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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 Hu[1] and Ni Tang[1]

[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

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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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[2]These authors contributed equally to this work.

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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.

Hepatocellular carcinoma (HCC) is the fifth most common cancer and ranks as the third leading cause of cancer-related death around the world.[1] Chronic infection with hepatitis B virus (HBV) is a major risk factor for cirrhosis and HCC in developing countries.[2] Many viral factors including high virus load, HBV genotype C, and core promoter mutations are associated with a high risk of HCC.[3–5]

Hepatitis B virus is a partially double-stranded circular 3.2-kb DNA that belongs to the orthohepadnavirus of the hepadnaviridae family. The HBV genome contains four overlapping ORFs that encode hepatitis B surface antigen, hepatitis B e-antigen (HBeAg), HBV polymerase, and HBx protein. The HBx protein, encoded by the HBV X gene, is a 154-amino acid (a.a.) polypeptide with a molecular weight of 17 kDa and plays a crucial role in hepatocarcinogenesis.[6] HBx is a promiscuous transactivator that can activate the NFAT, CREB/ATF, Wnt/β-catenin, and nuclear factor-κB signaling pathways.[7,8] In addition to transactivation, HBx has been implicated in crucial cellular events including inhibition of DNA repair,[9] modulation of apoptosis,[10] and regulation of the cell cycle.[11]

A1762T/G1764A (TA) mutations in the core promoter, which overlap with the HBx gene, are found commonly in HCC and are independent risk factors for the progression of HCC during chronic HBV infection.[12] *In vivo* studies suggest that the TA mutant increases the replication capacity of HBV and suppresses HBeAg serum levels.[13] A1762T/G1764A mutations are also predictors of postoperative survival in patients with HBV-related HCC.[14] A1762T/T1764A mutations in the HBx gene lead to Lys-Met 130 and Val-Ile 131 substitutions, which in turn affect the biological activities of HBx by abrogating its transcription activity and inhibitory effects on cell proliferation and transformation.[15] T1753A/A1762T/G1764A/T1768A (Combo) mutations in the basal core promoter (BCP) of HBV and combinations of multiple point mutations (G1613A, C1653T, T1753V, A1762T, and G1764A) are closely related to HCC in patients infected with HBV.[16,17] The Combo mutations in BCP result in four a.a. substitutions in HBx protein at a.a. 127 (isoleucine to asparagine/serine/threonine), 130 (lysine to methionine), 131 (valine to isoleucine), and 132 (phenylalanine to tyrosine), which might affect the biological activity of HBx.[18,19] However, the

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.[20] Mutations in the *CTNNB1*, *AXIN2,* and *APC* genes contribute to aberrant activation of Wnt/β-catenin signaling and HCC development.[21] HBx stabilizes cytoplasmic β-catenin through interaction with Wnt1 or activation of Erk.[22,23] 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 *Kpn*I and *Hind*III sites of the shuttle vector pAdTrack-TO4 (obtained from Dr. T.-C. He, University of Chicago, Chicago, IL, USA). The HBx mutations A1762T (T mutant), G1764A (A mutant), A1762T/G1764A (TA mutant), and T1753A/A1762T/G1764A/T1768A (Combo mutant) were constructed by overlapping extension PCR using pAd-HBx WT as the template, and subcloned into pAdTrack-TO4 (primer sequences are listed in Table S1). All HBx mutations were confirmed by DNA sequencing. Recombinant Ad-HBx (specifically, Ad-HBx WT, Ad-HBx T, Ad-HBx A, Ad-HBx TA, 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 AdR-siBC and a scrambled shRNA control (AdR-siControl) 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).[8,24] 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-7721cells were cultured in 25-cm^2 cell culture flasks and transfected with 3 μg β-catenin/Transcription Factor 4 (TCF4) 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 4×10^4 cells/well to the upper chamber. Then DMEM medium (600 μL) containing 10% FBS was added to the lower chamber as a chemoattractant. Cells were incubated at 37°C under 5% CO_2. After 12 h, cells were fixed with 4% formaldehyde and stained with crystal violet. The number of migrated cells was counted in five fields (200×) 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 (2×10^3) 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 every 24 h until 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 RQ1 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 Nuclear and Cytoplasmic Protein Extraction Kit (Beyotime Biotechnology) according to the manufacturer's instructions. Protein concentrations were determined using the BCA protein assay kit (Beyotime Biotechnology). Approximately 50 μg proteins were separated on 10% polyacrylamide gels and electrotransferred to PVDF membranes (Millipore, Billerica, MA, USA). The membranes were immunoblotted with the antibodies targeting β-catenin (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

(nt 1176–1823), including the core promoter region and overlapping X gene, was amplified by nested PCR. All primer sequences are listed in Table S1. The first PCR reaction was carried out in a 25-μL reaction volume using forward (HBVF; nt 1176–1195) and reverse (HBVR; nt 1823–1804) primers. Using HBVF′ (nt 1263–1282) and HBVR (nt 1823–1804) primers, a second round of PCR was carried out in a total volume of 25 μL containing 0.25 μL first-round product. The second-round PCR products were sequenced by BGI Biotechnology (Beijing, China).

Immunohistochemical staining. Liver tissues were fixed with 4% paraformaldehyde, embedded in paraffin, and sectioned according to standard procedures. Tissue slices were incubated with a β-catenin antibody at 4°C overnight. Subsequently, the slides were incubated with a secondary anti-rabbit IgG (ZSGB-BIO, Beijing, China) and visualized using 3,3′-diaminobenzidine (ZSGB-BIO).

Statistical analyses. Data are presented as the mean ± SD. Differences between groups were determined by ANOVA followed by Least Significant Difference Test. Mutation differences between two groups of samples were analyzed with the χ^2-test. All data were analyzed using SPSS 19.0 software (IBM, Chicago, IL, USA). In all experiments, $P < 0.05$ was considered statistically significant.

Results

HBx mutations enhance cellular migration and proliferation. To study the biological functions of HBx mutations in hepatoma cells, we first constructed recombinant adenovirus Ad-HBx WT (wild-type), Ad-HBx A1762T (T mutant), Ad-HBx G1764A (A mutant), Ad-HBx A1762T/G1764A (TA mutant), and Ad-HBx T1753A/A1762T/G1764A/T1768A (Combo mutant). Both the Ad-HBx WT and Ad-HBx mutants effectively infected hepatoma cells (Fig. S1).

We examined endogenous β-catenin expression in LO2, HepG2, Huh7, and SMMC-7721 cells. Western blot results indicated that the expression levels of β-catenin in SMMC-7721 and LO2 were similar to that in Huh7, without accumulation of the mutated form of β-catenin protein (Fig. S2a). Furthermore, we analyzed the endogenous β-catenin/transcription factor activity in hepatoma cells by using pTOP-luc luciferase reporter assay. As shown in Figure S2(b), endogenous reporter activity was maintained at a basal level in Huh7, SMMC-7721, and LO2 cells, whereas Wnt signaling was already activated in HepG2 cells, which harbor an oncogenic β-catenin mutation. Based on these results, SMMC-7721 and LO2 cells were suitable to examine whether HBx mutants can activate Wnt/β-catenin signaling activity.

Compared with HBx WT, both HBx TA and Combo mutants markedly promoted the migration of hepatoma cells ($P < 0.01$, Fig. 1a,b). The HBx T and HBx A mutants moderately accelerated the migration of hepatoma cells compared to the WT ($P < 0.05$). Compared with the GFP control, HBx WT slightly enhanced the migration of hepatoma cells.

The HBx TA and Combo mutants markedly promoted the proliferation of SMMC-7721 and SK-Hep1 cells ($P < 0.01$, Fig. 1c). In agreement with the migration results, HBx T and HBx A single mutations moderately accelerated tumor cell growth, whereas HBx WT had little effect on hepatoma cell proliferation. Similar stimulation of cell proliferation by HBx mutants was also observed in LO2 cells (Fig. S3). Together, these data suggest that HBx mutations enhance the migration and proliferation of hepatoma cells.

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.[25] 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-7721and 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*[24,26–28] that regulate cell migration and proliferation.[29] We next tested whether or not HBx mutations could influence the expression of Wnt/β-catenin downstream target genes in hepatoma cells. mRNA expression levels of c-Myc, CTGF, and WISP2 were 2.46-, 1.30-, and 1.55-fold higher in HBx TA-expressing cells, respectively, than those in HBx WT-transduced cells (Fig. 3a,b, $P < 0.05$). Moreover, the levels of c-Myc, CTGF, and WISP2 in HBx Combo-expressing SMMC-7721 cells were approximately 4.25-, 2.32-, and 2.64-fold higher, respectively, than those in SMMC-7721 cells infected with Ad-HBx WT ($P < 0.01$). HBx T and A mutants only slightly affected c-Myc, CTGF, and WISP2 expression. In addition, the protein expression levels of Wnt target genes were highly consistent with the RT-PCR and qRT-PCR results (Fig. 3c). Similar results were found in LO2 cells (Fig. 3). However, there was no significant increase in Cyclin D1 expression in hepatoma cells expressing HBx mutants. These results indicate that HBx Combo mutations activate the Wnt/β-catenin signaling pathway and upregulate target genes including *c-Myc*, *CTGF*, and *WISP2*.

HBx TA and Combo mutations upregulate β-catenin in HBV-related HCC tissues. HBx TA and Combo mutations increase the risk of HCC.[16] 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

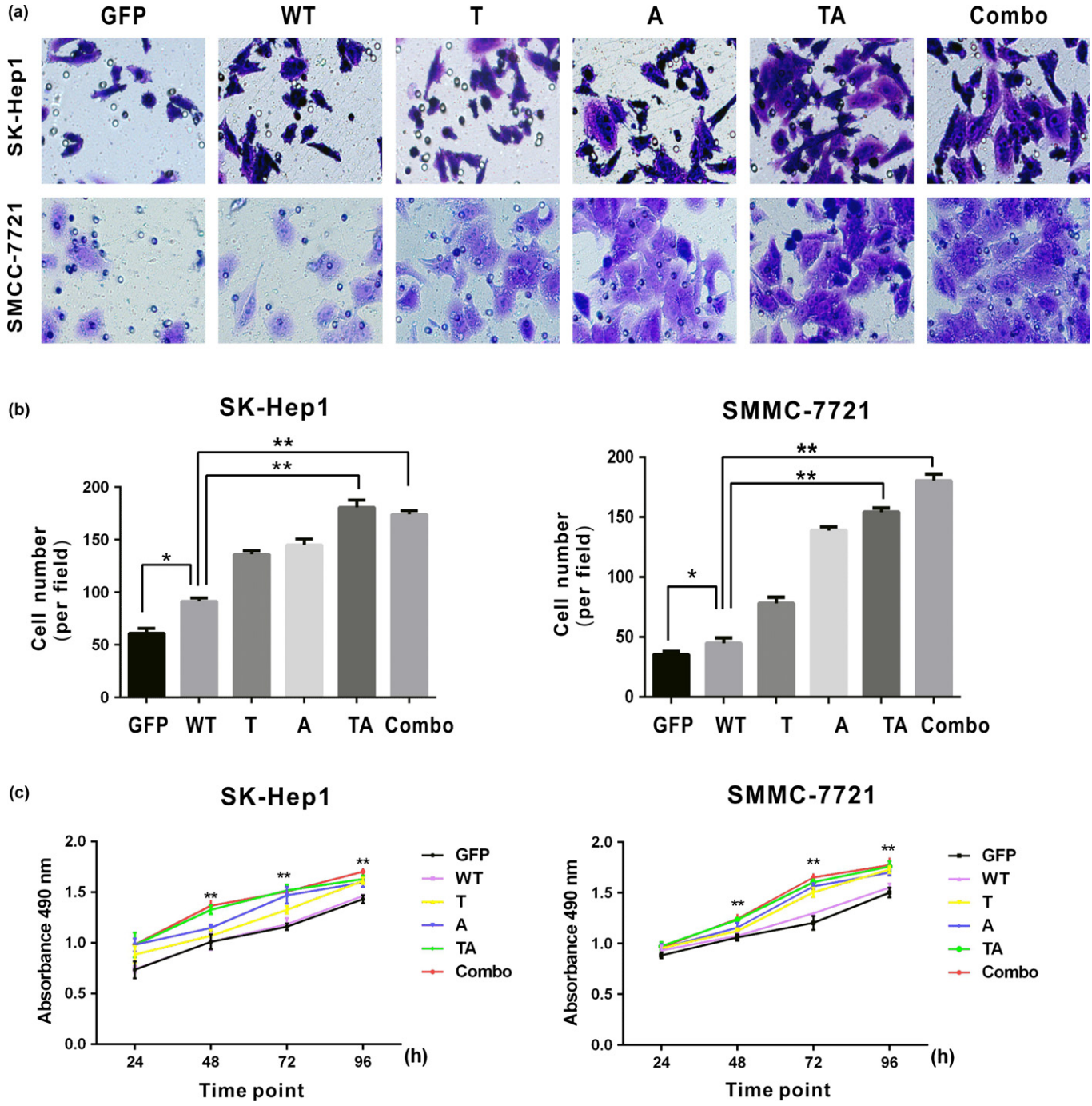

Fig. 1. HBx mutations (T1753V, A1762T, G1764A, and T1768A) promote migration and proliferation of hepatoma cells. (a) SMMC-7721 and SK-Hep1 cells were infected with recombinant adenoviruses Ad-GFP, Ad-HBx WT, or Ad-HBx mutants (T, A, TA, or Combo mutants). At 24 h after infection, cells were subjected to Transwell assay. (b) Quantitative evaluation of cell migration activity is presented as the mean ± SD of five randomly selected microscopic fields from three independent experiments. *$P < 0.05$ versus GFP control; **$P < 0.01$ versus WT. (c) Cell growth curves. Cells were treated as described in (a) then plated into a 96-well plate at 2×10^3 cells/well. The number of viable cells was counted for 4 days. Data are presented as the mean ± SD. **$P < 0.05$ versus WT.

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

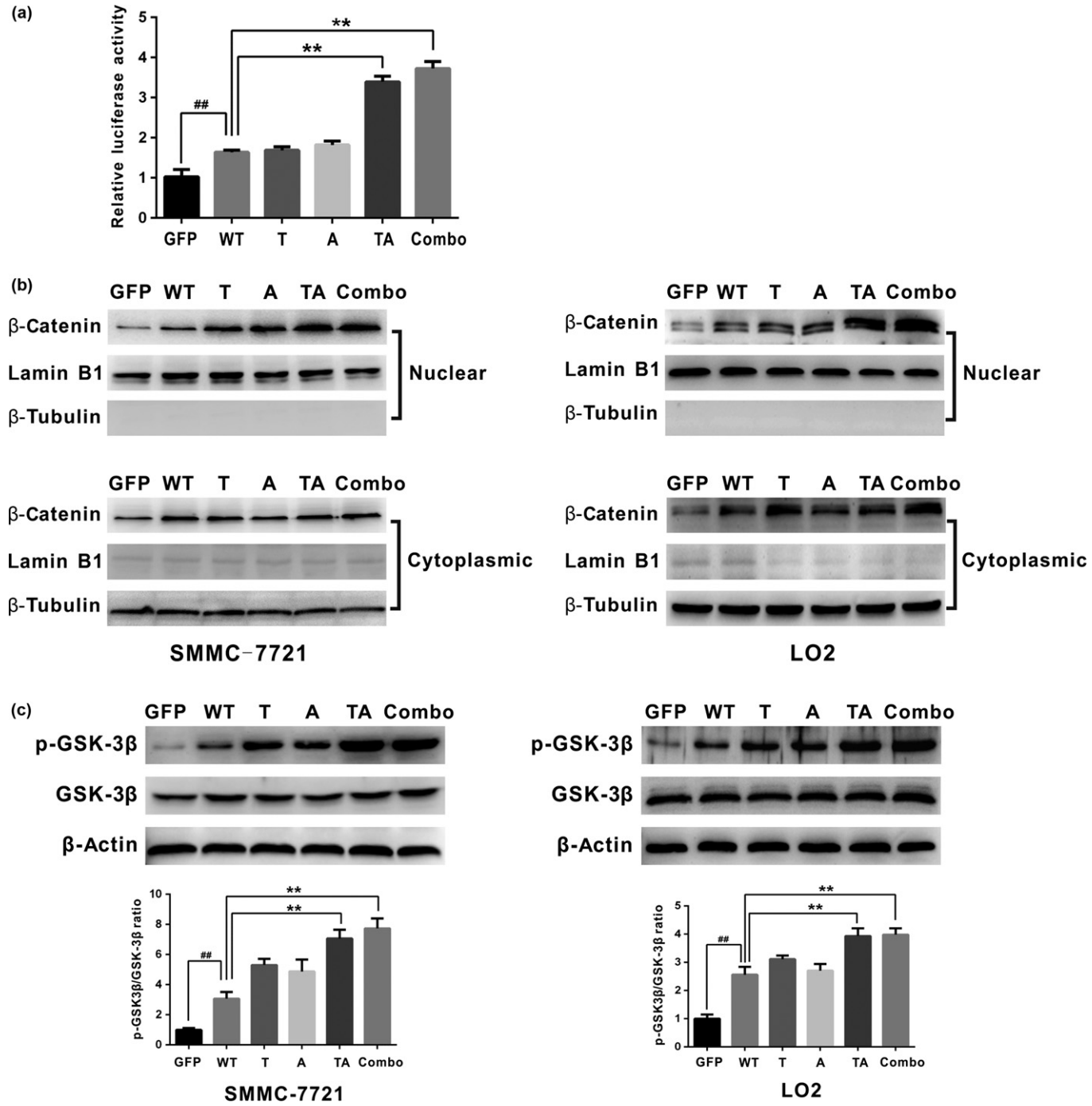

Fig. 2. HBx mutations (T1753V, A1762T, G1764A, and T1768A) activate the Wnt/β-catenin pathway in hepatocellular carcinoma cells. (a) HBx mutations increase TOP-luc luciferase reporter activity. SMMC-7721 cells were transfected with the Transcription Factor 4/Liver-Enriched Factor 1 reporter, pTOP-luc. At 24 h after transfection, cells were infected with recombinant adenoviruses Ad-GFP (control), Ad-HBx WT, or Ad-HBx mutants. At 36 h after infection, cells were collected for luciferase assays. Results are presented as the mean relative luciferase activity relative to the activity of the GFP-treated control sample ± SD of three independent experiments. $^{**}P < 0.01$ versus WT; $^{\#\#}P < 0.01$ versus GFP control. (b) β-Catenin levels in nuclear and cytoplasmic fractions. SMMC-7721 and normal liver LO2 cells were infected with Ad-GFP, Ad-HBx WT, or Ad-HBx mutants (T, A, TA, or Combo mutants). At 48 h after infection, cytosolic and nuclear proteins were extracted. Subsequently, both the cytosolic and nuclear fractions were immunoblotted with an anti-β-catenin antibody. The purity of the cytosolic and nuclear fractions was verified by immunoblot analysis using anti-β-tubulin and anti-lamin B1 antibodies, respectively. All data were acquired from three independent experiments, and representative results are shown. (c) HBx mutants inactivate glycogen synthase kinase-3β (GSK-3β) activity in hepatoma and liver cells. At 48 h following infection, whole cell lysates were subjected to immunoblotting with anti-GSK-3β and anti-phospho- (p-)GSK-3β antibodies. β-Actin was used as the loading control. Integrated density of GSK-3β and p-GSK-3β was quantitatively analyzed using ImageJ software. $^{**}P < 0.01$ versus WT; $^{\#\#}P < 0.01$ versus GFP control.

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

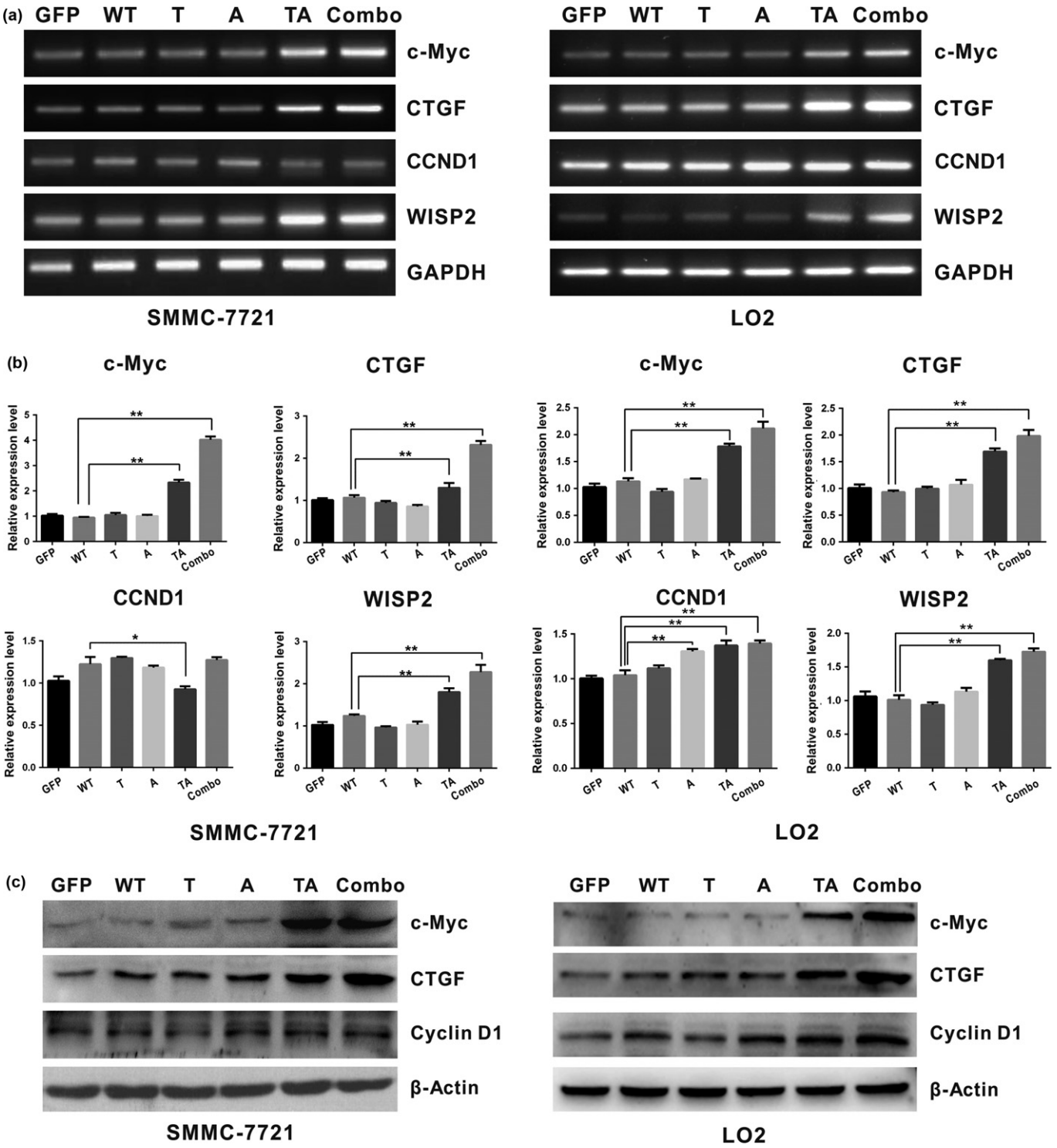

Fig. 3. HBx mutations upregulate Wnt/β-catenin target genes. (a) Semiquantitative RT-PCR analyses of mRNA expression for selected Wnt target genes. Hepatocellular carcinoma SMMC-7721 and normal liver LO2 cells were infected with recombinant adenoviruses Ad-GFP, Ad-HBx WT, or Ad-HBx mutants for 36 h. 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.

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

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-tumor tissues ($P > 0.05$).

upregulate β-catenin in HCC tissues, which may be crucial in the development of HCC.

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 mutations largely enhance the migration and proliferation of hepatoma cells. 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.

Discussion

The frequency of HBx mutations is significantly higher in patients with HCC than in patients with chronic hepatitis;[5] however, the detailed mechanisms of HBx mutations and hepatocarcinogenesis remain unclear. In our study, we found that HBx TA and Combo mutants markedly promoted 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

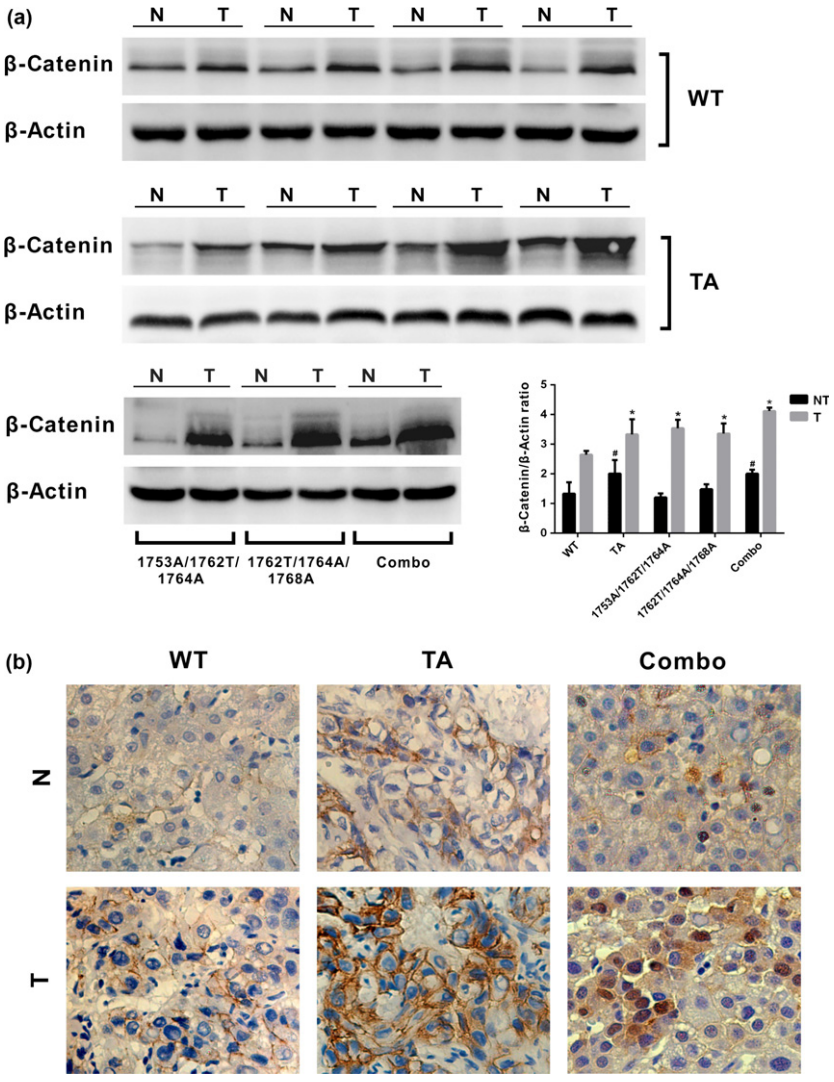

Fig. 4. HBx T1753A/A1762T/G1764A/T1768A (Combo) mutant upregulates β-catenin in hepatocellular carcinoma (HCC) tissue. (a) Western blot analyses of β-catenin expression in paired hepatitis B virus-related HCC samples. Tissue lysates were collected and blotted with a β-catenin antibody to determine the 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).

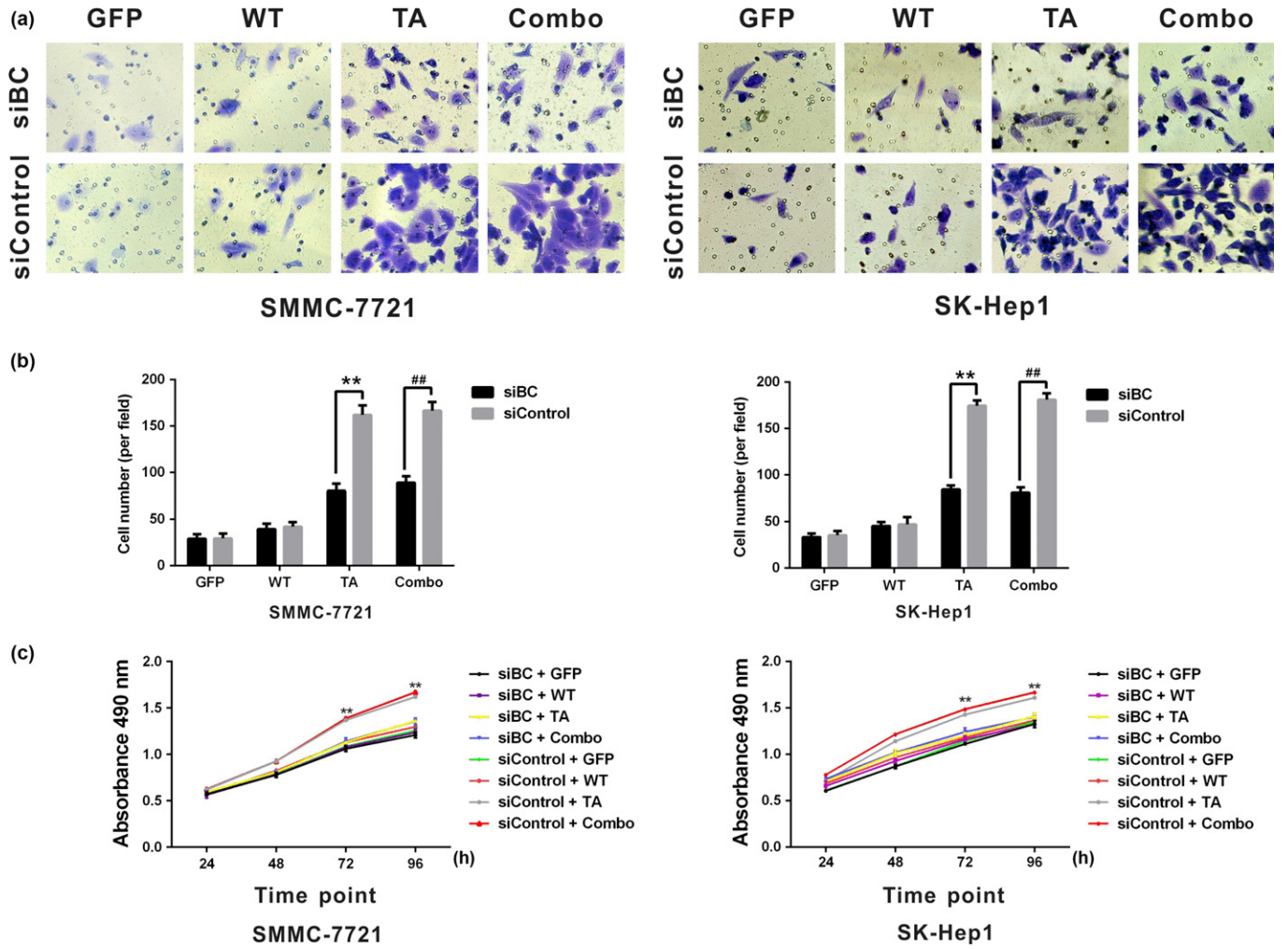

Fig. 5. HBx A1762T/G1764A (TA) and T1753A/A1762T/G1764A/T1768A (Combo) mutant-stimulated migration and proliferation are partially blocked by Wnt antagonists. (a) Hepatoma cells were infected with an optimal titer of β-catenin siRNA vector (siBC) and a scrambled shRNA control (siControl). After 24 h, the infected cells were replated and infected with recombinant adenoviruses Ad-GFP, Ad-HBx WT, or Ad-HBx mutants. Transwell and MTS assays were carried out. (b) Quantitative evaluation of cell migration activity. $^{**}P < 0.01$, siBC+TA mutant versus siControl+TA mutant; $^{\#\#}P < 0.01$, siBC+Combo mutant versus siControl+Combo mutant. (c) Cell growth curves. $^{**}P < 0.01$, siBC+HBx mutants versus siControl+HBx mutant.

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.[30,31] 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.[16]

The potential impact of naturally occurring BCP mutations on HBV viral biology has been extensively explored. A previous study reported that two core promoter mutations in the HBV BCP (C1768T and T1770A) resulted in enhanced viral encapsidation and replication.[32] The A1762T and G1764A double mutation (TA mutation) has been reported to prevent the binding of liver-enriched factor to the BCP region, thus inhibiting precore RNA transcription and HBeAg secretion.[13,33,34] Hussain *et al.* further explored the potential role of HBx alteration by overlapping BCP mutations on viral genome replication. Their results indicated that the A1762T and G1764A double mutation in the BCP, but not in the overlapping X gene, is responsible for the enhanced viral genome replication observed for BCP mutants.[35]

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.[36–38] In addition, the HBx Combo mutant largely abolished nuclear factor-κB activity and inhibited the pro-apoptotic effects of HBx.[16]

The HBx gene consists of two functional domains: an N-terminal negative regulatory domain and a C-terminal coactivation or transactivation domain.[39] A previous study reported that HBx mutations with a COOH-terminal truncation

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]

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 hot-spot mutations, including the A1762T and G1764A double mutation, were found selectively in tumor tissues.[40,42] This similar frequency of HBx point mutations in tumor and non-tumor tissues indicates that different HBV variants derived from 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.

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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 β-catenin and β-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 β-catenin was upregulated in HBx-mutated hepatocellular carcinoma tissues.

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

Table S1. Primer sequences.