HBx-K130M/V131I Promotes Liver Cancer in Transgenic Mice via AKT/FOXO1 Signaling Pathway and Arachidonic Acid Metabolism

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Abstract

Chronic hepatitis B viral (HBV) infection remains a high underlying cause for hepatocellular carcinoma (HCC) worldwide, while the genetic mechanisms behind this remain unclear. This study elucidated the mechanisms contributing to tumor development induced by the HBV X (HBx) gene in the liver. To compare the potential tumorigenic effects of K130M/V131I (Mut) HBx on HCC, the liver of mice carrying the HBx transgene was used to deliver HBx Mut and WT into the livers of fumarylacetoacetate hydrolase (Fah)-deficient mice and in the context of transformation related protein 53 (Trp53) deficiency. From our results, HBx Mut had a stronger tumorigenic effect than its WT variant. Also, inflammation, necrosis, and fibrosis were evident in HBx experimental animals. Reduction of forkhead box O1 (FOXO1) with increased phosphorylation of upstream serine/threonine kinase (AKT) was detected under HBx Mut overexpression. Thus, it is proposed that HBx Mut enhances disease progression by reducing FOXO1 via phosphorylation of AKT. At the metabolomic level, HBx altered the expression of genes that participated in arachidonic acid (AA) metabolism, as a result of inflammation via accumulation of proinflammatory factors such as prostaglandins and leukotriene in liver. Taken together, the increased rate of HCC observed in chronic hepatitis B patients with K130M/V131I-mutated HBx protein, may be due to changes in AA metabolism and AKT/FOXO1 signaling.

Implications: Our findings suggested that HBx-K130M/V131I-mutant variant promoted HCC progression by activating AKT/FOXO1 pathway and inducing stronger inflammation in liver via AA metabolism.

Introduction

Liver cancer is the fourth most common cause of cancer-related deaths worldwide, accounting for about 700,000 deaths annually. The major type of primary liver cancer is hepatocellular carcinoma (HCC), which accounts for 85% to 90% of total liver cancer cases (1–3). Chronic hepatitis B viral (HBV) infection has been shown to have a strong relationship with liver cirrhosis and HCC (4). HBV infection accounts for more than 50% of all HCC cases globally (5). HBV infection is an endemic disease, in which the prevalence of chronic HBV infection diverges widely in different parts of the world. About 50% of all new liver cancer cases diagnosed were from mainland China (1–3, 6, 7). HBV is categorized into 10 genotypes (A to J) and various subgenotypes according to its geographic distributions (3). HBV Genotypes B and C are widely distributed in East and Southeast Asia (3) and are predominantly found in the Han population in mainland China (8).

HBx is encoded by the X open reading frame of HBV. It plays a crucial role in hepatocarcinogenesis, functioning as a transcriptional activator that interacts with nuclear transcription factors and modulates cytoplasmic signaling pathways, such as the RAS/RAF/MAP and WNT/CTNNB1 signaling pathways (9, 10). HBx can also promote replication of HBV and regulate biological processes, such as host gene transcription, cell-cycle progression, apoptosis, and oxidative stress (3, 11, 12). HBx has also been reported to induce inflammation, angiogenesis, immune responses, and multidrug resistance, accelerating HCC progression (10, 13, 14).

Mutations in the HBV genome are very common in patients chronically infected with HBV and can be biomarkers for HCC development (15). Several HBx gene mutation hotspots,
including A12T, V44I, A66T, H86Y, E109D, and K130M/V131I can be found in HBV carriers. The dual amino acid changes at K130M/V131I, which overlaps both the HBx gene and basal core promoter (BCP), have been consistently shown to have the highest potential to promote HCC progression (10, 16–18). HBV patients with chronic hepatitis, fulminant hepatitis, or HCC are often detected with mutations at K130M/V131I sites and diagnosed as HBeAg negative (18–20). A recent meta-analysis of 54 studies examining the association between these mutations and HCC showed that X_K130M/V131I are detected in 66.5% of HCC cases, compared with 39.8% of non-HCC cases (21). These X protein changes are encoded by nucleotide changes in the BCP region A1762T/G1764A (BCP_A1762T/G1764A), and have been shown to be associated with decreased PreC/C RNA synthesis and decreased expression of HBeAg (20). However, the effect of the encoded protein changes on the tumorigenic potential of HBx is not well understood. Although several studies have reported the involvement of HBx in HCC development, the differential tumorigenicity of HBx mutations and the genetic mechanism(s) by which HBx induces and/or contributes to HCC development remains uncertain, and most studies have been performed using transformed liver cell lines such as Huh7.

The fumarylacetoacetate hydrolase (Fah)-deficient/Sleeping Beauty transposase 11 knock-in (Fah/SB11) transgenic mouse model has been established and used to test the oncogenic role of candidate liver cancer genes (9, 22–26). This mouse model allows for selective repopulation of hepatocytes with the expression of delivered transgenes. This Fah-deficient mouse model has a defect in hydrolyzing acetoacetate and fumarate in the tyrosine catabolic pathway. Therefore, it must be treated with nitisinone (NTBC) in the drinking water to prevent tyrosinemia (27). Codelivery of oncogenes and Fah cDNA in a transposon vector and removal of NTBC after gene delivery, the constitutively expressed transgenes in the expression vectors was coexpressed and allowed for selective repopulation of hepatocytes with stable transposon vector integration. These mice were allowed to age and sacrificed around 150 to 200 days posthydrodynamic injection (PHI; Supplementary Fig. S1B). All vectors used for hydrodynamic tail vein injections were prepared using EndoFree Plasmid Maxi Kit (Qiagen).

Plasmid construction

The PCR products of K130M/V131I mutant and WT variants of HBx (GOIs) were provided by N. Warner (Victorian Infectious Diseases Reference Laboratory, The Peter Doherty Institute for Infection and Immunity, Victoria, Australia). These GOIs were introduced into the Gateway entry clone pENTR 11 Dual Selection Vector (Life Technologies) to produce pENTR-GOI. The GOI in the entry clone was introduced into the destination vector (pT2/GD-DEST-EGFP for in vitro experiments or pPB/SB-DEST-EGFP for in vitro experiments) by LR clonase reaction using Gateway LR clonase Enzyme mix (Life Technologies) to give the pT2/GD-GOI-EGFP or pPB/SB-GOI-EGFP (Supplementary Fig. S1). A short hairpin RNA directed against the Trp53 gene was also incorporated into a transposon vector (shp53) for stable integration into the mouse hepatocyte genome (refs. 9, 24, 25, 28, Supplementary Fig. S1A). All vectors used for hydrodynamic tail vein injections were prepared using EndoFree Plasmid Maxi Kit (Qiagen).

Hydrodynamic tail vein injection

Twenty micrograms of pT2/GD-HBx-B_Mut-EGFP or pT2/GD-HBx-B_WT-EGFP was cojected with shp53-containing plasmid into 45-day old Fah/SB11 mice hydrodynamically. After injection, NTBC water was replaced with normal drinking water. The Fah cDNA in the expression vectors was coexpressed and allowed for selective repopulation of hepatocytes with stable transposon vector integration. These mice were allowed to age and sacrificed around 150 to 200 days posthydrodynamic injection (PHI; Supplementary Fig. S1B). Histological sections were taken from larger tumor nodules. Histology sections were then stained with antibodies used was as follows: pAKT (Ser473; Cell Signaling Technology) was diluted in ratio of 1:250, and p53 (DO-1; Santa Cruz Biotechnology) was diluted in ratio of 1:100. The histopathologic analyses.

Materials and Methods

Generation of Fah-deficient/Rosa26-SB11 transposase transgenic mice

All animal work was conducted under approved animal welfare protocols of The Chinese University of Hong Kong and The Hong Kong Polytechnic University (Hong Kong, SAR). Fah-deficient mice were bred with Rosa26-SB11/SB11 to obtain experimental animals that were deficient for Fah and transgenic for SB transposase (Fah/SB11). All Fah-deficient mice were maintained on 6 μg/ml 2-(2-nitro-4-trifluoromethylbenzoyl)-1,3-cyclohexadiene (NTBC, Swedish Orphan International AB) in the drinking water until hydrodynamically injected with transposon vector containing the Fah CDNA (27). The genotypes of Fah/SB11 transgenic mice were confirmed by PCR genotyping using primers specific for Fah alleles and Rosa26-SB11 as described previously (9).

Histologic analyses

Formalin-fixed, paraffin-embedded liver tissues were processed, sectioned at 5-μm using a standard microtome (Leica), mounted, and heat-fixed onto glass slides at 55°C overnight using standard protocols. These tissue slides were then stained with hematoxylin and eosin (H&E) following standard protocols, and IHC staining as described previously (9). Dilution of primary antibodies used was as follows: pAKT (Ser473, Cell Signaling Technology) were diluted in ratio of 1:250, β-Catenin (D10A8) XP (Cell Signaling Technology) was diluted in ratio of 1:500, Ki67 (Abcam) was diluted in ratio of 1:200, FAH (Abcam) was diluted in ratio of 1:250, and p53 (DO-1, Santa Cruz Biotechnology) was diluted in ratio of 1:300. Board-certified pathologist performed the histopathologic analyses.
Semiquantitative RT-PCR
RNA from liver tissues was extracted using TRIzol reagent (Life Technologies) following the protocol provided by the manufacturer. The first-strand cDNA was synthesized from 250 ng mRNA using the SuperScript First-Strand Synthesis System for RT-PCR (Life Technologies). Reactions with (RT+) and without (RT-) the reverse transcriptase were performed for all samples. This cDNA was used as template for subsequent PCR with various primer pairs (Supplementary Materials and Methods). PCR conditions were similar to PCR genotyping with reduced cycles to avoid amplicon saturation. Semiquantitative analyses of unsaturated amplicons were measured using ImageJ software. Intensity of bands was calculated as an arbitrary value relative to actin, beta (Actb) expression level.

Quantitative RT-PCR
qPCR was performed to determine the expression level of common downstream genes involved in cancer signaling pathways. The mRNA was extracted as mentioned previously using 1 μg of mRNA as cDNA synthesis template and qPCR was performed using the QuantiStudio 7 Flex Real-Time PCR System (University Research Facility in Life Sciences of The Hong Kong Polytechnic University, Hong Kong). PCR reaction mixture consisted of a 1:10 dilution of cDNA in the SYBR Green I containing qPCR Master Mix (GoTaq qPCR Master Mix, Promega) with specific primers (0.2 μmol/L final concentration of each primer). Primer sequences listed in Supplementary Materials and Methods.

Western blot analyses
Protein was extracted from liver sections of injected mice using Qproteome Mammalian Protein Prep Kit (Qiagen) following the protocol provided by the manufacturer. Protein concentration was detected using standard protein assay (Bio-Rad) and 30 μg of protein was loaded into the SDS-PAGE and transferred onto polyvinylidene difluoride membrane. Primary antibodies phospho-AKT, total AKT, non-phospho. (active) CTNNB1, total CTNNB1, FOXO1, and ACTB (Cell Signaling Technology) consisted of a 1:10 dilution of cDNA in the SYBR Green I containing qPCR Master Mix (GoTaq qPCR Master Mix, Promega) with specific primers (0.2 μmol/L final concentration of each primer). Primer sequences listed in Supplementary Materials and Methods.

Figure 1.
Tumor burdens in Fah/SB11 mice injected with WT and mutant (Mut) variants of HBx-B genotypes. A, Number of tumors found in experimental animals at about 160-days PH (HBxB-WT/shp53 (T), n = 10; HBxB-Mut/shp53 (T), n = 10; Empty/shp53 (T), n = 9). All mice were injected with shp53. Mean ± S.D.; n, number of animals; P, unpaired student t test. B, Representative image demonstrating diverse tumor burden observed in the HBxB-WT/shp53 and HBxB-WT/C6-shp53 experimental animals. Arrows indicate tumor nodules; the orientation of the livers were optimized to show the most tumor nodules; scale bars, 0.5 cm. C, Liver to whole body weight ratio of experimental animals. Mean ± S.D.; n, number of animals; P, Student t test. *P < 0.05. D, Representative IHC images for anti-Ki67 staining in controls and liver tumors of Fah/SB11 injected with various HBx and shp53 transgenes. Left column, no primary antibody was used. Right column, anti-Ki67 primary antibody used. Top and Middle panels, wild-type mouse liver and non-injected Fah/SB11 liver from animal under the treatment of NTBC water showing no detectable Ki67 activity, respectively. Bottom panels, Fah/SB11 animal injected with HBxB-Mut/shp53 showing strong nuclear staining of Ki67 in both tumor (T) and peripheral tissue (P) area. Scale bars, 100 μm. Magnified image shown in right corner (solid-lined box) of each slide. Scale bars, 1 μm. E, Representative H&E staining images demonstrating dysplastic hepatic morphology of the experimental animals. (i) Non-injected Fah/SB11 liver under continuous treatment of NTBC showing normal hepatic morphology. (ii) Fah/SB11 liver injected with empty vector control in the context of shp53. (iii) Multinucleated cells (indicated with green arrow) and pyknotic nuclei (indicated with yellow arrow) of hepatocytes were found in HBxB/shp53 injected liver. (iv) More mitotic figures (indicated with red arrow) were noted in liver sections injected with HBxB/shp53. Scale bars, 1 μm. (vii) Areas of lymphocytic inflammation (arrows) seen in Fah/SB11 mice with HBx gene co-injection of shp53. (viii) Fibrosis observed in Fah/SB11 animals with HBx-B genotype co-injected with shp53. (ix) Tumor nodule (T) observed in Fah/SB11 animals HBx gene co-injected with shp53 with dysplastic hepatic morphology seen in peripheral hepatocytes (P). Scale bars, 100 μm.

Cell culture and AKT inhibitor treatment assay
SNU-449 were cultured with RPMi medium (Gibco, Thermo Fisher Scientific) supplemented with 10% heat-inactivated FBS (Gibco, Thermo Fisher Scientific) and 1% antibiotic-antimycotic (Anti-Anti, Gibco, Thermo Fisher Scientific). Immortalized human hepatic cell line, HHLL7, was cultured with MEM supplemented with 10% FBS and 1% Anti-Anti. HHLL7 cells (5 x 10⁴ cells) were transfected with 1 μg of pPB/SB-GOI-EGFP and 0.5 μg of transposase vector using ViaFect transfection reagents (Promega) with a plasmid to reagent ratio of 1:3 in 6-well plate (Supplementary Fig. S1C). Puromycin was used for the selection of positively transfected cells. SNU-449 and positively transfected HHLL7 cells (4,000 cells) were treated with various concentrations (3.125, 6.25, 12.5, 25, 50, 100, and 200 μmol/L) of AKT protein kinase inhibitor (AZD3563, MedChemExpress) for 4 days in a 96-well plate. MTS assays were performed using the CellTiter 96 Aqueous One Solution Reagent (Promega) according to the manufacturer’s protocol (Supplementary Fig. S1C). Absorbance of each well was measured at 492 nm. Percentage of cell survival were calculated by dividing the absorbance of treated cohorts to the untreated control.

Serum metabolomic analyses
Blood from HBxB-Mut/shp53, WT/shp53, and Empty/shp53-injected mice was extracted during necropsy, kept at 4°C overnight and serum isolated. The procedure to prepare serum for Ultra Performance Liquid Chromatography-Orbitrap-Mass spectroscopy (UPLC-Orbitrap-MS), the UPLC separation method and mass spectrometer parameters can be found in Supplementary Materials (Supplementary Materials and Methods). An equal volume of each serum sample was pooled, vortexed, and aliquoted to provide pooled quality control (QC) samples and went through the same extraction protocols of LC-MS as described. They were injected to UPLC-Orbitrap-MS intermittently between samples for stability check of the instruments throughout the runs. The prepared serum samples were injected into a Waters ACQUITY UPLC system (Waters). Mass spectrometry analysis was achieved by Thermo Scientific Orbitrap Fusion Lumos Tribrid mass spectrometer equipped with a heated electrospray ionization source and a Scion Lynx ESI source.
ionization (H-ESI) interface (Thermo Fisher Scientific). Data were analyzed by Progenesis QI software 2.0 (Nonlinear Dynamics) and peak area was normalized by all compound with reference of QC. Quality screening was done by filtering out those unstable metabolites (coefficient of variation >30% in all pooled QC samples). The remaining normalized data matrix were exported to the Extended Statistical tool (EZinfo v2.0 software, Umetrics AB) for partial least squares-discriminant analysis (PLS-DA) so as to evaluate their classification performance. The parameter $Q^2$ is used to assess the goodness of prediction for performance of PLS-DA in a general acceptable threshold value of 0.5. The metabolites that had significant difference between HBx-B Mut/shp53 and WT/shp53 experimental samples were identified by comparing their $m/z$, mass fragmentation patterns, and chromatographic retention times with commercially available reference standards and/or online metabolite databases, such as Human Metabolome Database (http://metlin.scripps.edu), Metlin (http://metlin.scripps.edu), and literatures.

Statistical analyses

Values are given as mean ± SD. Statistical significance was assessed by two-tailed, unpaired Student $t$ test with $P$ values (Prism Software). $P$ values >0.05 were considered statistically significant.

Results

Divergent effect on tumor burden by mutant and WT variants of HBx-B

Expression vectors for pT2/CD-HBx-B, Mut-GFP and pT2/CD-HBx-B WT-GFP were coinjected with a short hairpin RNA that targeted Trp53 (shp53) into the tail vein of Fas/SB11 mice hydrodynamically and the number of GFP-positive tumors counted at around 160 days PHB. HBx-B WT/shp53 and HBx-B Mut/shp53 injected mice displayed a trend toward higher tumor burden than HBx-B WT/shp53 counterpart ($n = 10$; $P = 0.1272$) and both groups had significantly increased tumors relative to whole body weight ratio of Empty/shp53 control group ($P < 0.0001$; Fig. 1C).

Inflammation, proliferation, hepatocytomegaly, and necrosis phenotype in HBx-injected animals

IHC and H&E-stained liver sections displayed hepatic morphologic phenotypic changes as a result of HBx overexpression. As expected, normal nonproliferating livers were negative for the cell proliferation marker Ki67 by IHC, while liver tumor nodules isolated from experimental animals injected with HBx-B gene were strongly Ki67-positive (Fig. 1D). Hepatocyte nuclei were enlarged with apparent pyknotic changes, indicating the development of hepatocellular necrosis (Fig. 1E, iii). In addition, multi-nuclei and mitotic figures were found in these sections (Fig. 1E, iii and iv). Furthermore, different degrees of inflammation were observed on the basis of the amount of lymphocyte aggregation in the injected liver sections (Fig. 1E, vii). Histopathologic analyses revealed higher inflammation events in livers injected with HBx-B Mut/shp53 than its corresponding HBx-B WT/shp53 counterpart (Fig. 2A, i). Signs of necrosis and degenerative changes were also observed in liver sections of the injected animals (Fig. 2A, ii). Moreover, mild fibrosis was observed in mice injected with either WT or mutant HBx-B gene (Fig. 1E, vii and 2A, iii).

Transcriptional analyses of HBx-B–injected liver

to confirm that inflammation and tumor induction were driven by the expression of HBx genes, semiquantitative RT-PCR was performed to examine the expression of green fluorescent protein (Gfp), HBx, and Fah transgenes flanked by the transposon vectors. Both normal tissues and tumors isolated from HBx-B Mut/shp53 and HBx-B WT/shp53 mouse liver expressed the Gfp, HBx, and Fah transgenes (Fig. 2B), indicating the successful integration and expression of transgenes into the hepatocytes of injected experimental animals. Alpha-fetoprotein (Afp), a marker for HCC diagnosis, was detected in both HBx-B WT/shp53 and HBx-B WT/shp53 groups by semiquantitative RT-PCR. Afp expression level in mutant was higher than that of its corresponding adjacent microscopically normal liver samples ($P = 0.0599$). The mRNA expression levels of myelocytomatosis oncogene (Myc) and cyclin D1 (Condi), were analyzed in HBx-B/shp53–injected animals by qPCR. HBx-B WT/shp53 induced significantly higher expression of Condi than Empty/shp53, while there were no significant differences among all groups for Myc mRNA expression (Fig. 2C). The rescued activity of Fah in injected mice was also confirmed by IHC, indicating the successful integration, transcription, and translation of genes delivered by the transposon vector (Supplementary Fig. S2A). As expected, control WT mouse livers were strongly FAH-positive, while noninjected Fas/SB11 control animals (under NTBC treatment) were FAH-negative by IHC staining (Supplementary Fig. S2A). There was no detectable TRP53 in either HBx or Empty/shp53 control cohorts that were co-injected with shp53 by IHC staining (Supplementary Fig. S2B).

Increased AKT and CTNNB1 protein levels in HBx-injected mice

Both PI3K/AKT and WNT/CTNNB1 signaling pathways play vital roles in regulating cellular differentiation, proliferation,
and survival in HCC. To identify underlying mechanisms of HBx-induced HCC, activation of these two pathways were examined. Western blot analyses were performed on protein extracted from experimental cohorts using nonphospho (active) CTNNB1 (active CTNNB1), CTNNB1, phospho-AKT (ser473) (pAKT), AKT, and FOXO1 primary antibodies (Fig. 2D). The protein levels of total CTNNB1 and total AKT were significantly higher in HBx-B Mut/shp53 than that of Empty/shp53, while FOXO1 protein level was significantly lower in HBx-B Mut/shp53 than that of Empty/shp53, and there were a trend showing higher protein level of active CTNNB1 and phospho-AKT in HBx-B Mut/shp53 than that of Empty/shp53 (Fig. 2D, left). However, only phospho-AKT protein level was significantly increased in HBx-B WT/shp53 compared to Empty/shp53 and there were no significant differences in pAKT, active and total CTNNB1, and FOXO1 levels between HBx-B WT/shp53 and Empty/shp53 (Fig. 2D, right). Furthermore, IHC of liver tumor nodules isolated from mice injected with HBx-B Mut/shp53 and HBx-B WT/shp53 groups were strongly positive for nuclear pAKT and membranous active CTNNB1 (Supplementary Fig. S2C and S2D). The effect of suppressing AKT signaling pathway on cell survival was performed on SNU-449, a HBV-positive liver cancer cell line, and immortalized human hepatic cell line HHL7 transfected with HBx genotype B mutant and WT genes. Treatment with AKT inhibitor AZD5363 could significantly reduce the survival rate of SNU-449 and HBx-transfected HHL7 cell lines compared with nontreated cells (Fig. 2E).

**RNA-seq revealed crucial roles of metabolomic reprogramming in HBx-B–injected mice**

RNA-seq was performed to quantify the RNA expression profile of HBx-B-injected animals. RNA from HBx-B Mut/shp53 tumors (n = 3) and normal tissues (n = 4), HBx-B WT/shp53 normal tissues (n = 3), and Empty/shp53 normal tissues (n = 3) were analyzed by Illumina sequencing and expression level of each gene was calculated by fragments per kilobase of exon per million reads mapped (FPKM). Raw RNA-seq data were uploaded to Sequence Read Archive with the accession number PRJNA529044. On the basis of the gene expression level, differentially expressed genes (DEGs) were identified between groups. Fold change in gene expression between two samples was calculated by log2 FPKM ratio of two samples. Significant gene expression differences were filtered with log2 ratio greater or equal to 1 (for upregulation) and smaller or equal to –1 (for downregulation).

The number of DEGs between the normal tissues of HBx-B Mut/shp53 against Empty/shp53 control, HBx-B WT/shp53 against Empty/shp53 control, and HBx-B Mut/shp53 against HBx-B WT/shp53 were low (Supplementary Fig. S3A). Comparing RNA expression profiles of tumors (n = 3) and normal (n = 4) tissues of HBx-B Mut–injected mice, 377 DEGs were totally identified: 235 genes were upregulated and 142 genes were downregulated in the tumor compared with its normal tissues (Supplementary Fig. S3A and S3B). Insulin growth factor 2 (Igf2), Rous sarcoma oncogene (Src), p21 (RAC1) activated kinase 6 (Pak6), as well as well-known oncogenes, Myc and matrix metallopeptidases (Mmps), were upregulated in the tumors from the HBx-B Mut/shp53 cohort (Fig. 3; Supplementary Fig. S3A and S3B).
Supplementary Table S1). Transcriptional mRNA expression of targeted genes, such as Igf2, Src, Pak6, and Mmp7 were further confirmed by qPCR in nonsequenced tumor (n = 5) and normal (n = 5) samples. Significant upregulation of these targeted genes was detected in HBx-B Mut/shp53 injected cohort compared with empty control (Fig. 4A). Using KEGG pathway analyses, the DEGs show significant alterations for both canonical signaling and metabolic pathways. Among the canonical signaling pathways, most of the DEGs were involved in common cancerous signal transduction pathways such as Ras signaling, PI3K–Akt signaling, ECM–receptor interaction, MAPK signaling, and Notch signaling (Supplementary Table S2).

Apart from the canonical signaling pathways, a large number of genes were associated with various metabolic pathways (59/
519 genes. This suggests that metabolic changes played a significantly important role in promoting tumor progression. AA metabolism is one of the metabolic pathways with the highest number of DEGs involved (13 DEGs; Fig. 3; Supplementary Table S2). DEGs involved in AA metabolism, including prostaglandin-endoperoxide synthase 2 (Ptgs2), secretory phospholipase A2 (Pla2g), and arachidonate 12-lipoxygenase (Alox12) were upregulated, while cytochrome p450 monooxygenase, such as Cyp2p and Cyp4f, genes encoding xenobiotic monooxygenase and leukotriene-B4 (LTB4) 20-monooxygenase were downregulated (Fig. 3; Supplementary Table S1). The abundance of metabolites, such as prostaglandin B2, D2 and E2, LTB4, and 12-hydroxyeicosatetraenoic acid (12-HETE), from AA metabolism were shown to be significantly higher in the HBx genotype B mutant and WT experimental cohorts compared with the Empty/shp53 control (Fig. 4B).

**Discussion**

Using the Fah/SB11 mouse model, K130M/V131I mutant variant of HBx-B induced a higher tumor burden than its WT counterpart and significantly increased the liver weight of the experimental animals. Hepatocytes of HBx-B Mut/shp53- and WT/shp53-injected mice were larger than those of Empty/shp53 control and Fah/SB11 noninjected mice, indicating hepatomegaly. In another experimental cohort, in which Fah/SB11 mice were injected with HBx-B Mut and WT alone, the number of tumors induced were similar to the experimental cohorts that were coinfected with shp53 (data not shown). On the basis of this evidence, inactivation of Trp53 did not contribute to tumorigenesis at time of necropsy (between 160 and 200 PHI).

In addition, severe inflammation, mild necrosis, and fibrosis were observed in livers injected with HBx-B Mut/shp53 indicating the development of chronic hepatitis. From the RNA-seq analyses, the AA metabolism pathway associated with proinflammatory process was activated. From the metabolite profiles of the experimental cohorts, significantly higher abundance of the proinflammatory factors, such as prostaglandin B2, D2, E2, LTB4, and the procarcinogenic factor, 12-HETE, from AA metabolism were detected in the HBx-mutant and WT experimental cohorts compared with the Empty/shp53 control (Fig. 4B). These results suggest that HBx-B could induce liver inflammation through the activation of the AA metabolism pathway, producing more proinflammatory factors and attract the accumulation of leukocytes into the liver tissues.

Both IHC and Western blot analyses confirmed expression of either HBx-B Mut or WT could activate phosphorylation of AKT and overexpress CTNNB1 in our injected mice. Moreover, the protein level of FOXO1 was significantly reduced in HBx-B Mut/shp53 group. FOXO1 is a transcriptional factor that mediates the expression of downstream target genes involved in cell proliferation, apoptosis, cell-cycle arrest, and metabolism (30, 31). FOXO1 is also a downstream target of AKT, which can be phosphorylated by pAKT and translocated to the cytoplasm, and subsequently degraded via ubiquitination (30). Therefore, we propose that HBx-B–mutant variant increased cell proliferation and promoted cell-cycle progression by activating AKT phosphorylation, which in turn, phosphorylated and translocated FOXO1 to cytoplasm for ubiquitination, resulting in reduced FOXO1 abundance in cells. In transcriptional analysis, both HBx-B Mut/shp53 and WT/shp53 induced overexpression of Afp, Myc, and Ccnd1.

RNA-seq was performed to quantify the RNA expression profiles between tumor and its peripheral normal tissue of HBx-B Mut-injected mice. The sequencing results highlighted the importance of signal transduction pathways, such as PI3K–Akt signaling pathway, and metabolic pathways, including AA metabolism. The mRNA expression levels of Igf2, Src, Pak6, and Mmps were significantly increased in tumor of HBx-B Mut/shp53–injected animals comparing with that of normal tissues. Igf2 encodes for a mitogenic peptide, namely insulin-like growth factor 2 (IGF2). Other IGF components such as IGF2-mRNA binding protein (IGF2BP) and IGF-binding proteins (IGFBPs) are required to initiate the signaling cascade of IGF2 (32). Ingenuity Pathway Analysis upstream regulator analyses showed that expression of Igf2 was regulated by CTNNB1 and PI3K/AKT (Supplementary Table S3). In addition, Igfbp1, which was also upregulated in tumors of HBx-B Mut/shp53 cohort, was regulated by FOXO1 (Supplementary Table S3). These results are consistent with our translational and transcriptional analyses and further confirmed the activity of AKT/FOXO1 signaling pathway on regulating cell growth, proliferation, and survival via IGF2. Uproregulation of IGF2 was significantly associated with risk of hepatoblastoma development (32). On the basis of a previous study on gene expression profiling of patients with HCC acquired from various risk factors, IGF2 was one of the differentially expressed genes found exclusively in HBV-induced HCC cases (33). Activation of AKT and upregulation of Src in the HBx-B Mut cohort triggered downstream-cascaded events resulting in upregulation and activation of mDia1, which...
was responsible for actin regulation and associated with cell motility. A recent study published that high levels of Src could promote cell proliferation and invasion (34). In addition, protein tyrosine kinase 2 (PTK2)/Src complex could also activate MAPK, WNT, and PI3K–AKT signaling pathways responsible for cell proliferation and cell survival by overexpressing oncogenes such as Myc and Mmps, which were also upregulated in our DEGs list. Furthermore, Src is also an upstream target of PAK, which is encoded by Pak6 and is an effector of RAC proteins. Activation of Rac cascade triggers actin polymerization and induces membrane protrusion and cell spreading, resulting in mesenchymal cell morphology (35). PAK also possesses oncogenic roles that can regulate cell proliferation, cell survival, adhesion, and migration (36).

DEGs involved in AA metabolism were affected significantly; Ptg2, Pla2g, and Alox12 were upregulated, while Cyp2, Cyp4f, genes encoding xenobiotic monooxygenase, and LTB4 20-monooxygenase were downregulated. As mentioned previously, AA metabolism is highly associated with inflammation events in liver (37, 38). Thus, we hypothesized that HBx Mut could induce severe inflammation in liver via AA metabolism pathway by upregulating expression of Ptg2, which encodes for prostaglandin-endoperoxide synthase that converts the unstable product prostaglandin G2 (PGG2) to prostaglandin H2 (PGH2) that would be further converted to other prostaglandins (PGs), including PGE2, PGD2, and PGB2, resulting in overproduction of proinflammatory factors. Studies have shown that these PGs can promote tumor growth and are highly abundant in colon, lung, breast, and head and neck cancers (39–43). In addition, accumulated PGs could activate RAS/ERK and glycogen synthase kinase 3 beta (GSK3B)/CTNNB1 signaling pathways and hence induce cell proliferation (39). On the other hand, the gene encoding LTB4 20-monooxygenase was downregulated. LTB4 20-monooxygenase is an enzyme that converts LTB4 to 20-OH-LTB4, the down-regulation of gene encoding LTB4 20-monooxygenase caused the accumulation of LTB4 in cells. LTB4 is also known to be a proinflammatory factor that attracts leukocytes to the injured tissue (44). Moreover, LTB4 could also activate MEK/ERK and PI3K/AKT pathways through the interaction with the G protein–coupled receptors BLT2 in human pancreatic cancer cells (45, 46). Furthermore, ALOX12 has been described as a potential procarcinogenic enzyme (39). The higher abundance of 12-HETE, product of ALOX12, was detected in

**Figure 5.**
The schematic image of HBx/AKT/FOXO1 signaling pathway and AA metabolism–induced inflammation. HBx-B Mut-promoted tumor progression via phosphorylation of AKT in PI3K/AKT signaling pathway and reduced FOXO1 production. HBx-B Mut gene induced inflammation via AA metabolism by upregulating Ptg2 and downregulating gene coded for LTB4 20-monooxygenase, resulting in accumulation of proinflammatory factors, such as prostaglandins (PGs) and LTB4 around infected cells (47).
HBx-B–injected experimental cohorts compared with Empty/shp53 control, suggesting that HBx gene might also promote tumor progression by upregulation of Alox12.

All results shown herein confirm that HBx-B K130M/V131I–mutant variant displayed a stronger tumorigenic effect than its WT counterpart. The X_K130M/V131I amino acid changes are encoded by nucleotide changes at BCP_A1762T/G1764A, which also result in a decrease in PC/C mRNA and subsequent HBcAg expression. These changes have been strongly associated with the development of HCC in many clinical studies; however, whether the increased HCC is associated with the decrease in HBcAg levels, or mutation of the X protein remains unknown. To our knowledge, ours is the first study to show a direct effect of these X mutations on HBx-associated tumorigenesis. We suggest that HBx-B mutant variant promoted tumor progression via phosphorylation of AKT in the P38/akt signaling pathway and reduced FOXO1 production, resulting in upregulation of Igf2. Moreover, it significantly altered the metabolism of AA, which contributed to inflammation of HBx-B–injected animals. HBx Mut induced inflammation via AA metabolism by accumulation of proinflammatory factors such as the different types of PGs, LTBA, and 12-HETE to the infected tissues (Fig. 5; ref. 47). Taken together, these results provide a potential mechanism, whereby HBV encoding X_K130M/V131I (BCP_A1762T/G1764A) may contribute to the high rate of HCC observed clinically in patient cohorts containing these mutations.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions


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HBx-K130M/V131I Promotes Liver Cancer in Transgenic Mice via AKT/FOXO1 Signaling Pathway and Arachidonic Acid Metabolism

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