Dysregulation of β-catenin by hepatitis B virus X protein in HBV-infected human hepatocellular carcinomas

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1 Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignancies worldwide [1]. Epidemiologic studies have shown that more than 70% of HCC cases are linked to chronic infection with hepatitis B virus (HBV) or hepatitis C virus (HCV) [2]. After integration of the HBV DNA into the host genome, the expression of the viral protein, hepatitis B virus X protein (HBx), becomes deregulated and is still found in transformed hepatocytes, even in the absence of any other viral markers [3,4]. HBx is essential for viral infectivity and is a potential cofactor in viral carcinogenesis. It was previously reported that HBx could show oncogenic potential in some transgenic mice [5–7] and activate several signal pathways linking ras, raf, and mitogen-activated protein (MAP) kinase [8,9]. In addition, HBx has been shown to activate transcription factors, nuclear factor kappa B (NF-KappaB), activator protein 1 (AP-1) and possibly CCAAT/enhancer-binding protein (C/EBP) [10–12]. HBx is also able to interfere with the DNA repair and apoptosis mechanism [13–15]. Although some of the signaling pathways triggered by HBx are known to be involved in HCC development, the possible role of HBx in tumor progression and metastasis has not yet been fully understood.

Disruption in cell adhesion is thought to be the basis for tumor cell motility and invasiveness [16]. At adherens junction, E-cadherin/β-catenin complex is linked via α-catenin to the actin filament network and plays a fundamental role in the formation and maintenance of epithelial tissues. Changes in adhesion complexes lead to
alteration of cell polarity, proliferation, mobility and differentiation [17]. Altered expression of β-catenin and E-cadherin is frequently observed in the malignant progression of HCC [18,19]. In addition to its adhesive functions, β-catenin has also been found to serve as a key component in Wnt signaling [20,21]. Signal transduction via β-catenin involves its posttranslational stabilization and passage into the nucleus, where it interacts with transcription factors of T cell factor/lymphoid enhancer factor family to activate target genes involved in cell growth control and apoptosis such as c-myc and cyclin D1 [22,23]. Unstimulated cells harbor a cytoplasmic multiprotein complex composed of tumor suppressor proteins, adenomatous polyposis coli (APC), Axin, casein kinase Iα (CKI-α) and glycogen synthase kinase-β (GSK-3β). APC and Axin are thought to be scaffold proteins, facilitating the sequential phosphorylation of β-catenin at the N-terminal region by CKI-α and GSK-3β, thereby earmarking β-catenin for ubiquitination-dependent proteolysis [24]. When this signaling pathway is aberrantly activated, it can lead to malignant transformation [25]. Oncogenic mutations of β-catenin or Axin have been reported in approximately 20% of HCCs and implicated in the development of HCC [26–31].

In the current study, we reported for the first time the observation of HBx to reduce the expression levels of wild-type β-catenin and E-cadherin in hepatoma cell line HepG2 cells with subsequent disruption of cadherin-based cell junctions. This modification appears to be dependent on posttranslational mechanism mediated by HBx. Moreover HBx was able to upregulate β-catenin transcriptional activity by increasing the nuclear pool of truncated β-catenin. Immunohistology and Western blot analysis of HCC tissue samples revealed that β-catenin expression levels were decreased more frequently in HCC with HBV infection than in HBV-unrelated tumors, thus pointing to a new function of HBx in the HCC development and progression.

2 Materials and methods

2.1 Cell culture and transfection

All cell lines were grown in DMEM containing 10% fetal bovine serum (FBS). HepG2 cells stably transfected with pCDNA3.0 and pCDNA3.0-HBx (kindly provided by Dr. M.A. FEITELSON) have been described previously [32,33] and cultured in the presence of 200 μg/mL G418. Transient transfection of 293 cells was conducted using lipofectamine 2000 (Invitrogen) according to the manufacturer’s recommendation. For analysis of β-catenin stability, cells were treated with cycloheximide (150 μg/mL, Sigma) or LiCl (20 μmol/L, Sigma) for indicated time and subjected to immunoblotting.

2.2 Luciferase assay

Two different TCF-luciferase reporter genes were used in this study: An intact wild-type TCF-luciferase construct (pGL-OT) and a mutant TCF-luciferase reporter construct (pGL-OF) (both were gifts of B. VOGELSTEIN). A dual luciferase reporter assay was carried out according to manufacturer’s suggestions (Promega). pRL-TK (Promega) was co-transfected with each reporter construct to normalize for transfection. Luciferase activity was measured 48 h after transfection. All experiments were performed in triplicate.

2.3 cDNA constructs and antibodies

Expression construct of HBx has been described previously [33]. Antibodies specific for E-cadherin (H-108), β-catenin (E-5), γ-catenin (C-20) were purchased from Santa Cruz Biotechnology. A polyclonal antibody against β-catenin was used as described [34]. HBx monoclonal antibody was from Chemicon.

2.4 Northern blotting and RT-PCR

Total RNA was prepared using TRIzol (Invitrogen), RNA (40 μg) was separated and transferred to nitrocellulose membranes and probed using E-cadherin cDNA labeled by random oligonucleotide priming (Promega). RT-PCR was performed using the one-step RNA PCR kit (TaqKaRa). Quantification and equalization of the amount of cDNA was achieved using primers to amplify β-actin as an internal control. The sequences of primers used in the RT-PCR were as follows: β-catenin, 5′-TGGACAATGGCTACTCAAG-3′ (sense) and 5′-TTA-CAGGTCAGTATCAAACC-3′ (antisense); β-actin, 5′-GCCAACACAGTGCTGTCTG-3′ (sense) and 5′-CACATCTGCTGGGAAGGTGG-3′ (antisense).

2.5 Immunoprecipitation, immunoblotting and cell fractionation

Cell extracts were prepared using Nonidet P-40 (NP-40) lysis buffer [20 mmol/L Tris-HCL, 150 mmol/L NaCl, 1% NP-40, 1 mmol/L ethylenediaminetetraacetic acid (EDTA), 1 mmol/L ethyleneglycol-bis(8-aminoethyl ether) N,N′-tetraacetic acid (EGTA), 1 mmol/L Na2VO4, 1 mmol/L NaF, 2.5 mmol/L sodium pyrophosphate, 1 mmol/L β-glycerolphosphate, 1 mmol/L phenylmethyl-sulphonyl fluoride, 10 μg/mL aprotonin]. The supernatant was collected and incubated with antibodies at 4°C for 3 h, and then with protein A for an additional 3 h. The beads were washed three times with lysis buffer, and resuspended in sodium dodecyl sulfate (SDS) sample buffer. For immunoblot analysis, samples were separated by sodium...
2.9 Statistical analysis

Relationship between β-catenin and E-cadherin immunostaining or Western blot data and clinicopathologic factors was analyzed using χ² test. The significant level was defined as P < 0.05.

3 Results

3.1 HBx downregulated wild-type β-catenin expression in HepG2 cells

HepG2 cells contain truncated form of β-catenin and reduced expression of wild-type β-catenin [37]. To explore the potential role of HBx in β-catenin regulation, we used immunoblotting to evaluate the status of β-catenin in HBx stably transfected HepG2 cells. In comparison with the control cells, HepG2-HBx cells showed evidently decreased levels of wild-type β-catenin, with no or minimal changes in those of mutated β-catenin or γ-catenin. E-cadherin was also decreased in HBx-expressing HepG2 cells and the adherens junction was disrupted accordingly (Fig. 1a and 1b). Co-immunoprecipitation assay showed that the amounts of wild-type β-catenin and E-cadherin were both decreased in anti-β-catenin immunoprecipitates in HepG2-HBx cells (Fig. 1c), supporting the viewpoint that HBx might mediate adherens junction disruption through downregulation of cadherin/β-catenin complexes.

3.2 Reduction of wild-type β-catenin expression involved degradation of the protein

RT-PCR or Northern blot analysis of β-catenin and E-cadherin mRNA in HepG2-HBx cells compared with control cells showed that the effect of HBx was not transcriptionally mediated (Fig. 2a). This suggests that there is some posttranscriptional mechanism by which HBx may reduce wild-type β-catenin but not mutant β-catenin expression. We therefore examined the turnover of β-catenin and E-cadherin in HepG2-HBx cells and HepG2-pcDNA3.0 control cells. The cells were treated with cycloheximide to block new protein synthesis. Western blot showed that β-catenin and E-cadherin were degraded more rapidly in HBx stably transfected cells than in vector-transfected cells (Fig. 2b).

We then investigated whether lithium chloride (LiCl), a potent inhibitor of GSK-3β activity, could abolish the HBx induced decrease of wild-type β-catenin in HepG2 cells. Indeed, we found that treatment with LiCl for indicated time gradually increased the expression level of wild-type β-catenin in HBx expressing HepG2 cells with no effect on
the mutant form. Interestingly, increased E-cadherin was also observed after LiCl treatment and no or minimal change was found in control cells (HepG2-pcDNA3.0) (Fig. 2c).

To further confirm the effect of HBx on β-catenin degradation, we transiently transfected HBx into 293T cells. As expected, ectopically HBx expression evidently decreased the endogenous level of β-catenin in 293T cells in a dose-dependent manner, whereas the decrease was compromised in the presence of LiCl, indicating that HBx mediated β-catenin degradation was not restricted to a single cell type (Fig. 2d).

3.3 HBx enhanced β-catenin mediated signaling in HepG2 cells

Our results described above indicated that HBx was able to decrease levels of wild-type, but not the truncated β-
Fig. 2  β-catenin was destabilized in HBx expressing cells. (a) RT-PCR was performed to compare the mRNA levels of wild-type and mutant form of β-catenin in the stable cells, β-actin was used as a control for the same amount of RNA (left); Northern blot of total RNA showing comparable levels of E-cadherin transcription in HepG2-pcDNA3.0 and HepG2-HBx cells. The 18 S rRNA was used as the control for RNA loading (right); (b) The half-life of β-catenin was shortened in HepG2-HBx cells. Cells were incubated with cycloheximide to block new protein synthesis. Extracts were prepared at the indicated time after cycloheximide block and Western blot was carried out to show the alteration of β-catenin; (c) HepG2-pcDNA3.0 or HepG2-HBx cells were incubated with LiCl for indicated time. β-catenin expression was then determined; (d) HBx induced β-catenin degradation in 293T cells. 293T cells were transiently transfected with increasing amounts of pcDNA3.0-HBx plasmids without or with 20 mmol/L LiCl. 24 h later, cells were lysed and the extracts were subjected to Western blot for detection of β-catenin and HBx expression, β-actin was the loading control. All the experiments were repeated at least three times. GAPDH: glyceraldehyde-3-phosphate dehydrogenase. wt: wide-type; mut: mutant.
catenin protein through posttranscriptional mechanism. We then asked whether HBx expression could affect β-catenin dependent transactivation. To test this possibility, the reporter plasmid containing the wild-type Tcf4-binding motif (pGL3-OT) was transiently transfected into HepG2-pcDNA3.0 and HepG2-HBx cells. Reporter gene assay demonstrated that transcriptional activity was increased in cells expressing HBx protein, whereas no significant responses were seen with mutant (pGL-OF) reporter (Fig. 3a). However, the expression levels of β-catenin target genes such as cyclinD1 and c-Myc were not significantly changed, partly due to their strong endogenous expression (Data not shown).

Further evidence supporting a role for HBx came from experiments using shRNA to abolish HBx expression. We have developed an shRNA vector that expresses RNAi-
inducing shRNAs under the control of the U6 promoter (Fig. 3b). Transient transfection of anti-HBx shRNA (pGU6-HXR1) with pcDNA3.0-HBx into 293 cells efficiently abolished HBx expression (Fig. 3c). Anti-HBx shRNA significantly inhibited TCF4 reporter activity to the basal level in HBx-expressing HepG2 cells. The anti-HBx shRNA mediated inhibition was seemingly specific as another vector (pGU6-HXR2) targeting against a different sequence in HBx, which failed to block HBx expression, was unable to affect TCF4 reporter activity (Fig.3d and data not shown). We did not observe any significant effect on pGL-OF.

To further dissect the mechanism of HBx function in mediating β-catenin activation, the subcellular localization of β-catenin was determined by cell fractionation and immunofluorescence assays. As shown in Fig. 4a, confluent HepG2-pcDNA3.0 cells were uniformly stained along the membrane. In contrast, HepG2-HBx cells were intensively stained in the cytoplasm and nucleus. Cell fraction analysis showed that wild-type β-catenin was decreased by HBx mainly at the cytoplasmic level whereas nuclear accumulation of mutant β-catenin was slightly enhanced (Fig. 4b).

3.4 β-catenin and E-cadherin staining was decreased in HCCs with HBV infection

Since expression of HBx has been described at a high frequency in HBV-related HCCs (58.8% to 83%) [38,39], we then evaluated the β-catenin and E-cadherin expression by immunohistochemistry in 31 HCCs with HBV infection collected at the Eastern Hepatobiliary Surgery Hospital, Shanghai, China. In normal livers, a thin membranous β-catenin and E-cadherin signal delineated the hepatocytes (Fig. 5a and 5c). Normal and dysplastic cells from livers with chronic hepatitis did not display nuclear expression of β-catenin. HBx staining was observed in 71% (22 of 31 cases) of tumors analyzed (Fig. 5f). In 20 of these nodules (91%), membranous β-catenin as well as E-cadherin immunostaining was markedly decreased in comparison with adjacent liver tissues (Fig. 5a–5d). Nuclear staining could be observed in 32.2% (10 of 31) HCC nodules (Fig. 5e).

3.5 Comparative analysis of β-catenin and E-cadherin expression by Western blot

To further confirm the involvement of HBx in E-cadherin/β-catenin downregulation in HBV-related hepatocarcinogenesis, we performed Western blot using anti-E-cadherin and anti-β-catenin antibodies for 86 samples from 60 HBV-positive and 26 HBV-negative HCCs and compared the levels of the β-catenin and E-cadherin in cancerous tissue with those in the adjacent non-cancerous tissues. Protein concentration of tissue extracts was determined with BCA kit (Pierce) and a total of 50 μg protein was loaded in each
The relative expression levels of β-catenin and E-cadherin were further normalized by Poceau S staining and by using the actin protein level as the internal control (data not shown). The bands of the expected size 97 kD and 120 kD, for β-catenin and E-cadherin respectively, were detected in most of samples whereas a simultaneous loss of expression of β-catenin and E-cadherin was found in 6 samples (3 from HBV-positive and 3 from HBV-negative HCCs) (Fig. 6). In addition, a truncated β-catenin protein of approximately 65 kD, indicating a deleted β-catenin, was found in sample E82 (Fig. 6c).

Reduced expression of β-catenin was observed in 35 of the 60 cases (58.3%) in HBV-positive tumors (Fig. 6a) and in 5 of the 26 cases (19.2%) in HBV-negative tumors (Fig. 6b; Table 1), indicating that the β-catenin protein levels were significantly decreased in HBV-related HCCs ($P = 0.0008$). Comparative analysis of β-catenin and E-cadherin expression (Table 2) demonstrated that β-catenin and E-cadherin alteration was closely related in HBV-related HCCs but not significantly associated in non-HBV-related HCCs.

We then searched for association between β-catenin expression levels and available clinicohistological parameters. There was no significant difference between expression levels of β-catenin with tumor grade, size, cirrhosis, satellite nodule or AFP status. Unexpectedly, we...
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Paired samples of HCCs with HBV infection

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β-catenin
E-cadherin
Fig. 6 Western blot analysis of tissue homogenates. (a) Paired samples of HCCs with HBV infection (60 cases); (b) Paired samples of HCCs without HBV infection (26 cases); (c) Immunoblot showed a truncated β-catenin in cancerous tissue (sample E82). C: cancerous tissue; NC: adjacent non-cancerous tissue.

Table 1 Correlations between β-catenin expression levels and HBV infection status

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P = 0.0028

Table 2 Comparative analysis of β-catenin and E-cadherin alteration in HBV positive or negative tumors

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P = 0.2261

β-catenin-decreased or E-cadherin-decreased tumors include those with total loss of the proteins.
observed intravascular invasiveness less frequently in tumors with reduced β-catenin expression (2 of 38 cases, 5.1%, $P = 0.0134$; Table 3).

### 4 Discussion

HBx is often the only viral protein expressed by transformed hepatocytes [3,4] and its overexpression may alter signal transduction pathways important for HCC progression. In this work, we examined the mechanism by which HBx expression mediated tumor progression in light of two known aspects of β-catenin signaling function: cell adhesion and transcriptional activity in the nucleus. We found that HBx downregulated the wild-type β-catenin and E-cadherin expression in HepG2 cells, accompanied by the disruption of adherens junction. Because β-catenin and E-cadherin represent an essential requirement for formation of cadherin/catenin complex, it seemed likely that loss of β-catenin resulted in alteration of E-cadherin-mediated cell-cell adhesion. This effect was selective because HBx did not affect mutated β-catenin and γ-catenin.

Given that the mutated β-catenin present in HepG2 cells is unable to undergo phosphorylation by GSK-3β and degradation, we speculate that HBx signaling may induce the selective decrease in wild-type β-catenin by accelerating its degradation. Consistently, we observed that, LiCl was able to abrogate the effect of HBx. In addition, E-cadherin expression was rescued following LiCl treatment, indicating that its reduction was dependent on β-catenin turnover.

Disruption of intercellular adhesion is thought to be the first step required for tumor spreading. Impaired cadherin/β-catenin complexes expression or function has been frequently observed in HCC and correlated with tumor size, low grade of differentiation and capsular and vascular invasion [18]. Loss of E-cadherin expression in one third of tumors has been linked to transcriptional silencing caused by promoter methylation and to allelic deletions of the gene [19]. On the other hand, it has been proposed that binding of cadherins to β-catenin prevents recognition of degradation signals and that cadherin not associated with β-catenin may be targeted for degradation [40]. Consistently, our results presented here demonstrated a simultaneous alteration of β-catenin and E-cadherin, supporting the notion that their turnover was coupled.

In our study, reduced β-catenin was detected in 58.3%...
(35 out of 60) tumors with HBV infection by Western blot analysis, a frequency significantly higher than those in non-HBV-related HCC (5 of 26 cases, 19.2%; P = 0.0014). It is of interest that E-cadherin reduction was closely related to β-catenin alteration in HBV-positive tumors whereas no significant correlation was found in HBV-negative tumors. By using a commercially available HBx antibody, we observed positive HBx staining in 71% (22 of 31 cases) HBV-related HCCs. In combination with a previous study in which HBx has been demonstrated in 58.8% of HBV infected HCCs by different specific antibodies [38], we therefore propose that downregulation of β-catenin and E-cadherin in HBV related HCC is, at least in part, attributed to HBx expression.

Our observation that HBx can interfere with β-catenin signaling suggested that HBx expression during carcinogenesis not only affected cell adhesion but also became a switch in gene expression through upregulation of β-catenin/TCF activity. Interestingly, HBx increased β-catenin signaling with noticeable changes in cytosolic/nuclear levels of β-catenin protein, the major mechanism thought to control β-catenin signaling [41]. These data indicate that β-catenin/TCF dependent transcriptional activity is controlled by a finely tuned balance between transcription competent and incompetent pools of β-catenin complex. In line with this model, Gattardi et al. showed that a change in β-catenin activity cannot be accounted for by accumulation of either total or cadherin-free protein, since low levels of nuclear β-catenin comprising a small subfraction of the cellular β-catenin pool are sufficient for transcriptional activity [42]. Furthermore, Clevers et al. recently reported that changes in the phosphorylation states of N-terminus of β-catenin independently affect the signaling properties and half-life of β-catenin and that Wnt signals are transmitted through N-terminally dephosphorylated β-catenin [43]. Since the mutated β-catenin in HepG2 cells lacks the complete N-terminus for phosphorylation, it cannot contain negatively charged phosphate groups and is a potent transcriptional activator. Considering the frequent β-catenin mutation found in HCC, we proposed that HBx might accelerate HCC progression by upregulating transcriptional activity of the mutated β-catenin, inducing cellular changes that lead to the acquisition of metastatic and proliferational properties.

In conclusion, our results suggest that the expression of HBx contributes to the development of HCC, in part, by posttranscriptionally repressing the expression of wild-type β-catenin, thus providing tumors with metastatic potential. Since β-catenin or Axin mutation has been frequently found in HCC, we propose that HBx may not only have a role in downregulation of β-catenin levels, but also be responsible for enforcement of β-catenin signaling pathway by increasing transcription competent pool of β-catenin. The present studies extend our knowledge of the multiple functions of the HBx in activating the β-catenin signaling cascade in HCC development and progression.

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