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Original Article

Hepatitis B virus X protein promotes tumor invasion and poor prognosis in hepatocellular carcinoma via phosphorylation of paxillin at Serine 178 by activation of the c-Jun NH2-terminal kinase

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Abstract: Hepatitis B virus X protein (HBx) plays critical roles in hepatocellular tumorigenesis by activating different signaling pathways, including the c-Jun NH2-terminal kinase (JNK) pathway. Phosphorylation of paxillin (PXN) promotes cell migration via activation of the JNK signaling pathway, but PXN overexpression is not associated with poor outcome in patients with hepatocellular carcinoma (HCC). HBx gene manipulation and Western blotting indicated that phosphorylation of PXN at Serine 178 (p^{S178}-PXN) by HBx may promote invasiveness in HCC cells via HBx-mediated JNK activation. Immunohistochemical analysis indicated a positive correlation between p^{S178}-PXN and HBx expression levels in tumor specimens. The overall survival (OS) and relapse-free survival (RFS) were poorer in patients with high-p^{S178}-PXN expressing or high-HBx expressing tumors than in patients with low-p^{S178}-PXN expressing or low-HBx expressing tumors. In conclusion, phosphorylation of PXN at Serine 178 by HBx-mediated JNK activation may therefore play a critical role in tumor invasiveness and poor prognosis in patients with HBV-infected hepatocellular tumors. The expression levels of p^{S178}-PXN may be a reliable prognostic biomarker to predict the clinical outcomes in patients with HBV-associated HCC.

Keywords: HBx, HCC, PXN, tumor invasion and poor prognosis

Introduction

Hepatitis B virus X protein (HBx) is a major factor in hepatitis and hepatocellular carcinoma (HCC) caused by HBV infection. HBx localizes in the cytoplasm, where it participates in various cellular signaling pathways, including the Notch, PI3K/mTOR, Wnt/β-catenin, NF-κB, STAT, and Ras/Raf/MAPK pathways, which are related to the development, invasion, migration, and recurrence of HCC [1-3]. In the present study, we postulate that HBx may also be involved in the phosphorylation of paxillin (PXN), an adapter protein that recruits diverse cytoskeleton and signaling proteins into a complex and coordinates transmission of downstream signaling [4].

The functions of PXN include the regulation of cell spreading and cell mobility [4]. For example, PXN promotes tumor progression and metastasis in several cancers, including gastric, oral cavity squamous cell, lung, breast, and colorectal cancers [4-9]. PXN overexpression may also predict poor prognosis in non-small cell lung cancer (NSCLC) via de-targeting of microR-

NA-218 [6]. However, PXN expression itself has no apparent prognostic significance in HCC [10]. We therefore suggest that PXN phosphorylation, rather than PXN overexpression, may be responsible for tumor progression and metastasis in HCC.

The phosphorylation of PXN at Serine 178 is mediated by the c-Jun NH2-terminal kinase (JNK) and is essential for migration of rat bladder tumor epithelial cells (NBT-II) [11, 12]. JNK is generally through to be involved in inflammation, proliferation, and apoptosis [11-13], which raises the possibility that HBx may activate the JNK signaling pathway in HBV-associated hepatocellular tumorigenesis. In the present study, we hypothesized that activation of the JNK signaling by HBx may promote the phosphorylation of PXN at Serine 178, thereby promoting tumor invasion and poor prognosis in HBV-associated HCC.

Materials and methods

Cell lines

The HepG2, Hep3B, Huh 7, and PLC/PRF/5 cell lines were obtained from the American Type Culture Collection (ATCC) and the culture conditions were as described elsewhere. Cells were cultured and stored according to the supplier's instructions and used at passages 5 to 20. Once resuscitated, the cell lines were routinely authenticated (once every 6 months; the cells used in the present study were last tested in November 2015) through cell morphology monitoring, growth curve analysis, species verification by isoenzymology and karyotyping, identity verification using short tandem repeat profiling analysis, and contamination checks.

Study subjects

This study enrolled 110 patients with HCC. The inclusion criteria for patients were: primary diagnosis with hepatocarcinoma; no metastatic disease at diagnosis; no previous diagnosis of carcinoma; no neoadjuvant treatment before primary surgery; and no evidence of disease within one month of primary surgery. Tumor specimens collected from surgically-resected liver cancer patients were stored at -80°C in Department of General Surgery, Taichung Veterans General Hospital (Taichung, Taiwan), between 2009 and 2012. Patients were asked to submit written informed consent, and the study was approved by the Institutional Review Board (CE14323A).

The tumor stage of each specimen was histologically determined according to the WHO classification system. Cancer relapse data were obtained by chart review and confirmed by surgeons. Clinical parameters and overall survival (OS) data were collected from chart review and the Taiwan Cancer Registry, Ministry of Health and Welfare, Executive Yuan, Taiwan, ROC. Survival time was defined as the period from the date of primary surgery to the date of death.

Antibodies

Anti-HBx antibody was purchased from *Abcam* (London, UK). Anti-PXN antibody was obtained from NeoMarker (Fremont, CA). Anti-phospho-S178-PXN (pS178-PXN) was obtained from ECM Bioscience (Versailles, KY). All other antibodies were purchased from Santa Cruz Biotechnology (Dallas, TX).

Plasmid constructs and transfection

The PXN-overexpression plasmid was kindly provided by Dr. Salgia (The University of Chicago, USA). Mutated PXN expression constructs containing point mutations (S178A) were constructed by the QuickChange site-directed mutagenesis system (Stratagene, USA). The HBx-overexpression plasmid was constructed in a pAcGFP1-N1 vector. Different concentrations of expression plasmids were transiently transfected into the liver cancer cells (1×10^6 cells) using the Turbofect reagent (Glen Burnie, MD). After 48 h, the cells were harvested and whole cell extracts were assayed in subsequent experiments.

Silencing of endogenous HBx expression by small interfering RNA (siRNA)

The first HBx siRNA (UGUGCACUUCGCUUCACCU), the second HBx siRNA#2 (CCGACCUUGAGGCAUACUU), FAK siRNA (GUAUUGGACCUGCGAGGGA) and JNK1/2 siRNA (JNK1: GACCAUUUCAGAAUCAGACUU; JNK2: GAUGCUAACUUAUGUCAGGUU) were designed according to the cDNA sequence of indicated genes in previous studies [14-16]. The procedures and methods were as described previously [17].

Western blotting

Equal amounts of protein were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred onto a polyvinylidene difluoride membrane (PerkinElmer, Norwalk, CT). After blocking, the membranes were reacted with specific antibody at 4°C overnight, followed by incubation with horseradish peroxidase-conjugated secondary antibody for 1 h. The immunoblotted proteins were detected using an enhanced chemiluminescence kit (PerkinElmer).

Immunohistochemistry (IHC) analysis

The immunohistochemical procedures and quantification methods were described previously [17]. The signal intensities were evaluated independently by three observers. Immunostaining scores were defined as the cell staining intensity (0 = nil; 1 = weak; 2 = moderate; and 3 = strong) multiplied by the percentage of labeled cells (0-100%), leading to scores from 0 to 300. A score over 150 was rated as "high" immunostaining, while a score less than 150 was rated as "low" immunostaining.

Boyden chamber invasiveness assays

A Boyden chamber with a pore size of 8 μm was used for cell invasiveness assays. Cells (1×10^4 cells) in 0.5% serum containing culture medium (HyClone, Ogden, UT) were plated in the upper chamber and 10% fetal bovine serum was added to culture medium in the lower chamber as a chemoattractant. The upper side of the filter was covered with 0.2 mg/ml Matrigel (Collaborative Research, Boston, MA) diluted in RPMI-1640. After 16 h, cells on the upper side of the filter were removed and cells that adhered to the underside of membrane were fixed in 95% ethanol and stained with 10% Giemsa dye. The number of invasive cells was counted in ten contiguous light microscope fields.

Statistical analysis

The SPSS statistical software program (Version 18.0; SPSS Inc.) was used for statistical analyses. The association between HBx and p^{S178}-PXN expression was analyzed by the Chi-square test. Survival plots were generated using the Kaplan-Meier method, and differences between patient groups were determined by the log-rank test. Multivariate Cox regression analysis was performed to determine the overall survival (OS) and relapse-free survival (RFS). The analysis was stratified for all known variables (age, gender, and tumor stage) and protein expression.

Results

HBx may promote PXN phosphorylation at Serine 178 through activation of the JNK signaling pathway to promote cell invasiveness in HCC cells

The possibility that HBx could activate the JNK signaling pathway to promote phosphorylation of PXN at Serine 178 (p^{S178}-PXN) was examined by collecting HBx-negative HepG2 and HBx-positive Hep3B cells for HBx gene manipulation using an HBx expression vector and a small interfering RNA for HBx (siHBx). Two doses of HBx expression vector (1 and 5 μg) and two types of siHBx (si-1 and si-2) were transfected into HepG2 and Hep3B cells, respectively. Western blotting showed the expected dose-dependent increase and decrease in HBx expression in HBx-overexpressing HepG2 and HBx-knockdown Hep3B cells (**Figure 1A**). The levels of phosphorylated JNK (p-JNK) and PXN (p-PXN) protein in HBx-overexpressing HepG2 and HBx-knockdown Hep3B cells were also concomitantly increased and decreased, respectively, in a dose-dependent manner. However, the levels of JNK and PXN protein were unchanged by HBx gene manipulation in both cell types (**Figure 1A**).

A specific inhibitor of the JNK signaling pathway, SP600125, was used to test whether activation of the JNK signaling pathway by HBx could be responsible for phosphorylation of PXN in HCC cells. Western blotting indicated that the expression levels of p-JNK and p^{S178}-PXN were markedly reduced by SP600125 treatment in HBx-overexpressing HepG2 cells (**Figure 1B**). As expected, the p-JNK expression levels were decreased by SP600125 in HepG2 cells transfected with an empty vector (HepG2 control cells), but the JNK and PXN protein levels were unchanged in HBx-overexpressing HepG2 cells and in its control cells in the presence or absence of SP600125 treatment (**Figure 1B**). Similar findings were observed in Huh7 and PLC/PRF/5 cells (Supplementary

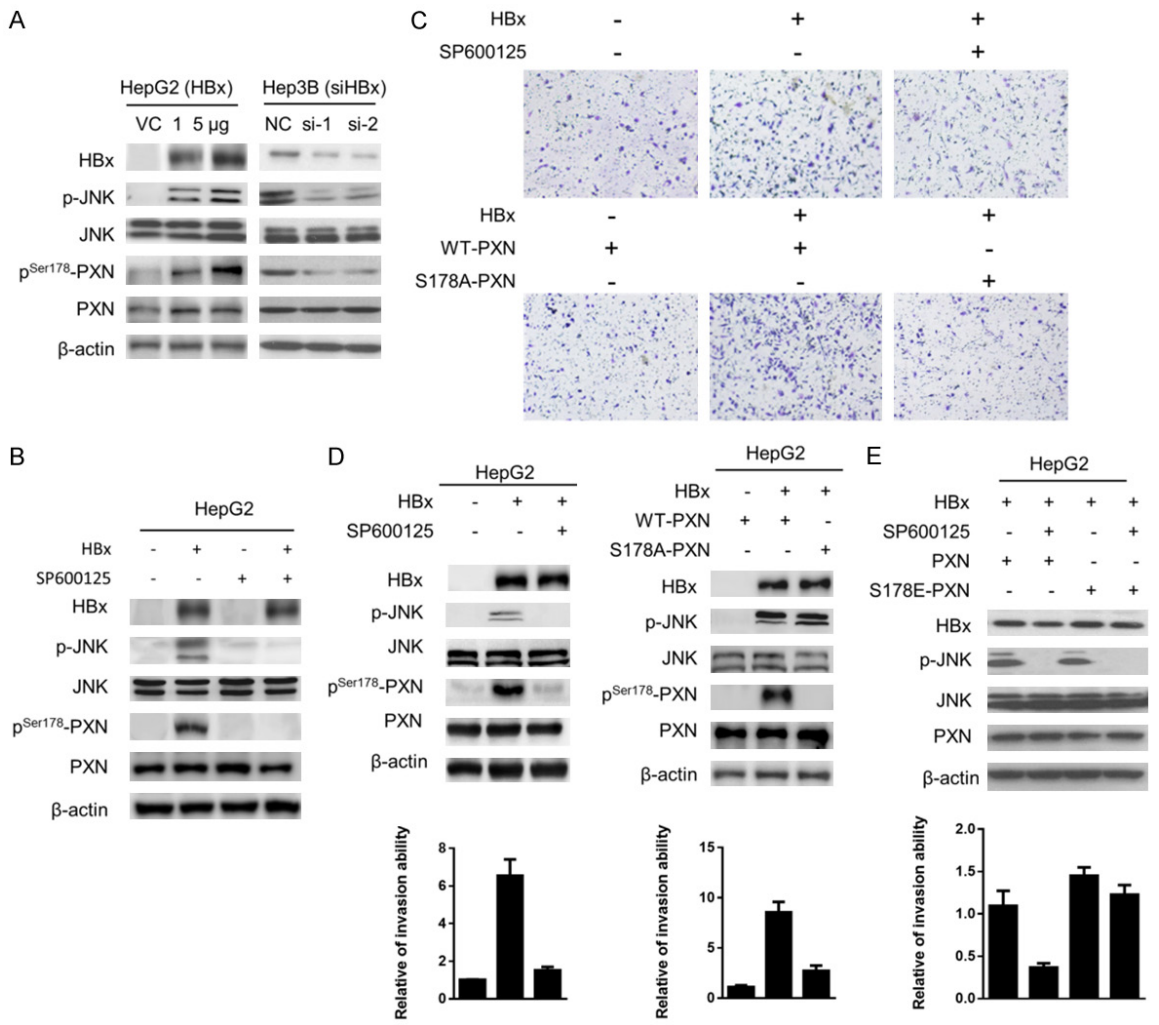

Figure 1. Phosphorylation of PXN at Serine 178 by HBx-mediated JNK activation may be responsible for cell invasiveness in HepG2 and Hep3B cells. (A) HBx-negative HepG2 and HBx-positive Hep3B cells were transfected with two doses of HBx expression vector (1, 5 μg) and two types of siHBx (si-1 and si-2), respectively. Western blotting was conducted to evaluate the expression levels of p-JNK, JNK, p^{S178}-PXN, and PXN in HBx-overexpressing HepG2 and HBx-knockdown Hep3B cells when compared with HepG2 with transfection of empty vector (VC) and Hep3B with transfection of non-specific RNAi (NC). β-Actin was used as protein loading controls. (B) HepG2 cells were transfected with HBx expression vector and/or treatment of a JNK inhibitor SP600125. All protein expressions were determined by Western blotting and β-actin was used as protein loading controls. (C) Boyden Chamber assay was conducted to evaluate the relative invasive ability of HepG2 cells transfected with HBx expression vector and/or treatment of SP600125 when compared with VC cells. HepG2 cells were transfected with HBx expression vector and/or WT-PXN or p^{S178A}-PXN or p^{S178E}-PXN expression vector. The protein expression levels and the relative invasive ability were presented in (D) and (E).

Figure 1). These results suggest that HBx may activate the JNK signaling pathway in HCC cells to promote phosphorylation of the PXN protein at Serine 178.

Phosphorylation of PXN at Serine 178 by HBx-mediated JNK activation may be responsible for cell invasiveness in HCC cells

We examined the possibility that PXN phosphorylation at Serine 178 by HBx-mediated JNK activation could be responsible for cell invasiveness in HCC cells. HepG2 cells were transfected with the expression vector of HBx, wild-type (WT)-PXN, and/or mutant p^{S178A}-PXN. Western blotting indicated a substantial increase in the p-JNK protein levels by HBx and/or WT-PXN overexpression in HepG2 cells (**Figure 1D**). In addition, p^{S178}-PXN expression levels were markedly increased by the combined transfection of HBx plus WT-PXN, whereas p^{S178}-PXN expression levels were unchanged in

Table 1. Relationships of HBx and p^{S178}-PXN expression with clinicopathological parameters in patients with HCC

Parameters	Case no.	HBx		P	p^{S178}-PXN		P
		Low (%)	High (%)		Low (%)	High (%)	
	110	60 (54.5)	50 (45.5)		60 (54.5)	50 (45.5)	
Age							
≤ 61	59	33 (55.9)	26 (44.1)	0.604	34 (57.6)	25 (42.4)	0.485
> 61	51	26 (51.0)	25 (49.0)		26 (51.0)	25 (49.0)	
Genders							
Female	16	5 (31.2)	11 (68.8)	0.052	10 (62.5)	6 (37.5)	0.489
Male	94	54 (57.4)	40 (42.6)		50 (53.2)	44 (46.8)	
Stage							
1	37	21 (56.8)	16 (43.2)	0.640	24 (64.9)	13 (35.1)	0.122
2+3+4	73	38 (52.1)	35 (47.9)		36 (49.3)	37 (50.7)	
T							
1+2	38	22 (57.9)	16 (42.1)	0.515	24 (63.2)	14 (36.8)	0.188
3+4	72	37 (51.4)	35 (48.6)		36 (50.0)	36 (50.0)	
N							
0	107	57 (53.3)	50 (46.7)	1.000	59 (55.1)	48 (44.9)	0.590
1	3	2 (66.7)	1 (33.3)		1 (33.3)	2 (66.7)	
α-fetoprotein							
≤ 20	45	29 (64.4)	16 (35.6)	0.059	24 (53.3)	21 (46.7)	0.832
> 20	65	30 (46.2)	35 (53.8)		36 (55.4)	29 (44.6)	
Vascular invasion							
Absence	48	33 (68.8)	15 (31.3)	0.005	32 (66.7)	16 (33.3)	0.025
Presence	62	26 (41.9)	36 (58.1)		28 (45.2)	34 (54.8)	
Tumor number							
≤ 1	89	53 (59.6)	36 (40.4)	0.010	52 (58.4)	37 (41.6)	0.092
> 1	21	6 (28.6)	15 (71.4)		8 (38.1)	13 (61.9)	
HBx							
Low	59				40 (67.8)	19 (32.2)	0.003
High	51				20 (39.2)	31 (60.8)	

mutant p^{S178A}-PXN- or p^{S178E}-PXN-overexpressing HepG2 cells (**Figure 1D** and **1E**).

The Boyden chamber invasiveness assays indicated that the cell invasive ability was greatly elevated by HBx overexpression in HepG2 cells, but this elevation of cell invasive ability was almost completely suppressed by treatment with SP600125 when compared with control cells without SP600125 treatment (**Figure 1C** and **1D**). The invasive ability in HepG2 cells was also markedly increased by transfection with both HBx and WT-PXN, but the invasiveness was nearly unchanged by transfection with both HBx and mutant p^{S178A}-PXN or p^{S178E}-PXN when compared with control cells (**Figure 1C-E**). Moreover, siJNK and siFAK were used to direct silence JNK and FAK signaling pathway. Western blot and Boyden chamber invasiveness assay indicated that JNK activation by HBx was responsible for cell invasiveness via phosphorylation of PXN at Serine 178 (Supplementary Figure 1). These results clearly indicated that phosphorylation of PXN at Serine 178 by the JNK signaling pathway may be responsible for HBx-mediated cell invasiveness in HCC cells.

Relationships of HBx and p^{S178}-PXN expression levels with the clinicopathological parameters in patients with HCC

We enrolled 110 tumor specimens from patients with HCC for immunohistochemical anal-

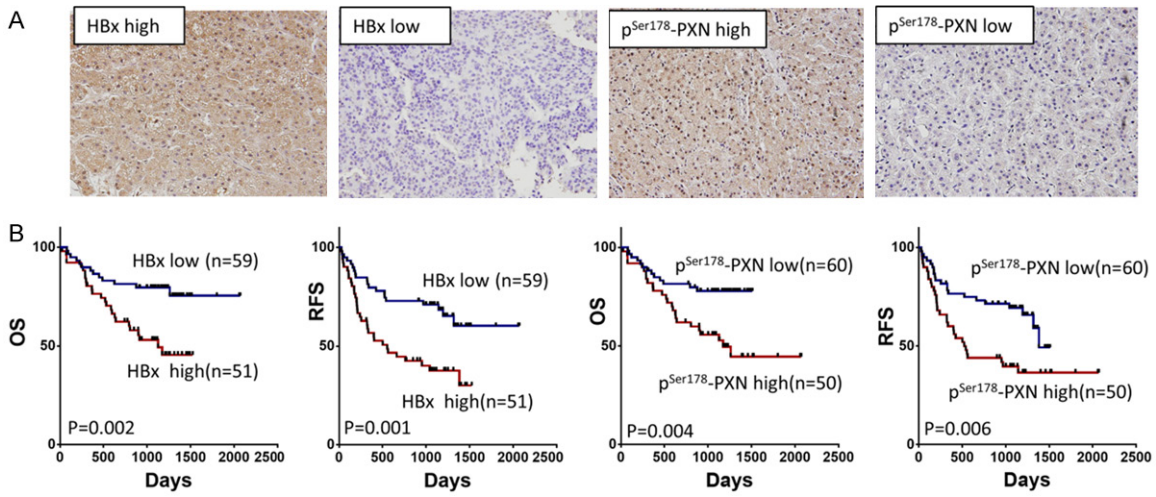

Figure 2. The expression levels of HBx and p^{S178}-PXN protein in tumor specimens from patients with HCC were evaluated by immunohistochemical analysis (A). Kaplan-Meier analysis was conducted to assess the survival curves of HBx and p^{S178}-PXN expression on OS and RFS in patients with HCC (B).

ysis of HBx and p^{S178}-PXN protein levels. High HBx expression was marginally more prevalent in female than in male patients (68.8% vs. 42.6%, P = 0.052; **Table 1**). High p^{S178}-PXN expression was more commonly observed in high fibrosis score tumors than in low fibrosis score tumors (52.8% vs. 31.6%, P = 0.034; **Table 1**). The α-fetoprotein levels were relatively higher in patients with high-HBx expressing tumors than in patients with low-HBx expressing tumors (53.8% vs. 35.6%, P = 0.059; **Table 1**). The HBx expression levels were not associated with the clinical parameters of age, tumor size (T), nodal metastasis (N), and tumor stage. The p^{S178}-PXN expression levels were not associated with the clinical parameters of age, gender, tumor size (T), nodal metastasis (N), tumor stage, and α-fetoprotein levels. Interestingly, high p^{S178}-PXN expression was more frequently observed in patients with high-HBx expressing tumors than in patients with low-HBx expressing tumors (60.8% vs. 32.2%, P = 0.003; **Table 1**). Interestingly, the presence of vascular invasion and a higher tumor number (> 1) was commonly observed in HBx-positive tumors than in HBx-negative tumors (vascular invasion: 58.1% vs. 31.3%, P = 0.005; tumor number: 71.4% vs. 40.4%, P = 0.010; **Table 1**). In addition, the presence of vascular invasion was more frequently seen in high p^{S178}-PXN expressing tumors than in low p^{S178}-PXN expressing tumors (54.8% vs. 33.3, P = 0.025); however, high tumor number was marginally observed in high p^{S178}-PXN expressing tumors than in low p^{S178}-PXN expressing tumors (61.9% vs. 41.6%, P = 0.092; **Table 1**). These observations in patients with HCC seemed to support the proposed cell model for an involvement of HBx in the phosphorylation of PXN at Serine 178 in HCC.

High expression of HBx and p^{S178}-PXN proteins may be associated with poor overall survival (OS) and poor relapse-free survival (RFS) in patients with HCC

Representative immunostaining results for HBx and p^{S178}-PXN protein expression are presented in **Figure 2A**. Kaplan-Meier analysis indicated that a poorer OS and RFS in patients with high-HBx expressing tumors and high-p^{S178}-PXN expressing tumors than in patients with low-HBx expressing tumors and low-p^{S178}-PXN expressing tumors (OS: P = 0.002 for HBx, P = 0.004 for p^{S178}-PXN; RFS: P = 0.001 for HBx, P = 0.006 for p^{S178}-PXN; **Figure 2B**). Cox-regression analysis further showed that patients with high-HBx expressing tumors had hazard ratios of 2.84 and 2.59 for OS and RFS, respectively, when compared to patients with low-HBx expressing tumors (95% CI 1.44-5.62, P = 0.003 for OS, 95% CI, 1.46-4.61, P = 0.001 for RFS; **Table 2**). The p^{S178}-PXN expression also had prognostic significance for OS and RFS in this study population (HR, 2.36, 95% CI, 1.20-4.62, P = 0.013 for OS; HR, 2.03, 95% CI, 1.16-3.55, P = 0.013 for RFS; **Table 2**). The HR values for OS and RFS were also higher for patients with high-HBx plus

Table 2. Cox-regression analysis for the prognostic value of HBx or p^{Ser178}-PXN expression on OS and RFS in patients with HCC

Variables	OS				RFS			
	Adjusted		95% CI	P	Adjusted		95% CI	P
	No.	HR*			No.	HR*		
HBx								
Low	59	1			59	1		
High	51	3.30	1.65-6.59	0.001	51	2.85	1.56-5.21	0.001
p^{S178}-PXN								
Low	60	1			60	1		
High	50	2.59	1.30-5.17	0.007	50	2.05	1.14-3.66	0.016
HBx/p^{S178}-PXN								
Low/Low	40	1			40	1		
High/Low	20	0.51	0.11-2.42	0.399	20	1.24	0.47-3.26	0.661
Low/High	19	0.38	0.08-1.77	0.217	19	0.83	0.31-2.23	0.712
High/High	31	4.03	1.87-8.68	< 0.001	31	4.12	1.98-8.55	< 0.001

*The HR values were stratified for all variables including age, gender, tumor stage, vascular invasion and tumor numbers.

high-p^{S178}-PXN expressing tumors (high/high) than for patients with low-HBx plus low-p^{S178}-PXN expressing tumors (low/low) (HR, 3.82, 95% CI, 1.80-8.13, P < 0.001 for OS; HR, 3.87, 95% CI, 1.94-7.73, P < 0.001 for RFS), but no prognostic significance was seen for the other two combinations (high/low and low/high) when compared with low/low tumors. In addition, multivariate Cox-regression analysis indicated that the prognostic significance was revealed for tumor stage (OS: HR, 2.59, 95% CI, 1.18-5.64, P = 0.017; RFS: HR, 1.92, 95% CI, 1.05-3.51, P = 0.033), but not for age and genders in this study population (Supplementary Table 1). These results suggested that HBx-mediated JNK activation of PXN phosphorylation at Serine 178 may play a critical role in tumor progression and metastasis of HBV-associated HCC.

Discussion

The findings presented here provided evidence that HBx may activate the JNK pathway to mediate PXN phosphorylation at Serine 178, thereby promoting cell invasiveness in HBV-associated HCC cells. A prognostic significance of HBx and/or p^{S178}-PXN expression levels on the OS and RFS of patients with HCC was supported by Kaplan-Meier and Cox-regression models. However, the prognostic significance of p^{S178}-PXN expression on OS and RFS was not observed in patients with non-HBx HCC (Supplementary Table 2). Therefore, we suggest that PXN phosphorylation at Serine 178, in response to HBx-mediated JNK activation, may play a crucial role in HBV-associated hepatocellular tumorigenesis.

The HBx protein is known to induce transcription factor AP-1 by activation of the ERK and JNK signaling pathways [18]. HBx activation of the ERK and JNK signaling pathways is also thought to play a role in viral transformation and pathogenesis [18]. Previous studies have indicated that the induction of phosphorylation of PXN at Serine 178 by the JNK signaling pathway may promote the migration of bladder tumor epithelial cells, Schwann cells, and pancreatic cancer cells [11-13]. In the present study, altering FAK-PXN interaction by transfecting siFAK or treatment of its inhibitor (PF-562271) markedly reduced cell invasion (Supplementary Figure 2), and this observation was consistent with a previous report [19], but a decrease in cell invasion by siFAK or PF-562271 was not mediated through JNK activation and PXN phosphorylation. More interestingly, activation of the JNK signaling pathway may increase the expression of p21-activated protein kinase (PAK1), which in turn may enhance HCC metastasis by PXN phosphorylation at Serine 178. These observations support the findings of the present study and suggest that PXN phosphorylation at Serine 178 by HBx-mediated JNK activation may play an important role in HBV-associated HCC tumorigenesis.

In our cell model experiments, HBx gene manipulation did not change the PXN expression levels in either HepG2 or Hep3B cells (**Figure 1A**), whereas this gene manipulation altered the p^{S178}-PXN expression levels in both cell types. HBx gene manipulation also caused concomitant changes in the expression levels of p-JNK and p^{S178}-PXN in both cell types (**Figure 1A**). Previous work has shown that PXN expression itself is not associated with the clinical outcomes in patients with HCC[10]; however, in the present study, p^{S178}-PXN expression was associated with poor prognosis on OS and RFS in these patients (**Figure 2** and **Table 2**). More interestingly, HBx and pS178-PXN expressions were positively correlated with vascular invasion and tumor number in patients with HCC (**Table 1**). These observations were similar with previous studies [20, 21]. These results support the hypothesis that HBx may promote tumor progression and metastasis in HBV-associated HCC by activating JNK signaling to induce PXN phosphorylation at Serine 178.

In summary, PXN phosphorylation at Serine 178 by HBx-mediated JNK activation may promote tumor invasion and predict poor OS and RFS in HBV-associated HCC. We therefore suggest that p^{S178}-PXN expression might be a useful prognostic biomarker for predicting the clinical outcomes in patients with HBV-associated HCC.

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Disclosure of conflict of interest

None.

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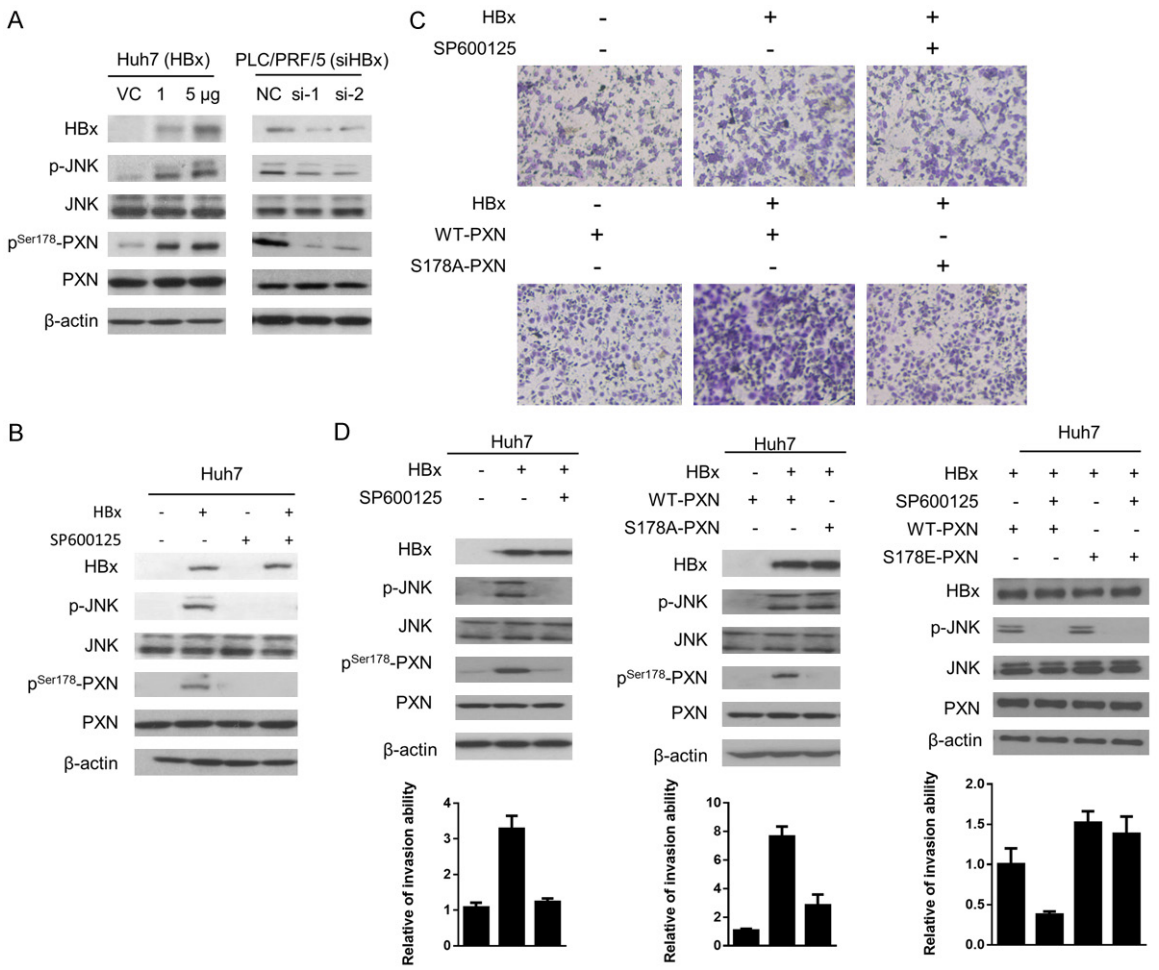


Supplementary Figure 1. Phosphorylation of PXN at Serine 178 by HBx-mediated JNK activation may be responsible for cell invasiveness in Huh7 and PLC/PRF/5 cells.

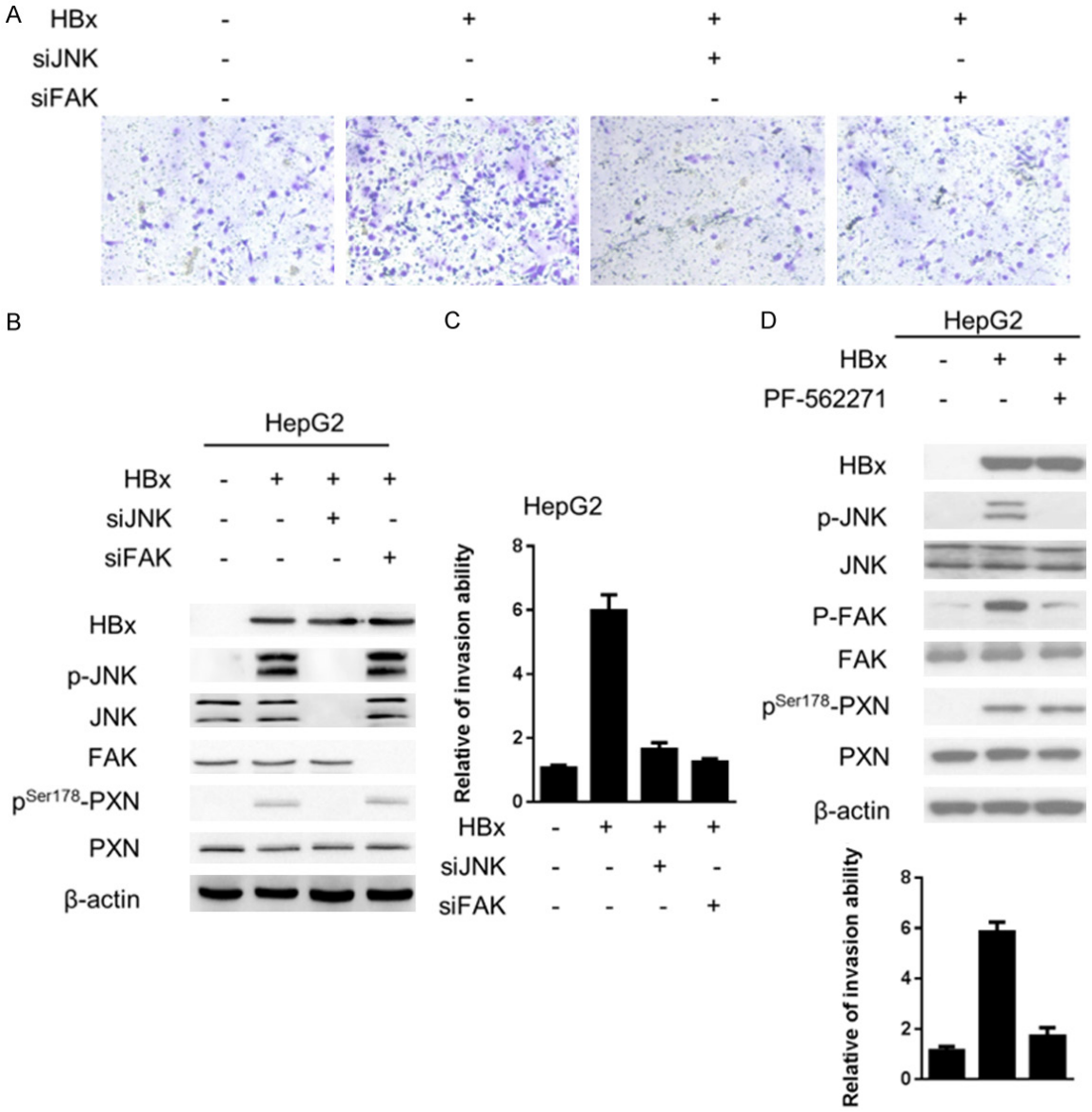
Supplementary Table 1. Cox-regression analysis for the prognostic value of age, genders, and tumor stage on OS and RFS in patients with HCC

	OS				RFS			
Variables	No.	HR	95% CI	P	No.	HR	95% CI	P
Age								
≤ 61	59	1			59	1		
> 61	51	1.04	0.55-1.98	0.903	51	1.11	0.65-1.92	0.698
Genders								
Female	16	1			16	1		
Male	94	1.03	0.35-2.97	0.964	94	1.08	0.46-2.54	0.858
Stage								
1	37	1			37	1		
2+3+4	73	5.36	1.93-14.89	0.001	73	2.33	1.11-4.89	0.026
Vascular invasion								
Absence	48	1			48	1		
Presence	62	0.62	0.31-1.23	0.171	62	0.91	0.49-1.69	0.771
Tumor number								
≤ 1	89	1			89	1		
> 1	21	0.80	0.35-1.87	0.612	21	1.37	0.70-2.71	0.359

Supplementary Table 2. Cox-regression analysis for the prognostic value of HBx or p^{Ser178}-PXN expression on OS and RFS in patients with non-HBx expressed HCC

	OS				RFS			
	Adjusted		95% CI	P	Adjusted		95% CI	P
Variables	No.	HR*			No.	HR*		
p^{S178}-PXN								
Low	40	1			40	1		
High	19	0.42	0.09-1.98	0.269	19	0.82	0.29-2.31	0.703

*The HR values were stratified for all variables including age, gender, tumor stage, vascular invasion and tumor numbers.



Supplementary Figure 2. Phosphorylation of PXN at Serine 178 induced by HBx may be responsible for promoting cell invasion via activation of the JNK signaling (A) Boyden chamber assay was used to evaluate invasion ability in HBx-transfected HepG2 cells, but the invasive cell numbers induced by HBx was markedly decreased by JNK- and FAK-knockdown cells. (B) Western blot was used to evaluate the expression levels of p-JNK, JNK, FAK, pS178-PXN, and PXN modulated by HBx and/or siJNK or siFAK transfection in HepG2 cells. β-actin was used as protein loading control. (C) The cell invasive ability induced by HBx in HepG2 was markedly reduced by siJNK or siFAK transfection when compared with vehicle control. (D) Western blot was used to evaluate the expression levels of p-JNK, JNK, p-FAK, FAK, pS178-PXN modulated by HBx and/or FAK inhibitor PF-562271 (upper panel). The invasive ability induced by HBx in HepG2 cells was rescued by PF-562271 treatment (lower panel).