HBx mutations promote hepatoma cell migration through the Wnt/β-catenin signaling pathway

Zhen Chen,1,2 Jia Tang,1,2 Xuefei Cai,1,2 Yao Huang,1 Qingzhu Gao,1 Li Liang,1 Ling Tian,1 Yi Yang,1 Yaqiu Zheng,1 Yuan Hu1 and Ni Tang1

1 The Second Affiliated Hospital and the Key Laboratory of Molecular Biology of Infectious Diseases designated by the Chinese Ministry of Education, Chongqing Medical University, Chongqing, China

HBx mutations (T1753V, A1762T, G1764A, and T1768A) are frequently observed in hepatitis B virus (HBV)-related hepatocellular carcinoma (HCC). Aberrant activation of the Wnt/β-catenin signaling pathway is involved in the development of HCC. However, activation of the Wnt/β-catenin signaling pathway by HBx mutants has not been studied in hepatoma cells or HBV-associated HCC samples. In this study, we examined the effects of HBx mutants on the migration and proliferation of HCC cells and evaluated the activation of Wnt/β-catenin signaling in HBx-transfected HCC cells and HBV-related HCC tissues. We found that HBx mutants (T, A, TA, and Combo) promoted the migration and proliferation of hepatoma cells. The HBx Combo mutant potentiated TOP-luc activity and increased nuclear translocation of β-catenin. Moreover, the HBx Combo mutant increased and stabilized β-catenin levels through inactivation of glycogen synthase kinase-3β, resulting in upregulation of downstream target genes such as c-Myc, CTGF, and WISP2. Enhanced activation of Wnt/β-catenin was found in HCC tissues with HBx TA and Combo mutations. Knockdown of β-catenin effectively abrogated cell migration and proliferation stimulated by the HBx TA and Combo mutants. Our results indicate that HBx mutants, especially the Combo mutant, allow constitutive activation of the Wnt signaling pathway and may play a pivotal role in HBV-associated hepatocarcinogenesis.

Key words
GSK-3β, HBx mutants, hepatitis B virus, hepatocellular carcinoma, Wnt/β-catenin signaling pathway

Correspondence
Ni Tang, Key Laboratory of Molecular Biology for Infectious Diseases (Ministry of Education), Department of Infectious Diseases, Institute for Viral Hepatitis, The Second Affiliated Hospital, Chongqing Medical University, 1 Yixueyuan Road, Chongqing, 400016, China.
Tel: +86-23-68486780; Fax: 86-23-68486780;
E-mail: nitang@cqmu.edu.cn

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potential role of HBx Combo mutations in hepatocarcinogenesis is largely unknown.

Aberrant Wnt/β-catenin signaling has been implicated in tumorigenesis of multiple cancers such as colon, breast, and ovarian cancer. Mutations in the CTNNB1, AXIN2, and APC genes contribute to aberrant activation of Wnt/β-catenin signaling and HCC development. In the current study, we explored whether HBx mutations play roles in activation of the Wnt/β-catenin pathway and are involved in hepatoma cell proliferation and motility. We also investigated β-catenin activation in HBV-related HCC samples within HBx mutants, which might be helpful for the diagnosis and treatment of HCC in clinical settings.

Material and Methods

Plasmids and recombinant adenoviruses. The HBx wild-type fragment (nt 1374-1838) was amplified by PCR from plasmid pcDNA3.1-HBV 1.1 (HBV genotype C) and inserted into KpnI and HindIII sites of the shuttle vector pAdTrack-T04 (obtained from Dr. T.-C. He, University of Chicago, Chicago, IL, USA). The HBx mutants A1762T (T mutant), G1764A (A mutant), A1762T/G1764A (T/A mutant), and A1762T/G1764A/T1768A (Combo mutant) were constructed by overlapping extension PCR using pAd-HBx WT as the template, and subcloned into pAdTrack-T04 (primer sequences are listed in Table S1). All HBx mutations were confirmed by DNA sequencing. Recombinant Ad-HBx (specifically, Ad-HBx WT, Ad-HBx A, Ad-HBx T, Ad-HBx T/A, and Ad-HBx Combo) were generated successfully in HEK293 cells using the AdEasy system (obtained from Dr. T-C. He). All recombinant adenoviruses expressed GFP as a marker for monitoring of infection efficiency. An analogous adenovirus (Ad-GFP) expressing only GFP was used as a control. The β-catenin siRNA vector Ad-rsiBC and a scrambled shRNA control (Ad-rsiControl) expressing red fluorescent protein (RFP) for monitoring of infection efficiency were kindly provided by Dr. T. C. He.

Cell culture. The human HCC cell lines SK-Hep1, SMMC-7721, and normal human liver cells (LO2) were initially obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). Hepatoma cell line SMMC-7721 was derived from hepatoma tissue of a male patient with HCC. All cells in the experiment were maintained in DMEM (HyClone, Logan, UT, USA). All culture media were supplemented with 10% FBS (Gibco, Rockville, MD, USA), 100 units/mL penicillin, and 100 μg/mL streptomycin (HyClone).

Patient samples. The HBV-related HCC tumor tissues and paired non-tumorous tissues were acquired from 50 patients who underwent surgery at the 2nd Affiliated Hospital of Chongqing Medical University (Chongqing, China) between 2012 and 2015, with the approval of the Institutional Review Board of Chongqing Medical University. Patients provided informed consent and had not received chemotherapy or radiation therapy before surgery. All liver specimens were immediately collected after surgery and stored at −80°C until further use.

Luciferase assay. SMMC-7721 cells were cultured in 25-cm² cell culture flasks and transfected with 3 μg β-catenin/Transcription Factor 4 (TCF-4) responsive luciferase reporter pTOP-luc using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) following the manufacturer’s instructions. The pRL-TK plasmid (Promega, Madison, WI, USA) was added as an internal control. At 24 h after transfection, cells were seeded into 24-well plates (Life Sciences, Tewksbury, MA, USA). At 8 h following replating, cells were infected with Ad-GFP, Ad-HBx WT, or Ad-HBx mutants. Cells were harvested 36 h post-infection and subjected to the Dual-Luciferase Reporter Assay (Promega). Each assay was carried out in triplicate and repeated three times.

Cell migration assay. Cell migration was measured using Transwell units with a polycarbonate filter (BD, San Jose, CA, USA). Cells were infected with Ad-GFP, Ad-HBx WT, or Ad-HBx mutants. At 24 h after infection, cells were suspended in 200 μL serum-free medium and added at 4 × 10⁵ cells/well to the upper chamber. DMEM medium (600 μL) containing 10% FBS was added to the lower chamber as a chemoattractant. Cells were incubated at 37°C under 5% CO₂. After 12 h, cells were fixed with 4% formaldehyde and stained with crystal violet. The number of migrated cells was counted in five fields (200x) on each membrane, and the average per field was calculated.

Cell proliferation assay. Cell proliferation was measured using the CellTiter 96 AQ One Solution Cell Proliferation Assay (MTS) (Promega). Infected cells (2 × 10⁵) were replated in 96-well plates, 20 μL MTS reagent was added to each well, and the plates were incubated for 2 h at 37°C. The absorbance at 490 nm was measured on day 4 using a microplate reader (Bio-Tek, Winooski, VT, USA).

RNA extraction and quantitative real-time PCR. Total cellular RNA was extracted from infected cells using TRIZOL Reagent (Invitrogen) according to the manufacturer’s instructions. RNA was treated using QI1 DNase (Promega) and converted to cDNA by AMV reverse transcriptase (Promega). The cDNA was diluted five-fold and used as the template in the quantitative real-time (qRT)-PCR reaction. All primer sequences, including c-Myc, Connective Tissue Growth Factor (CTGF), WNT1 Inducible Signaling Pathway Protein 2 (WISP2), and cyclin D1 are listed in Table S1. SYBR Green qRT-PCR was carried out using the CFX Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA). Duplicate reactions were measured for each sample and GAPDH was used as a normalization control.

Western blot analysis. Proteins were extracted from cells and tissues with cell lysis buffer (Beyotime Biotechnology, Jiangsu, China) containing 1 mM PMSF (Beyotime Biotechnology). Nuclear and cytoplasmic proteins were separated using the Nucleo and Cytoplasmic Protein Extraction Kit (Bioworld, Louis Park, MN, USA). Endogenous β-actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA), glycogen synthase kinase-3β (GSK-3β), (phospho-S9)-GSK-3β, CTGF, c-Myc, and cyclin D1 (all purchased from Bioworld, Louis Park, MN, USA). Secondary goat anti-rabbit IgG (H+L)-HRP antibodies were purchased from Bioworld. Endogenous β-actin (Bioworld) expression was used as the normalization control. Western blots were quantitated by densitometric analysis by using the ImageJ software program (https://imagej.nih.gov/ij/).

Amplification and sequence analysis of BCP region of HBV genome. The HBV genome was extracted from liver tissues using the Wizard Genomic DNA Purification Kit (Promega) according to the manufacturer’s instructions. The HBV fragment
HBx mutations activate Wnt/β-catenin signaling and stabilize β-catenin levels by inactivating GSK-3β. The pTOP-luc luciferase reporter assay was undertaken to determine whether HBx mutations could potentiate Wnt/β-catenin activity in hepatoma cells. HBx WT induced a 1.64-fold increase in luciferase activity compared with the GFP control in SMMC-7721 cells (P < 0.01, Fig. 2a). Compared to the control, luciferase activity increased 3.4-fold in the TA mutant group (P < 0.01) and 3.73-fold in the Combo mutant group (P < 0.01). Moreover, the HBx TA and Combo mutants increased luciferase activity by 2.07- and 2.27-fold, respectively, compared with HBx WT (P < 0.01).

The characteristic activation of the canonical Wnt pathway involves stabilization of β-catenin in the cytoplasm and its translocation to the nucleus. To examine the activation of Wnt/β-catenin signaling by HBx mutants, hepatoma cells SMMC-7721 and human hepatocyte cells LO2 were infected with Ad-GFP or Ad-HBx (Fig. S1), and the accumulation and localization of intracellular β-catenin was measured in the nuclear and cytoplasmic fractions. HBx increased the expression of β-catenin in both the cytoplasm and nucleus in SMMC-7721 and LO2 cells. Furthermore, HBx TA and Combo mutants significantly enhanced β-catenin accumulation in the nucleus of SMMC-7721 and LO2 cells (Fig. 2b).

Previous studies suggested that GSK-3β plays a crucial role in the phosphorylation of β-catenin, which causes β-catenin degradation through the ubiquitin–proteasome system. To explore the possible mechanism through which HBx mutants induced intracellular β-catenin accumulation, we assessed the expression and activation of GSK-3β in hepatoma cells. HBx mutants (especially TA and Combo mutants) markedly enhanced p-GSK-3β levels in SMMC-7721 and LO2 cells (Fig. 2c), whereas total GSK-3β levels were unchanged. These results suggest that HBx mutants enhance β-catenin accumulation in hepatoma cells partially through inactivation of cellular GSK-3β.

HBx mutations increase expression of Wnt/β-catenin downstream target genes. Aberrant activation of Wnt/β-catenin leads to upregulation of downstream target genes such as c-Myc, CCND1, CTGF, and WISP2 that regulate cell migration and proliferation. We next tested whether or not HBx mutations could influence the expression of Wnt/β-catenin downstream target genes in hepatoma cells. HBx mutants (especially TA and Combo mutants) markedly enhanced p-GSK-3β levels in SMMC-7721 and LO2 cells (Fig. 2c), whereas total GSK-3β levels were unchanged. These results suggest that HBx mutants enhance β-catenin accumulation in hepatoma cells partially through inactivation of cellular GSK-3β.

HBx TA and Combo mutations upregulate β-catenin in HBV-related HCC tissues. HBx TA and Combo mutations increase the risk of HCC. To explore the role of HBx mutations in HCC tumorigenesis, we measured the expression of β-catenin in 50 HCC and paired adjacent non-tumorous tissues from
HBV-positive patients. The genetic variations in the core promoter region were determined by the nested PCR method. The most frequently occurring mutation in this region was A1762T/G1764A, present in 20/50 (40%) HCC tissues and 19/50 (38%) non-tumorous tissues. T1753V or T1768A mutation always occurred along with the TA double mutation (8/50, 16%; 1/50, 2%, respectively) (Table 1). T1753A/A1762T/G1764A/T1768A mutations were rarely found in these samples (2–4%). No significant differences were found in the distribution or frequency of HBx mutants between tumor and adjacent non-tumorous tissues.

We were interested in investigating whether HBx mutations increased β-catenin levels in HCC tissues. Therefore, we examined β-catenin expression in 11 paired HCC samples by Western blot and immunohistochemical staining assays. As expected, the expression of β-catenin in tumorous tissues was
much higher than that in non-tumorous tissues. In addition, β-catenin levels in tumor tissues bearing the HBx TA, 1753A/1762T/1764A, 1762T/1764A/1768A, and Combo mutations were significantly increased (Fig. 4a, \( P < 0.05 \)). We also examined the protein expression of β-catenin by immunohistochemical staining. β-Catenin was located mainly in the
cytoplasm and cytoplasmic membranes in HBx WT samples. The 1753A/1762T/1764A and 1762T/1764A/1768A mutations moderately enhanced β-catenin expression in HCC tissues. However, the HBx Combo mutation markedly enhanced β-catenin expression and caused partial nuclear accumulation of β-catenin (Figs 4b, S4). Overall, HBx Combo mutations

Fig. 3. HBx mutations upregulate Wnt/β-catenin target genes. (a) Semiquantitative RT-PCR analyses of mRNA expression for selected Wnt target genes. Hepatocellular carcinoma SMMC-7721 and normal liver LO2 cells were infected with recombinant adenoviruses Ad-GFP, Ad-HBx WT, or Ad-HBx mutants for 36 h. Total RNA was isolated for RT-PCR analyses using primers specific for WISP2, CCND1, CTGF, and c-Myc. All samples were normalized to GAPDH. (b) mRNA expression levels of c-Myc, cyclin D1, CTGF, and WISP2 were determined by quantitative real-time PCR. Data are shown as means ± SD of three independent experiments. *P < 0.05 versus WT; **P < 0.01 versus WT. (c) HBx mutations enhance the expression of Wnt/β-catenin downstream genes at the protein level. Cells were infected with Ad-GFP, Ad-HBx WT, or Ad-HBx mutants. Total cell lysates were obtained 48 h after infection. β-Actin was used as the loading control. Experiments were carried out in triplicate, and representative results are shown.
Knockdown of β-catenin effectively abrogated cell migration and proliferation stimulated by HBx TA and Combo mutants. Our experiments showed that HBx TA and Combo mutants markedly promote hepatoma cell migration and proliferation. Additionally, HBx mutants can activate Wnt/β-catenin signaling. To explore the potential relevance of these effects, we silenced β-catenin expression by the siRNA approach to block the Wnt/β-catenin pathway (Fig. S5). The stimulated migration and proliferation of cells with HBx TA and Combo mutations were largely inhibited by siBC (Fig. 5). Therefore, we concluded that HBx TA and Combo mutations enhance the Wnt/β-catenin signaling to promote hepatoma cell tumorigenicity.

Table 1. Prevalence of hepatitis B virus core promoter/X mutations in tumor and non-tumorous tissues from patients with hepatitis B virus-related hepatocellular carcinoma

| Mutation                  | Tumor (n = 50), n (%) | Non-tumor (n = 50), n (%) |
|---------------------------|-----------------------|---------------------------|
| Wild-type                 | 20 (40)               | 20 (40)                   |
| A1762T/G1764A             | 20 (40)               | 19 (38)                   |
| T1753V/A1762T/G1764A      | 8 (16)                | 8 (16)                    |
| A1762T/G1764A/T1768A      | 1 (2)                 | 1 (2)                     |
| V1753A/A1762T/G1764A/T1768A | 1 (2)               | 2 (4)                     |

There were no significant differences between tumor and non-tumorous tissues (P > 0.05).

Discussion

The frequency of HBx mutations is significantly higher in patients with HCC than in patients with chronic hepatitis; however, the detailed mechanisms of HBx mutations and hepatocarcinogenesis remain unclear. In our study, we found that HBx TA and Combo mutants markedly promote hepatoma cell migration and proliferation. Depletion of β-catenin largely blocked cell migration and proliferation promoted by HBx mutants. We also found that the HBx Combo mutant enhanced pTOP-luc activity and caused partial nuclear translocation of β-catenin. HBx TA and Combo mutants induced β-catenin accumulation through inactivation of GSK-3β, thus contributing to upregulation of the Wnt target genes c-Myc, CTGF, and WISP2. Activation of canonical Wnt signaling was also shown in HBV-related HCC tissues bearing HBx TA and Combo mutants. Based on these results, we concluded that HBx TA

Fig. 4. HBx T1753A/A1762T/G1764A/T1768A (Combo) mutant upregulates β-catenin in hepatocellular carcinoma (HCC) tissue. (a) Western blot analyses of β-catenin expression in paired hepatitis B virus-related HCC samples. Tissue lysates were collected and blotted with a β-catenin antibody to determine the expression of β-catenin in HCC tumor (T) and paired adjacent non-tumorous tissues (N). *P < 0.05 versus WT in tumor tissues; **P < 0.05 versus WT in non-tumorous tissues. (b) Immunohistochemical staining of β-catenin in tumor (T) and paired adjacent non-tumorous tissues (N). Tissue samples were incubated with a β-catenin antibody and then visualized with 3,3′-diaminobenzidine. β-Catenin-positive cells stained brown. Representative images are shown (magnification, ×400).
and Combo mutations promote hepatoma cell tumorigenicity by activating the Wnt/β-catenin signaling pathway.

HBx mutations are associated independently with HBV-related HCC. Hepatitis B virus core promoter mutations are related to severe hepatic inflammation and liver cirrhosis. Hepatitis B virus mutations in the BCP occur in a sequential and accumulative manner. The TA double mutations always accompanied the T1753V or T1768A point mutations. In addition, the prevalence of HBx Combo mutations was significantly higher in HCC patients than in patients with chronic hepatitis. Previous studies indicated that the HBx Combo mutant upregulates S-Phase Kinase Associated Protein 2 (SKP2) through activation of the Rb/E2F1 pathway, thus accelerating p21 and p53 degradation. The Combo mutant has been shown to promote cellular proliferation by increasing the proportion of cells in S phase and the extent of cellular DNA synthesis. In addition, the HBx Combo mutant largely abolished nuclear factor-κB activity and inhibited the pro-apoptotic effects of HBx.

The HBx gene consists of two functional domains: an N-terminal negative regulatory domain and a C-terminal coactivation or transactivation domain. A previous study reported that HBx mutations with a COOH-terminal truncation and Combo mutations promote hepatoma cell tumorigenicity by activating the Wnt/β-catenin signaling pathway.

However, how BCP mutations in the HBx gene contribute to the progression of liver diseases such as HCC remains largely unknown. Combo mutations result in a.a. substitutions (I127N/K130M/V131I/F132Y) that may lead to aberrant biological activity of HBx. Previous studies indicated that the HBx Combo mutant upregulates S-Phase Kinase Associated Protein 2 (SKP2) through activation of the Rb/E2F1 pathway, thus accelerating p21 and p53 degradation. The Combo mutant has been shown to promote cellular proliferation by increasing the proportion of cells in S phase and the extent of cellular DNA synthesis. In addition, the HBx Combo mutant largely abolished nuclear factor-κB activity and inhibited the pro-apoptotic effects of HBx.

The HBx gene consists of two functional domains: an N-terminal negative regulatory domain and a C-terminal coactivation or transactivation domain. A previous study reported that HBx mutations with a COOH-terminal truncation
HBx with a COOH-terminal deletion mutation lost its transcription activity and inhibitory effects on cell proliferation and transformation. This COOH-shortened HBx may further activate the ras and myc oncogenes, thus promoting tumor migration and proliferation. (19)

In our study, we found that the HBx TA and Combo mutants upregulate cytoplasmic and nuclear β-catenin by inactivating GSK-3β. However, the detailed mechanism by which HBx mutations activate Wnt/β-catenin signaling is unclear. The potential mechanisms should be considered: HBx may suppress GSK-3β activity through activation of Src kinase or Erk kinase. (22,23) or HBx may interact with the suppressor APC to activate Wnt/β-catenin. (41) These possibilities will be explored in future studies.

In the current study, we did not find a significant difference in the distribution or frequency of HBx mutants between tumor and adjacent non-tumorous tissues. Our findings were consistent with two previous studies reporting that no tumor-specific and adjacent non-tumorous tissues. Our findings were consistent in the distribution or frequency of HBx mutants between tumor and non-tumor pairs might have evolved from the same ancestor viral strain. (43) Furthermore, progressive accumulation of naturally occurring viral mutants, especially the A1762T/G1764A double mutation accompanied by T1753A/T1768A point mutations, occurred only at or near the stage of HCC, indicating that the mutation combination plays a synergistic role in enhancing HBV carcinogenesis. (5) Another possibility, however, is that activation of the Wnt/β-catenin pathway might play an essential role in the early stage of malignant transformation but not in the late stage of HCC carcinogenesis. Further studies are required to elucidate the activation of Wnt/β-catenin in different stages of HBV infection, such as chronic hepatitis, cirrhosis, and HCC. In conclusion, our findings provide detailed information regarding the roles of HBx TA and Combo mutants in the progression of HCC and suggest a novel therapeutic strategy to prevent HBV-related HCC.

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Disclosure Statement
The authors have no conflict of interest.

References
1 Parkin DM. International variation. *Oncogene* 2004; 23: 6329–40.
2 Brechot C, Gozueck D, Murakami Y, Paterlini-Brechot P. Molecular bases for the development of hepatitis B virus (HBV)-related hepatocellular carcinoma (HCC). *Semin Cancer Biol* 2000; 10: 211–31.
3 Chan HL, Hui AY, Wong ML et al. Genotype C hepatitis B virus infection is associated with an increased risk of hepatocellular carcinoma. *Gut* 2004; 53: 1494–8.
4 Chen CL, Wong HL, Li et al. Risk of hepatocellular carcinoma across a biological gradient of serum hepatitis B virus DNA level. JAMA 2006; 295: 65–73.
5 Guo X, Jin Y, Qian G, Tu H. Sequential accumulation of the mutations in core promoter of hepatitis B virus is associated with the development of hepatocellular carcinoma in Qdong, China. *J Hepatol* 2008; 49: 718–25.
6 Tang H, Oishi N, Kaneko S, Murakami S. Molecular functions and biological roles of hepatitis B virus x protein. *Cancer Sci* 2006; 97: 977–83.
7 Wei Y, Neuveut C, Tiollais P, Buendia MA. Molecular biology of the hepatitis B virus and role of the X gene. *Pathobiol Paris* (2010); 58: 267–72.
8 Xie Q, Chen L, Shan X et al. Epigenetic silencing of SFRP1 and SFRP5 by hepatitis B virus X protein enhances hepatoma cell tumorigenicity through Wnt signaling pathway. *Int J Cancer* 2014a; 135: 635–46.
9 Matsuda Y, Ichida T. Impact of hepatitis B virus X protein on the DNA damage response during hepatocarcinogenesis. *Med Mol Morphol* 2009; 42: 138–42.
10 Elmore LW, Hancock AR, Chang SF et al. Hepatitis B virus X protein and p53 tumor suppressor interactions in the modulation of apoptosis. *Proc Natl Acad Sci USA* 1997; 94: 14707–12.
11 Ahuja R, Kapoor NR, Kumar V. The HBxnoncoprotein of hepatitis B virus engages nucleophosmin to promote RNA transcription and cellular proliferation. *Biochim Biophys Acta* 2015; 1853: 1783–95.
12 Kuasakabe A, Tanaka Y, Inoue M et al. A population-based cohort study for the risk factors of HCC among hepatitis B virus mono-infected subjects in Japan. *J Gastroenterol* 2011; 46: 117–24.
13 Leng XH, Chen EQ, Du LY et al. Biological characteristics of the A1762T/G1764A mutant strain of hepatitis B virus in vivo. *Med Mol Rep* 2015; 1: S141–8.
14 Xie Y, Liu S, Zhao Y, Guo Z, Xu J. X protein mutations in hepatitis B virus DNA predict postoperative survival in hepatocellular carcinoma. *Tumour Biol* 2014; 35: 3143–50.
15 Sirma H, Giannini C, Poussin K, Paterlini P, Kremsdorff D, Brechot C. Hepatitis B virus X mutants, present in hepatocellular carcinoma tissue occur more frequently in HCC than in non-tumor tissues. (40) HBx with a COOH-terminal deletion mutation lost its transcription activity and inhibitory effects on cell proliferation and transformation. This COOH-shortened HBx may further activate the ras and myc oncogenes, thus promoting tumor migration and proliferation. (19)
30 Chen CH, Hung CH, Lee CM et al. Pre-S deletion and complex mutations of hepatitis B virus related to advanced liver disease in HBeAg-negative patients. Gastroenterology 2007; 133: 1466–74.
31 Liang TJ, Hasegawa K, Rimon N, Wands JR, Ben-Porath E. A hepatitis B virus mutant associated with an epidemic of fulminant hepatitis. N Engl J Med 1991; 324: 1705–9.
32 Baumert TF, Marrone A, Vergalla J, Liang TJ. Naturally occurring mutations define a novel function of the hepatitis B virus core promoter in core protein expression. J Virol 1998; 72: 6785–95.
33 Buckwold VE, Xu Z, Yen TS, Ou JH. Effects of a frequent double-nucleotide basal core promoter mutation and its putative single-nucleotide precursor mutations on hepatitis B virus gene expression and replication. J Gen Virol 1997; 78(Pt 8): 2055–65.
34 Yu H, Zhu R, Zhu YZ, Chen Q, Zhu HG. Effects of mutations in the X gene of hepatitis B virus on the virus replication. Acta Virol 2012; 56: 101–10.
35 Hussain Z, Jung HS, Lim SO, Yu DY, Jung G. Hepatitis B viral X protein interacts with tumor suppressor adenomatous polyposis coli to activate Wnt/beta-catenin signaling. Cancer Lett 2011; 300: 162–72.
36 Huang Y, Tong S, Tai AW, Hussain M, Lok AS. Hepatitis B virus core promoter mutations contribute to hepatocarcinogenesis by deregulating SKP2 and its target, p21. Gastroenterology 2011; 141: 1412–21, 1421 e1–5.
37 Huang Y, Tai AW, Tong S, Lok AS. HBV core promoter mutations promote cellular proliferation through E2F1-mediated upregulation of S-phase kinase-associated protein 2 transcription. J Hepatol 2013; 58: 1068–73.
38 Yan J, Yao Z, Hu K et al. Hepatitis B virus core promoter A1762T/G1764A (TA)/T1753A/T1768A mutations contribute to hepatocarcinogenesis by deregulating Skp2 and P53. Dig Dis Sci 2015; 60: 1315–24.
39 Gong DY, Chen EQ, Huang FJ, Leng XH, Cheng X, Tang H. Role and functional domain of hepatitis B virus X protein in regulating HBV transcription and replication in vitro and in vivo. Viruses 2013; 5: 1261–71.
40 Wang D, Cai H, Yu WB, Yu L. Identification of hepatitis B virus X gene variants between hepatocellular carcinoma tissues and pericarcinoma liver tissues in Eastern China. Int J Clin Exp Pathol 2014; 7: 5988–96.
41 Hsieh A, Kim HS, Lim SO, Yu DY, Jung G. Hepatitis B viral X protein interacts with tumor suppressor adenomatous polyposis coli to activate Wnt/beta-catenin signaling. Cancer Lett 2011; 300: 162–72.
42 Zhang AY, Lai CL, Poon RT et al. Hepatitis B virus full-length genomic mutations and quasispecies in hepatocellular carcinoma. J Gastroenterol Hepatol 2016; doi: 10.1111/jgh.13316.
43 Wilbe K, Salminen M, Laukkonen T et al. Characterization of novel recombinant HIV-1 genomes using the branching index. Virology 2003; 316: 116–25.

Supporting Information
Additional Supporting Information may be found online in the supporting information tab for this article:

Fig. S1. Validation of infection efficiency in hepatoma cells.

Fig. S2. Endogenous expression of beta-catenin and beta-catenin/transcription factor (Tcf) activity in hepatoma cells.

Fig. S3. Migration and proliferation were enhanced by HBx mutants in hepatocytes.

Fig. S4. Expression level of beta-catenin was upregulated in HBx-mutated hepatocellular carcinoma tissues.

Fig. S5. Infection efficiency of adenoviruses and knockdown of beta-catenin by beta-catenin siRNA vector (siBC).

Table S1. Primer sequences.