PDK1-WNK1 signaling is affected by HBx and involved in the viability and metastasis of hepatic cells

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Abstract. Hepatitis B virus (HBV)-encoded X antigen (HBx) contributes to the development of hepatocellular carcinoma (HCC). Although HBx has been implicated in the progression of HCC, its precise function in HBV-associated HCC remains unclear. In the present study, HBx affected 3-phosphoinositide-dependent protein kinase-1 (PDK1) and with-no-lysine (K) kinase (WNK1) signaling, which was identified to be involved in the viability and metastasis of hepatic cells. The phosphorylation of WNK1 was decreased when the hepatic cells were treated with a PDK1 inhibitor. The inhibition of PDK1 activity inhibited the viability and migration of hepatic cells. To the best of our knowledge, the present study is the first to identify the activation of PDK1 in HCC tissues, confirmed using western blot analysis. PDK1-WNK1 signaling may be a potential therapeutic target in HBV-associated liver cancer.

Introduction

Hepatocellular carcinoma (HCC) is a major malignancy worldwide and exhibits increased incidence of tumor recurrence and metastasis (1,2). Hepatitis B virus (HBV)-encoded X antigen (HBx) acts as a multifunctional regulator in HBV-associated HCC development (3). HBx-transgenic mice exhibited increased expression of HBx, which may lead to HCC (4,5). However, the mechanism of the direct function of long-term expression of HBx in the development of HCC remains obscure.

3-Phosphoinositide-dependent protein kinase-1 (PDK1) is a crucial kinase that functions as the downstream effector molecular regulator of phosphoinositide 3-kinase (PI3K) and activates members of the activated protein kinase-dependent, cyclic guanosine monophosphate-dependent and protein kinase C family of protein kinases, including protein kinase B (Akt), protein kinase C, p70 ribosomal protein S6 kinases and serum- and glucocorticoid-dependent kinase (6-9). Hyperactivation of PDK1 was identified in several types of human cancer (10-12). Downregulation of PDK1 inhibits metastasis of human breast cancer cells (12). Clinical data suggested that PDK1 exhibit increased expression in liver cancer and was associated with a significantly decreased postoperative overall survival rate and increased recurrence rates in patients with HCC following curative resection (13). Nevertheless, the molecular mechanism by which PDK1 contributes to carcinogenesis of liver cancer (particularly of HBV-associated liver cancer) remains unclear.

With-no-lysine (K) kinases (WNKs) are a group of serine-threonine protein kinases with an atypical placement of the catalytic lysine residue compared with other protein kinases (14,15). WNK1 is the main downstream effector of the PDK1-associated pathway (16), which serves an important function in cell proliferation and migration (17). Previous studies demonstrated that WNK1 may also be an important kinase involved in various types of cancer (18-21).

The results of the present study indicated the increased expression of phospho (p)-PDK1 in HBV-associated HCC. Additionally, the activation of PDK1/WNK1 signaling was investigated in stable hepatic cell lines expressing HBx. An inhibitor of PDK1 significantly suppressed the viability and metastasis of hepatic cells.

Materials and methods

Cell culture. Human hepatoma cells SK-Hep1 and hepatic cells LO2 (Cell Resource Center, Shanghai Institute of Life Sciences, Chinese Academy of Sciences, Shanghai, China) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin at 37˚C in a

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humidified atmosphere containing 5% CO_2. All reagents were obtained from Corning Incorporated (Corning, NY, USA).

Transient transfection and generation of stable cell lines. pHAGE-HBx vector expressing full-length HBx was constructed. The synthesized full-length HBx gene was inserted into the pHAGE vector to generate pHAGE-HBx plasmid. The construct was confirmed by DNA sequencing. LO2 and SK-Hep1 cells were transfected using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) using 2.5 μg plasmids in a 6-well plate, according to the manufacturer's protocol. For stable transfection, LO2 and SK-Hep1 cells were transfected using pHAGE-HBx (or pHAGE-vector) for 48 h and maintained in DMEM containing 2 μg/ml puromycin. After 3-4 weeks of selection, individual colonies were isolated and expanded. The increased expression of target genes in these colonies was confirmed using the reverse transcription-quantitative polymerase chain reaction (RT-qPCR).

RT-qPCR. Total RNA was extracted from HBx transfected or the control cells using an Ultrapure RNA kit (CW0586S; CW Biotech, Beijing, China). A total of 400 ng RNA was transcribed into cDNA using the PrimeScript RT Mastermix (cat. no. RR036Q; Takara Biotechnology Co., Ltd., Dalian, China), according to the manufacturer's protocol. The cDNA was amplified using the SYBR-Green PCR Master Mix (Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. The thermocycling conditions were as follows: Initial denaturation of 95˚C for 5 min followed by 40 cycles of 94˚C for 15 sec and final extension of 55˚C for 1 min. The following primers were used: HBx forward, 5'-ACTCTCAGCAATGTCAACG-3' and reverse, 5'-ATTTATGCCTACAGCCTCC-3'; GAPDH forward, 5'-TGAAGGTCGGAGTCAACGGA-3' and reverse 5'-CCTGGAAGATGGTGATGGGAT-3'. Reactions were performed in triplicate. Relative expression of HBx was determined using the $2^{-\Delta\Delta Cq}$ method (22).

Western blot analysis. Protein lysates from tissues were prepared using a 1% protease and phosphatase inhibitor cocktail [Halt™ Protease and Phosphatase Inhibitor Cocktail, EDTA-Free (100X); cat. no. 78441; Thermo Fisher Scientific, Inc.] in a lysis buffer (4% SDS, 0.1 M dithiothreitol and 0.1 M Tris/HCl, pH 7.6). Cells were lyzed in radioimmunoprecipitation assay buffer (50 mM Tris/HCl, 1% Triton X-100, 0.5% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA and 0.1% SDS) containing a protease and phosphatase inhibitor cocktail (as above). The total protein concentration was measured by BCA assay and samples containing equal amounts of total protein (30 μg) were separated by 10% SDS-PAGE and transferred onto nitrocellulose membranes. The membranes were then blocked with 5% non-fat milk in Tris-buffered saline at room temperature for 1 h. Following blocking, membranes were incubated overnight at 4˚C with the following primary antibodies: Rabbit anti-WNK1 antibody (1:500; cat. no. 4979S; Cell Signaling Technology, Inc., Danvers, MA, USA), anti-p-PDK1 (Ser^{241}) antibody (1:500; cat. no. 3061S; Cell Signaling Technology, Inc.), rabbit anti-PDK1 antibody (1:500; cat. no. 3062S; Cell Signaling Technology, Inc.), anti-p-WNK1 (Thr^{60}) antibody (1:200; cat. no. AF4720; R&D Systems, Inc., Minneapolis, MN, USA) and anti-GAPDH antibody (1:2,000; cat. no. TA08; OriGene Technologies, Inc., Beijing, China). Membranes were then washed and incubated with secondary antibodies Goat Anti-Mouse IgG, HRP Conjugated (1:5,000; cat. no. CW0102; CW Biotech, Beijing, China) or Goat Anti-Rabbit IgG, HRP Conjugated (1:5,000; cat. no. CW0103; CW Biotech) at room temperature for 1 h. The protein bands were visualized using enhanced chemiluminescence Western Blotting Substrate (cat. no. 32106; Pierce; Thermo Fisher Scientific, Inc.)

Cell viability assay. Hepatic cell viability was detected using a Cell Counting kit-8 (CCK-8; Dojindo Molecular Technologies, Inc., Kumamoto, Japan). Cells ($5x10^3$ cells/well in 100 μl medium) were seeded in a 96-well plate. Cells were treated with 0, 5 or 20 μM PDK1 inhibitor II (cat. no. 521276; Merck KGaA, Darmstadt, Germany) in a humidified incubator (37˚C, 5% CO_2). Following incubation for the indicated durations, a 1/10 volume of the CCK-8 solution was added to each well and cells were incubated for a further 1 h. Cell viability was determined by measuring the absorbance at 450 nm using a plate reader (Spectramax Paradigm, Molecular Devices, LLC, Sunnyvale, CA, USA).

Transwell migration assay. For the Transwell migration assay, $2x10^4$ cells [suspended in DMEM containing 0.5% fetal bovine serum (FBS)] were trypsinized and plated in the upper chamber of fibronectin-coated plates (8 μm pore filter, Corning Incorporated). The cells were then treated with 0, 5 or 20 μM PDK1 inhibitor II. The lower chamber of the Transwell contained DMEM supplemented with 2.5% FBS. At 20 h, the filters were removed and the cells located on the membrane were fixed with methanol for 2 h. The cells that had migrated to the underside of the membrane were stained using 0.5% crystal violet at room temperature for 1 h and examined under a light microscope (Nikon Eclipse 80i; Nikon Corporation, Tokyo, Japan).

Clinical HCC samples. Primary HCC tissue and the paired normal liver tissue specimens were obtained from 21 patients with primary liver cancer who underwent surgical resection at China-Japan Union Hospital of Jilin University (Jilin, China). The samples were collected between March 2012 and February 2015. Of the total 21 patients, 17 (81%) were male and 4 (19%) were female. The median age was 57.8 years (range, 42-73 years). All participants provided written informed consent. Ethical approval was obtained from the research ethics committee of China-Japan Union Hospital of Jilin University.

Statistical analysis. Data were analyzed using GraphPad Prism (version 6.0) software (GraphPad Software, Inc., La Jolla, CA, USA). The relevant data are expressed as the mean ± standard deviation. Statistical significance between two groups was determined using Student's t-test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

HBx affects the phosphorylation of PDK1/WNK1 in hepatic cells. To evaluate the association between HBx and PDK1,

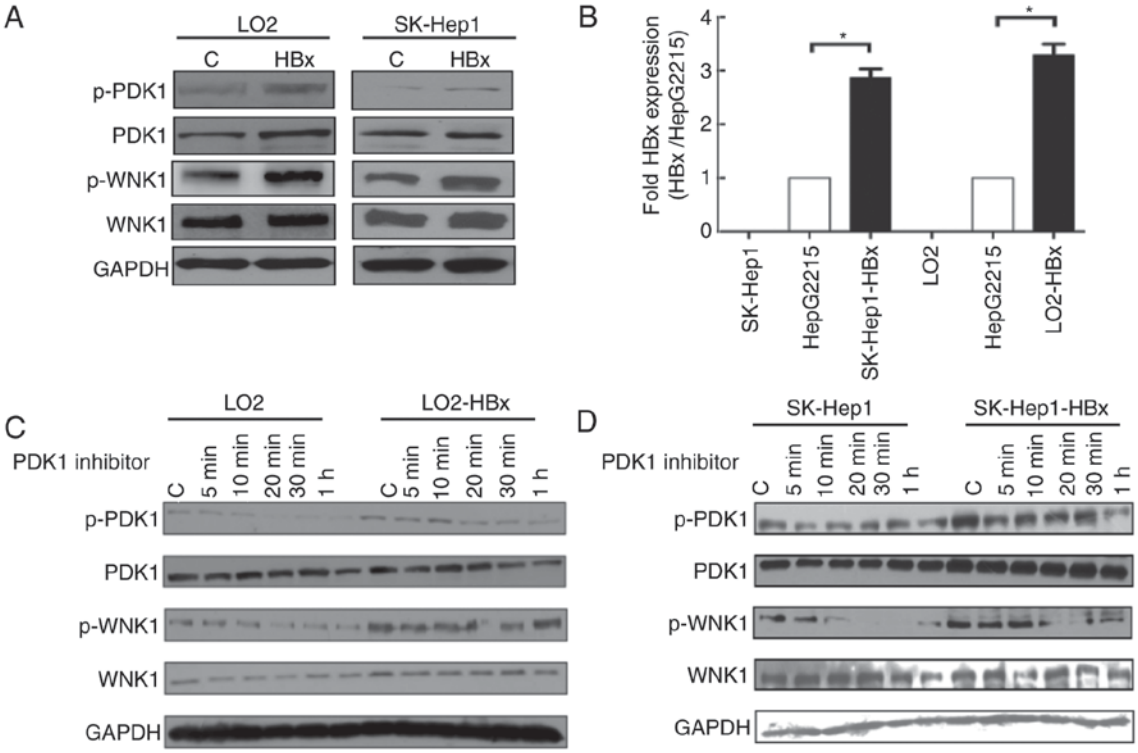

Figure 1. PDK1 and WNK1 are activated in HBx-transfected hepatic cells. (A) The expression of PDK1/p-PDK1 and WNK1/p-WNK1 in LO2 and SK-Hep1 cells following HBx-gene transfection was evaluated using western blot analysis. (B) HBx expression in LO2-HBx and SK-Hep1-HBx cells determined using the reverse transcription-quantitative polymerase chain reaction. HepG2.2.15 was used as positive control. *P<0.05. Western blot analysis of the expression of PDK1/p-PDK1 and WNK1/p-WNK1 in response to a PDK1 inhibitor in (C) LO2/LO2-HBx and (D) SK-Hep1/Hep1-HBx cells. PDK1, 3-phosphoinositide-dependent protein kinase-1; WNK1, with-no-lysine (K) kinase 1; p, phospho; C, control.

the expression of PDK1 and WNK1 was examined in HBx-overexpressing hepatic cell lines. Hepatic cell lines SK-Hep1 and LO2 were transfected with pHAGE-HBx or pHAGE-vector constructs and the overexpression of HBx in the pHAGE-HBx construct was confirmed using RT-qPCR (data not shown). Expression levels of p-PDK1 (Ser^{241}) and p-WNK1 (Thr^{60}) were markedly upregulated in HBx-transfected hepatic cells compared with in the control group (Fig. 1A). These results indicate that HBx may affect the phosphorylation of PDK1 and WNK1 in hepatic cells.

PDK1/WNK1 pathway is suppressed in response to the PDK1 inhibitor in vitro. SK-Hep1 and LO2 cell lines were employed to establish HBx-stable expression cell lines in order to study the mechanism underlying HBx-mediated regulation of PDK1. Stable expression of HBx by gene transfection was confirmed using RT-qPCR. HepG2.2.15 was used as an HBx-positive control cell line (Fig. 1B). Next, the expression of PDK1 and WNK1 was evaluated in transfected or non-transfected SK-Hep1 and LO2 cell lines treated with 20 μM PDK1 inhibitor for 0, 5, 10, 20, 30 and 60 min (Fig. 1C and D). The phosphorylation of PDK1 and WNK1 were inhibited when SK-Hep1 and LO2 cells were treated with a PDK1 inhibitor (Fig. 1C and D). The effects of the inhibitor were decreased in stable cell lines expressing HBx compared with the control (Fig. 1C and D). These results demonstrate that WNK1 is one of the downstream effectors of PDK1. Additionally, HBx may attenuate the effect of the PDK1 inhibitor on hepatic cells and thus regulate the PDK1/WNK1 signaling pathway.

HBx inhibits the viability of hepatic cells in response to the PDK1 inhibitor. The LO2-HBx cell line with stable expression of HBx exhibited increased viability compared with the control (data not shown). To investigate how HBx affects hepatic cell viability, a CCK-8 assay was performed on LO2/LO2-HBx and SK-Hep1/SK-Hep1-HBx cells. Hepatic cells were treated with 0, 5, 20 μM PDK1 inhibitor for 72 h (Fig. 2A and B) and with 20 μM PDK1 inhibitor for 0, 24, 48 and 72 h (Fig. 2C and D). The viability of all cells was significantly suppressed at 48 and 72 h, whereas the suppression in the HBx stable-expression cells was weaker compared with the control cells. Thus, the results of the present study suggest that HBx may attenuate the effect of PDK1 inhibitor on the viability of hepatic cells.

PDK1 inhibitor suppresses the migration of hepatic cells. To investigate the function of the PDK1/WNK1 signaling pathway in the metastasis of hepatic cells, a Transwell assay was employed to examine the migratory ability of LO2/LO2-HBx (Fig. 3A and B) and SK-Hep1/SK-Hep1-HBx cells (Fig. 3C and D). The results indicated that the migration of cells was gradually decreased in response to increasing doses of the PDK1 inhibitor in LO2/LO2-HBx (Fig. 3B) and SK-Hep1/SK-Hep1-HBx cells (Fig. 3D). No significant differences in the migratory ability were identified between the control and corresponding stable HBx-expressing cells.

p-PDK1 is overexpressed in human HCC tissues. Since activation of PDK1 is associated with overexpression of HBx, p-PDK1 expression was evaluated in primary HCC and paired

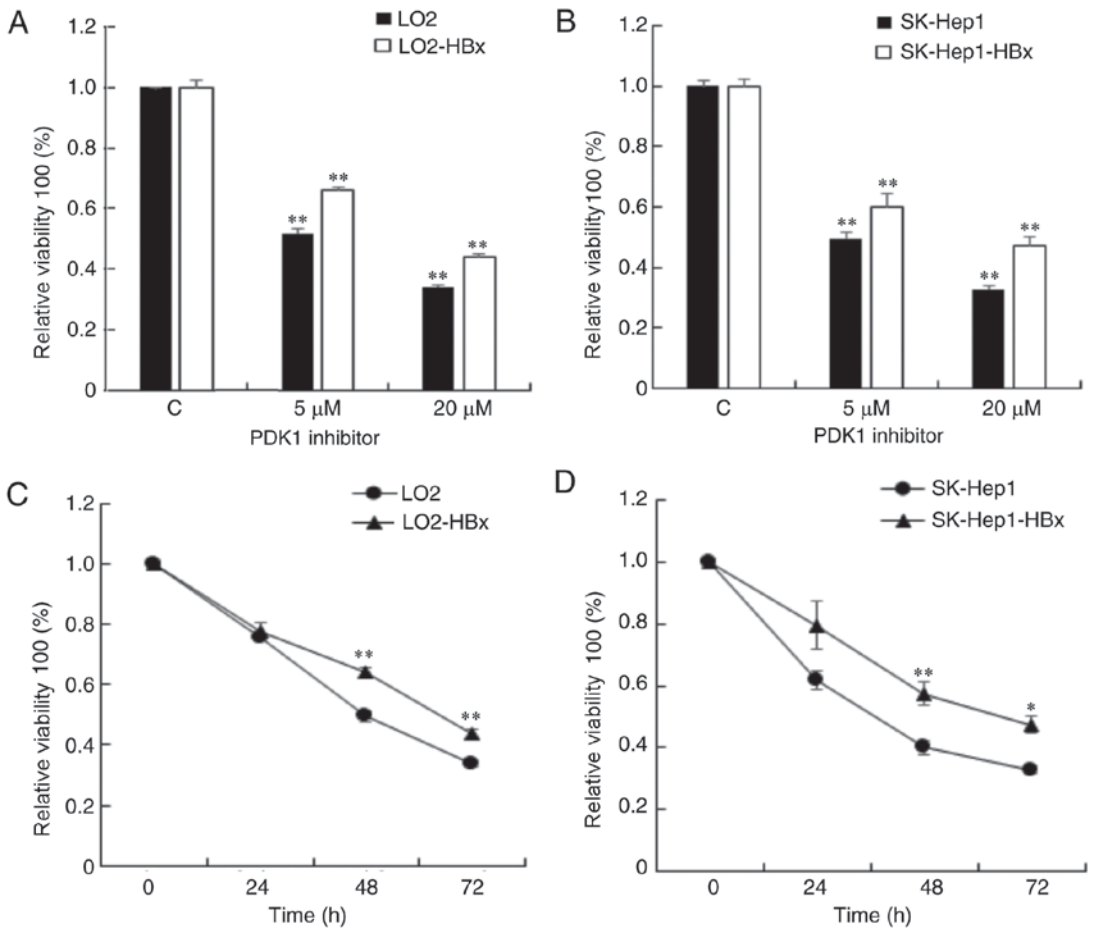

Figure 2. HBx attenuates the effect of a PDK1 inhibitor on the viability of hepatic cells. (A) Viability of LO2/LO2-HBx cells in response to 0, 5 or 20 μM PDK1 inhibitor. (B) Viability of SK-Hep1/SK-Hep1-HBx cells in response to 0, 5 or 20 μM PDK1 inhibitor using a Cell Counting kit-8. *P<0.05; **P<0.01 vs. C. (C) Viability of LO2/LO2-HBx cells in response to 20 μM PDK1 inhibitor for 0, 24, 48 or 72 h. (D) Viability of SK-Hep1/SK-Hep1-HBx cells in response to 20 μM PDK1 inhibitor for 0, 24, 48 or 72 h. *P<0.05; **P<0.01 vs. HBx-non-expressing group. Experiments were performed in triplicate at least three times. PDK1, 3-phosphoinositide-dependent protein kinase-1; WNK1, with-no-lysine (K) kinase 1; C, control.

normal liver tissue specimens (Fig. 4A). Increased expression of p-PDK1 was identified in 71% (15/21) of the HCC samples compared with in the corresponding non-tumor livers (Fig. 4B). These results indicate that p-PDK1 may be associated with the development of HBV-associated HCC.

Discussion

Chronic infection in HBV is a major cause of liver cancer, accounting for 55% of cases worldwide (23). The molecular mechanism underlying HBV-induced HCC remains unclear. However, evidence suggests that HBx protein serves a pivotal function in the development of HCC (24). HBx may regulate several signaling pathways. For example, HBx together with Akt may be important for cell proliferation and tumorigenic transformation of cells (25). Previous studies have demonstrated that HBx activated Wnt/β-catenin signaling and induced the Janus kinase/signal transducer and activator of transcription signaling pathway (26-29). The molecular mechanisms involved in the development of HBV-associated HCC are complex. Although a number of molecules and signaling pathways have been identified, further research is required.

The PI3K signaling pathway is one of the most important pathways in cell proliferation and may be a suitable target for cancer therapeutic intervention (30-32). Previous studies demonstrated that downregulation of PDK1 may suppress the progression of breast cancer (33,34). PDK1 is highly expressed in liver cancer and is associated with prognosis (13). PDK1 may also be a suitable target for HBV-induced cancer.

In the present study, the function of PDK1/WNK1 signaling in HBx-overexpressing hepatic cell lines was explored. The results suggested an overexpression of p-PDK1 in the majority of HCC tissues. Additionally, overexpression of p-PDK1 was significantly associated with the expression of HBx which is an important HBV-encoded protein involved in the genesis and development of HCC (3). The results of the present study demonstrated that upregulation of p-PDK1 occurs in HBV-associated HCC. In the present study, the expression of HBx in human HCC tissues was not examined since all patients were HBV-positive (indicating the expression of HBx). Nevertheless, it has been demonstrated previously that the protein expression of HBx is not always detectable in HBV-associated HCC (35), thus further investigation is required.

The molecular mechanism underlying the upregulation of p-PDK1 in HCC remains unclear. However, several studies identified that PDK1 may be a potential therapeutic target to improve melanoma and angiosarcoma treatment (10,11,36). The results of the present study demonstrated that the inhibition

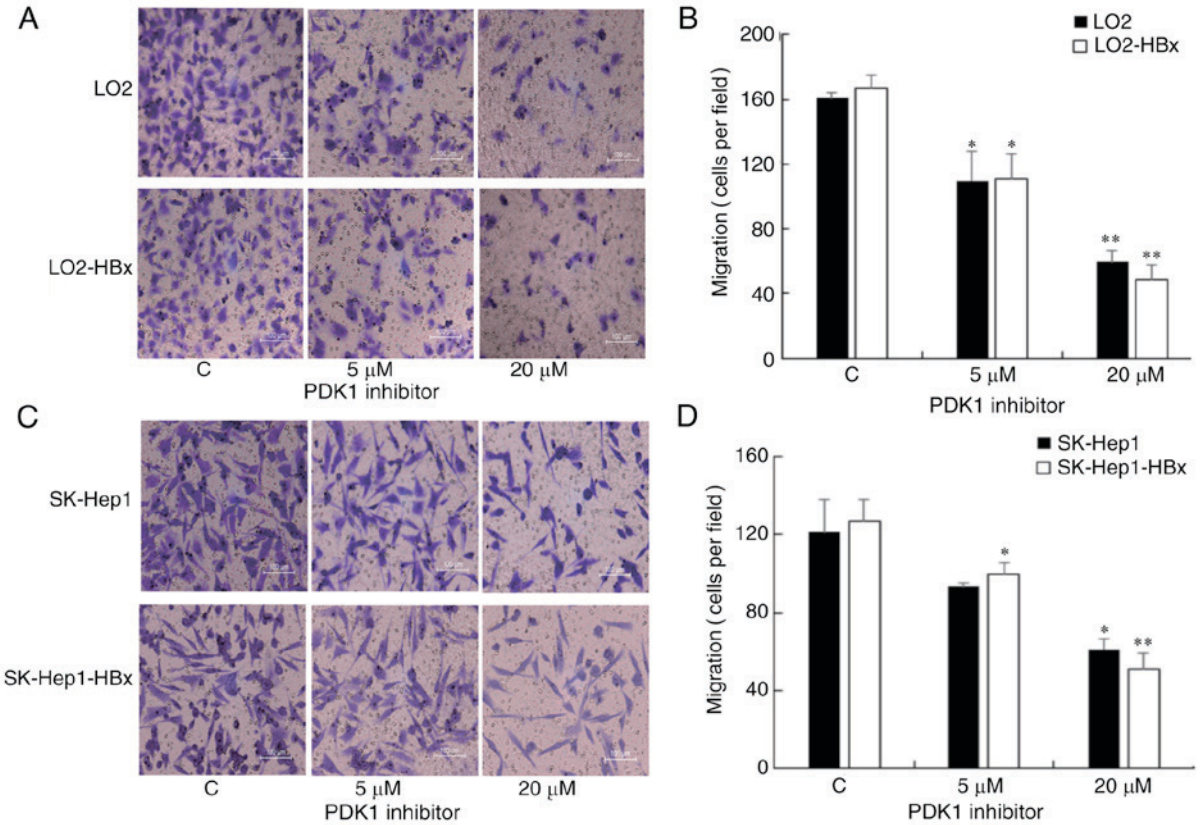

Figure 3. PDK1 inhibitor suppresses the migration of hepatic cells. (A) Representative images of migrated LO2/LO2-HBx cells in response to 0, 5 or 20 μM PDK1 inhibitor (scale bar, 100 μm). (B) Cell migration rate of LO2/LO2-HBx cells in response to 0, 5 or 20 μM PDK1 inhibitor. (C) Representative images of migrated SK-Hep1/SK-Hep1-HBx cells in response to 0, 5 or 20 μM PDK1 inhibitor (scale bar, 100 μm). (D) Cell migration rate of SK-Hep1/SK-Hep1-HBx cells in response to 0, 5 or 20 μM PDK1 inhibitor. *P<0.05; **P<0.01 vs. C. PDK1, 3-phosphoinositide-dependent protein kinase-1; C, control.

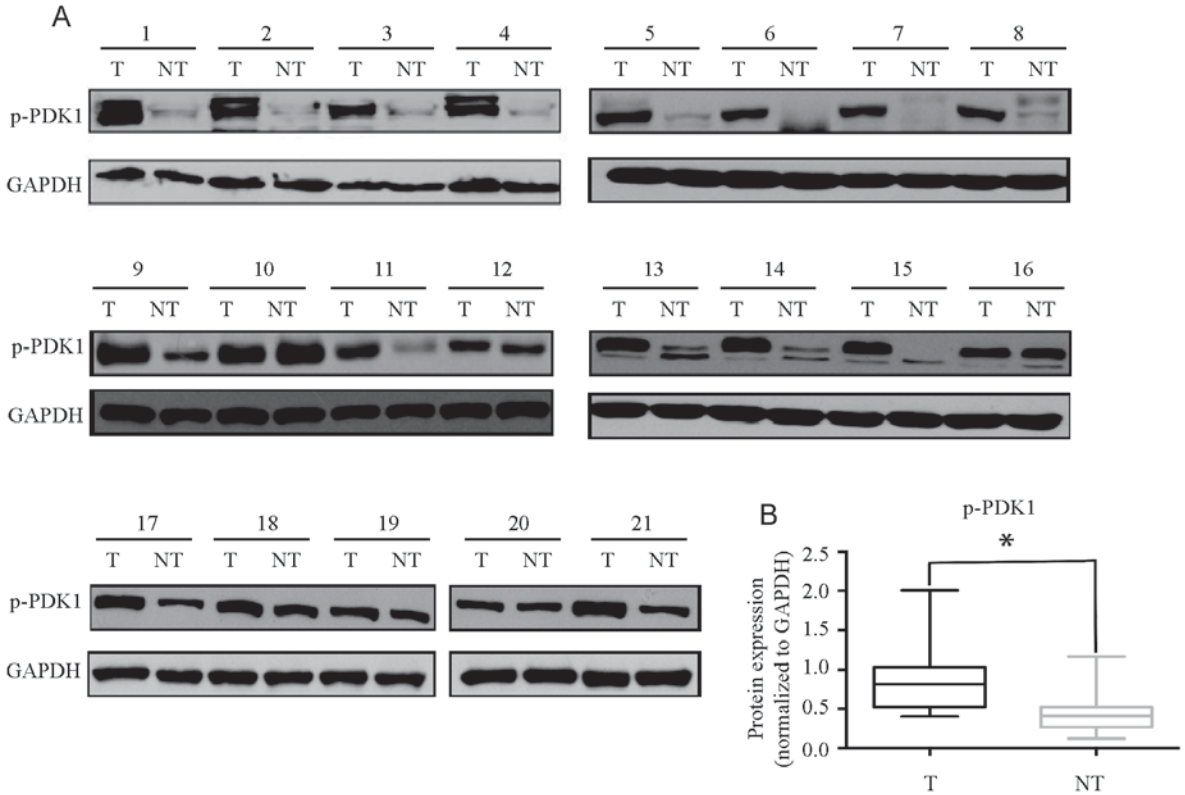

Figure 4. p-PDK1 is upregulated in HCC tissues. (A) Expression levels of p-PDK1 in 21 primary HCC and paired normal liver tissue specimens. (B) Western blot analysis of the expression of p-PDK1 in HCC and paired non-tumor samples. *P<0.05. HCC, hepatocellular carcinoma; PDK1, 3-phosphoinositide-dependent protein kinase-1; p, phospho; C, control; T, tumor; NT, non-tumor.

of the kinase activity of PDK1 may decrease the viability and metastasis of hepatic cells. Additionally, no differences were observed in the migration of control and stable HBx-expressing cells, thus suggesting that the regulation of the PDK1/WNK1 signaling pathway by HBx may not be pivotal for liver cancer metastasis. Evidence suggests that a PDK1 inhibitor may regulate metastasis mainly through additional PDK1-mediated signaling pathways (12).

The results of the present study demonstrated that the expression of p-PDK1 and p-WNK1 was upregulated in HBx-overexpressing HCC cells, which is in accordance with the phosphoproteomic data of HCC samples (data not shown). Evidence suggests that the PDK1/WNK1 signaling pathway is associated with HBx-associated HCC. In the present study, it has been demonstrated that the expression of p-PDK1 and p-WNK1 were upregulated in stable HBx-expressing hepatic cells. Additionally, the upregulation of p-PDK1 and p-WNK1 was inhibited in response to a PDK1 inhibitor in LO2-HBx and SK-hep1-HBx cell lines in a dose- and time-dependent manner. Stable expression of HBx reversed the effects of PDK1 inhibitor on cell viability compared with the control.

In conclusion, p-PDK1 is upregulated in human HCC tissues and in stable HBx-expressing hepatic cell lines. The overexpression of p-PDK1 serves an important function in HBx-associated cell viability. PDK1 may be a potential target for combating HBV-induced liver cancer.

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Competing interests

The authors declare that they have no competing interests.

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