CDC20. However, this could not explain why Ct-HBx and M3-HBx upregulate the expression of CDC20 in HepG2 and HeLa cells and in the liver of corresponding SB mice. The reason that the effect of M3-HBx on the growth of HeLa cells is attenuated by silencing CDC20 remains to be elucidated. The mechanisms by which the HBx mutants upregulate the expression of the four key molecules remain unknown. Our reporter assays indicated that the HBx mutants do not *trans*-activate the promoter activities of *PAI1*, *CDC20*, and *p21*. It is possible that the HBx mutants *trans*-activate their enhancers or stabilize their mRNAs via epigenetic modifications.

For the first time, we found that PAI1 whose expression is significantly upregulated by Ct-HBx or M3-HBx in cell lines and in the SB mice functions as a hub. Most importantly, silencing PAI1 attenuates the effect of M3-HBx or Ct-HBx on the growth of HepG2 cells and significantly decreases the growth of HeLa cells with Ct-HBx. Thus, PAI1 is an important player in HBx mutants-induced carcinogenesis. PAI1 (also known as SERPINE1) is a multifaceted proteolytic factor that not only has anti-fibrinolytic and anti-plasminogen activation activities but also plays a pro-tumorigenic role via its pro-angiogenic and anti-apoptotic activities [40]. In the tumor microenvironment, PAI1 expression is upregulated in tumor-associated macrophages after being stimulated with cancer-associated fibroblasts-generating CXCL12 and promotes the malignant behavior of the HCC cells by mediating epithelial–mesenchymal transition [41]. High levels of PAI1 and its genetic predisposition have been consistently reported to predict poor prognosis in several types of human cancers including HBV-HCC [41–44]. Thus, the HBx mutants upregulate the expression of PAI1, facilitating the generation of the cancer-promoting microenvironment in affected livers. Thus, PAI1 is a promising candidate as a predictive and/or prognostic biomarker and a therapeutic target in HBV-HCC.

Our study has limitations. First, we did not elucidate the mechanism by which the HBx mutants upregulate the expression of PAI1, CDC20, and p21, although we exclude the possibility that the HBx

mutants *trans*-activate their promoters. Second, the effect of the 3'-terminal of HBx on the expression of p21 was not investigated. Third, the mechanisms by which M2-HBx, M3-HBx, and Ct-HBx induced cancer-promoting inflammation remain to be investigated.

Conclusions

Conclusively, the HBx combo mutation C1653T + T1674G + A1762T/G1764A promotes carcinogenesis via upregulating the expression of PAI1 and CDC20 and inducing cancer-promoting inflammation. The oncogenic effect of Ct-HBx also depends on the upregulation of PAI1. Thus, PAI1 should be an important predictive and prognostic biomarker and promising therapeutic target in HBV-HCC.

Abbreviations

HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HBx, hepatitis B virus X; SB, Sleeping Beauty; PAI1, plasminogen activator inhibitor-1; CDC20, cell division cycle 20; M1, A1762T/G1764A; M2, T1674G + T1753C + A1762T/G1764A; M3, C1653T + T1674G + A1762T/G1764A; Ct-HBx, carboxylic acid-terminal truncated HBx; WT, wild-type; Fah, fumaryl acetoacetate hydrolase; NTBC, 2-(2-nitro-4-trifluoromethylbenzoyl)-1,3-cyclohexanedione; IHC, Immunohistochemistry; H&E, hematoxylin-eosin; GEO, Gene Expression Omnibus; IL-4, interleukin-4; IFN- α , interferon- α ; TNF- α , tumor necrosis factor- α ; GM-CSF, granulocyte macrophage colony stimulating factor; TGF β , transforming growth factor β ; VEGF, vascular endothelial growth factor; DMEM, Dulbecco's modified Eagle's medium; STR, short tandem repeat; siRNAs, Small interfering RNAs; P21(CDKN1A), cyclin dependent kinase inhibitor 1A; qRT-PCR, real-time quantitative reverse transcription PCR; CCK8, Cell Counting Kit-8; GSEA, gene set enrichment analysis; PPI, protein-protein interaction; GAPDH, Glyceraldehyde-3-phosphate dehydrogenase; CK18, cytokeratin 18; Afp, alpha-fetoprotein; Ctnna3, catenin alpha 3; Fhit, fragile histidine triad gene; MDM2, E3 ubiquitin protein ligase; RCF3, replication factor C subunit 3; PLK1, polo like kinase 1; CDC6, cell division cycle 6; EGR1, early growth response 1; SKP2, S-phase kinase associated protein 2.

Declarations

Ethics approval and consent to participate

All animal studies were conducted under the animal welfare protocol approved by the ethics committee of Second Military Medical University.

Consent for publication

All authors agree to submit the article for publication.

Availability of data and materials

Data of HBV-capture sequencing were uploaded to the Sequence Read Archive (SRA) database under Accession Number PRJNA743432. The gene profiling data of the SB mouse models and HeLa cells were uploaded to the Gene Expression Omnibus (GEO) database under Accession Numbers GSE179125 and GSE179126, respectively.

Competing interests

The authors declare that they have no competing interests.

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Author Contributions

Cao GW conceived and supervised the study. Pu R, Zhou XY, Hou XM, Cai SL, Chen LP, Wu JF, and Yang F carried out the experiments. Chen X did the bioinformatics analysis. Liu WB and Zhou XY conducted the statistical analyses and drafted the manuscript. Cao GW interpreted the data, discussed the results, and extensively revised the manuscript. Wang X provided the Sleeping Beauty mouse model. Tan XJ and Yin JH guided on the experiments. All authors read and approved the final manuscript.

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Figures

Figure 1

Figure 1

Representative features of H&E and IHC stainings of the liver and tumors from the Sleeping Beauty (SB) mouse models. The first column shows representative gross features indicating the tumors in the livers. The second column shows representative H&E stainings indicating the histological features of the livers. The third to fifth columns show representative IHC stainings for HBx, CK-18, and AFP. Vector, the mice injected with the empty vector; WT, M1, M2, M3, and Ct, the mice injected with vectors carrying WT-HBx, M1-HBx, M2-HBx, M3-HBx, and Ct-HBx, respectively.

Figure 2

Figure 2

Tumor incidence and HBx insertion sites of the SB mouse models. (A) Incidence of tumors in the mouse models. V, the mouse models injected with the empty vector, $n=7$; WT, the mouse models injected with WT-HBx, $n=7$; M1, the mouse models injected with M1-HBx, $n=4$; M2, the mouse models injected with M2-HBx, $n=7$; M3, the mouse models injected with M3-HBx, $n=8$; Ct, the mouse models injected with Ct-HBx, $n=7$. (B) The number of HBx insertion sites in different functional regions and different tissue types. T, tumors from the mice with tumor nodules; N, liver tissues from the tumor-free mice. (C) The total number of HBx insertion sites between two of the three groups. ** $P < 0.05$.

Figure 3

Figure 3

The serum levels and the positive rates of cytokines in the SB mouse models. (A) the serum level of IFN- γ ; (B) the serum level of TNF- α ; (C) the positive rate of IL-1 β ; (D) the positive rate of IL-12; (E) the serum level of IL-5; (F) the serum level of IL-6; (G) the serum levels of IFN- γ , TNF- α , IL-5, and IL-6 in the mouse models with or without tumor; and (H) the positive rates of IL-1 β and IL-12 in the mouse models with or without tumor. N, the tumor-free mice; T, the mice with tumors. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Figure 4

Figure 4

Effects of HBx mutations on malignant phenotypes of cancer cells. (A) Ectopic expression of the HBx mutants significantly increased the proliferation of HepG2 cells, compared to WT-HBx. (B) Ectopic expression of the HBx mutants increased the rate of cells in the S phase of HepG2 cells, compared to WT-HBx. (C) Ectopic expression of the HBx mutants had no significant effect on the migration of HepG2 cells, compared to WT-HBx. (D) Ectopic expression of the HBx mutants significantly increased the proliferation

of HeLa cells, compared to WT-HBx. (E) Ectopic expression of the HBx mutants increased the rate of cells in the S phase of HeLa cells, compared to WT-HBx. (F) Ectopic expression of the HBx mutants had no significant effect on the migration of HeLa cells, compared to WT-HBx. (G) Tumor weights among different groups. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$.

Figure 5

Figure 5

The four cancer-related gene sets identified both in human cell cDNA microarrays and in mouse tissue cDNA microarrays. (A) BERENJENO_TRANSFORMED_BY_RHOA_UP gene set including *PAI1*; (B) CHIANG_LIVER_CANCER_SUBCLASS_PROLIFERATION_UP gene set including *CDC20*; (C) MARKEY_RB1_CHRONIC_LOF_UP gene set including *P21*; and (D) VERNELL_RETINOBLASTOMA_PATHWAY_UP gene set including *SKP2*.

Figure 6

Figure 6

Effects of the HBx mutants on the expression of functional molecules in vitro. (A) Ectopic expression of Wt-HBx or HBx mutants affects the mRNA levels of PAI1, CDC20, P21, and SKP2 in HeLa cells. (B) Ectopic expression of Wt-HBx or HBx mutants affects the mRNA levels of PAI1, CDC20, P21, and SKP2 in HepG2 cells. (C) Ectopic expression of Wt-HBx or HBx mutants affects the protein levels of PAI1, CDC20, P21, and SKP2 in HeLa cells. (D) Ectopic expression of Wt-HBx or HBx mutants affects the protein levels of PAI1, CDC20, P21, and SKP2 in HepG2 cells. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$.

Figure 7
A

B

Figure 7

The expression levels of PAI1, CDC20, and p21 in the mouse tumor tissues of the SB mouse models injected with WT-HBx or each of the four HBx mutants. (A) Representative IHC images of PAI1 staining, CDC20 staining, and p21 staining. (B) Histograms of the IHC scores for the protein expression of PAI1, CDC20, and p21. * P < 0.05; *** P < 0.001.

Figure 8

Figure 8

Silencing functional molecules influences the oncogenic effects of the HBx mutants. (A) Silencing PAI1, CDC20, and p21 influences the effects of HBx mutants on the proliferation of HepG2 cells. (B) Silencing PAI1, CDC20, and p21 influences the effects of HBx mutants on the proliferation of HeLa cells. (C) Silencing PAI1, CDC20, and p21 influences the effects of HBx mutants on the cell cycle in HepG2 cells.

(D) Silencing PAI1, CDC20, and p21 influences the effects of HBx mutants on the cell cycle in HeLa cells. WT-HBx served as a control group in all comparisons.

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