

Hepatitis B virus X protein increases microRNA-21 expression and accelerates the development of hepatoma via the phosphatase and tensin homolog/phosphoinositide 3-kinase/protein kinase B signaling pathway

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Abstract. Hepatitis B virus (HBV) X protein (HBx) is a key regulatory protein that is involved in HBV infection, replication and carcinogenesis of hepatocellular carcinoma (HCC). The aim of the present study was to investigate the role of HBx in the progression and metastasis of liver cancer cells and to determine the underlying molecular mechanism of HBx in metastatic liver cancer cells. HBx protein expression was detected by western blot analysis, and microRNA (miR)-21 levels were determined by reverse transcription-quantitative polymerase chain reaction in the highly metastatic MHCC-97H low metastatic MHCC-97L and SMMC-7721 liver cancer cell lines. The results demonstrated that the levels of HBx and miR-21 were significantly increased in MHCC-97H cells compared with MHCC-97L and SMMC-7721 cells. In addition, three pairs of small interfering (si)RNA specific to HBx were designed and synthesized to interfere with endogenous HBx in liver cancer cells, and the results demonstrated that knockdown HBx was associated with a corresponding decrease in miR-21 expression. The MTT assay results demonstrated that cell viability significantly decreased in HBx-siRNA cells compared with scramble siRNA-transfected cells. In addition, transfection with an miR-21 inhibitor inhibited MHCC-97H cell proliferation. Furthermore, Transwell assay results revealed that downregulation of HBx and treatment with miR-21 inhibitors contributed to the inhibition of MHCC-97H cell invasion and metastasis. Western blot analysis demonstrated that miR-21 inhibitors and HBx-siRNA treatment led to the upregulation of phosphatase and tensin homolog (PTEN), and decreased levels of phosphoinositide 3-kinase (PI3K),

phosphorylated protein kinase B (Akt) and matrix metalloproteinase (MMP)-2. The results of the present study indicated that HBx was positively associated with miR-21 expression, and downregulation of miR-21 and HBx suppressed MMP-2 activity via the PTEN/PI3K/Akt signaling pathway. Therefore, HBx and miR-21 may represent novel therapeutic targets for the treatment of HCC.

Introduction

Primary hepatocellular carcinoma (HCC) is one of the most predominant diseases worldwide, particularly in China (1,2). Hepatitis B virus (HBV), hepatitis C virus (HCV), aflatoxin B1, water pollution and alcoholism all contribute to the development of liver cancer (3). In China, the leading cause of liver cancer is cirrhosis due to HBV infection (4-6). The nonstructural HBV X protein (HBx) is comprised of 154 amino acids, with a molecular mass of ~17.5 kDa (7). It is a key regulatory protein of HBV, and has been associated with HBV infection, replication, pathogenesis and potentially carcinogenesis (8,9). Recently, Kouwaki *et al* (10) reported that HBx regulates HBV replication by interacting with jumonji-C domain containing-5 (JMJD5), a novel binding partner to HBx, which facilitates HBV replication via the hydroxylase activity of JMJD5. In addition, anti-HBx short hairpin RNAs have been demonstrated to effectively inhibit HBV replication *in vivo* by targeting conserved sequences in the oncogenic HBx open reading frame (11). Kim *et al* (12) revealed that HBx altered host gene expression, leading to the development of HCC in transgenic mice. Notably, a previous study revealed that enhanced cell motility was alleviated by mutations in the proline rich domain located in HBx, which provided novel insights on the underlying mechanism of HCC development associated with chronic HBV infection (13). However, the exact underlying mechanism of HBx in HCC development and progression remains unclear.

MicroRNAs (miRNAs) are a newly identified class of conserved RNA molecules 21 to 23 nucleotides in length, and regulate the stability or translational efficiency of target mRNAs. miRNAs serve important roles in the progression and metastasis of liver cancer (14). Epithelial-mesenchymal

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transition (EMT) has been recognized as a critical event that initiates cancer invasion and metastasis. It involves the loss of tumor cell polarity and is associated with alterations in intercellular adhesion, cytoskeletal reorganization and cellular signaling pathway. Yin *et al* (15) reported that liver metastasis-associated miRNAs, including serum miRNA (miR)-126, miR-141 and miR-21, may be novel biomarkers for the clinical diagnosis of early stage liver-metastatic colorectal cancer. A previous study determined that miR-21 may act as a key regulator of fibrogenic EMT in hepatocytes via PTEN/Akt pathway (16). Zhang *et al* (17) additionally demonstrated that miR-21, miR-17 and miR-19a, induced by a phosphatase of regenerating liver-3, are involved in the proliferation and invasion of colon cancer cells. A number of studies have revealed that miR-21 is overexpressed in human tumors and that it may be involved in the regulation of metastasis in several tumor types, including melanoma (18), breast cancer (19,20), keratinocytes (21), colorectal cancer (22) and HCC (23). However, it is unclear whether miR-21 is involved in the progression and metastasis of HBV-positive liver cancers.

The aim of the present study was to investigate the role of miR-21 in HBx-positive liver cancer cells, and to determine the association between HBx and miRNAs in the development and metastasis of liver cancer cells. The results of the present study may provide novel ideas and effective targets for future HCC therapy.

Materials and methods

Cell line and agents. The human HCC cell lines, MHCC97H and MHCC-97L, were obtained from the Liver Cancer Institute of Fudan University (Shanghai, China). The SMMC-7721 liver cancer and immortalized L02 human liver cell lines were used as negative controls and were purchased from Cell Resource Center of Shanghai Institutes for Biological Sciences (Chinese Academy of Sciences, Shanghai, China). The liver cancer cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1% penicillin and 1% streptomycin. DMEM and FBS were purchased from Hyclone; GE Healthcare Life Sciences (Logan, UT, USA).

MTT assay. MTT assay was performed as previously described (24,25). Briefly, the SMMC-7721 human liver cancer cells at a density of 3,000 cells/well were transfected with HBx-small interfering (si)RNA1, HBx-siRNA2 or HBx-siRNA3 for 48, 72 or 96 h, respectively. Proliferation in the different groups was detected by MTT assay. In the other treatment group, MHCC-97H cells were plated into 96-well plates at a density of 3,000 cells/well and treated with miR-21 mimics or miR-21 inhibitors for 48, 72 or 96 h. The cell viability was determined by MTT assay.

Transfection. Three pairs of siRNA specific to HBx were designed and synthesized by Jima Corporation (Shanghai, China). Scramble siRNA was used as the negative control (NC) for siRNA experiments. Transfection of cells was performed using Oligofectamine (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) according to the manufacturer's

protocol. The Opti-MEM medium was replaced 6 h following transfection. RNA was extracted 48 and 72 h following siRNA treatment for reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and cell lysates were prepared for western blot analysis.

The sequences for the chemically synthesized HBx-siRNAs were as follows: HBx-siRNA1 forward, 5'-GGUCUUACA UAAGAGGACUdTdT-3' and reverse, 5'-AGTCCTCTTATG TAAGACCDdTdT-3'; HBx-siRNA2 forward, 5'-CCGACCUUG AGGCAUACUdTdT-3' and reverse, 5'-AAGUAUGCCUCA AGGUCGGdTdT-3'; HBx-siRNA3 forward, 5'-GGACGU CCUUUGUUUACGUdTdT-3' and reverse, 5'-ACGTAAACA AAGGACGTCCdTdT-3'. The miR-21 inhibitor, anti-miR-21 (sequence, 5'-UCAACAUCAGUCUGAUAAAGCUA-3'), is a chemically modified single strand RNA and a competitive inhibitor of miR-21. The sequence of the miR-21 mimic used was: 5'-UAGCUUAUCAGACUGAUGUUGAAACAUCAG UCUGAUAAAGCUAUU-3'. The miR-21 mimics and miR-21 inhibitors were all obtained from Shanghai GenePharma Co., Ltd. (Shanghai, China).

RT-qPCR for quantitative analysis of miR-21. Total RNA was extracted from liver cancer cells using TRIzol® (Takara Bio, Inc., Otsu, Japan) according to the manufacturer's protocol. The cDNA samples were transcribed using the PrimeScript-RT reagent kit (Takara Biotechnology Co., Ltd., Dalian, China) in a 20 ml final reaction volume according to the manufacturer's instructions. An RT-qPCR assay was performed to evaluate miR-21 levels using SYBR Premix ExTaq™ II (Takara Biotechnology Co., Ltd.) on an ABI 7500 system according to the manufacturer's instructions, the thermocycling conditions were 40 cycles of 12 sec at 95°C and 1 min at 58°C. Each sample was analyzed in triplicate and U6 small nuclear RNA was used for normalization. No template or reverse transcription were included as negative controls. The sequence of primers for RT-qPCR were as follows: MiR-21 RT: 5'-CGTCGCTACATCGAGTGTAGCATATGCGACGTC AACATC-3'; miR-21 forward, 5'-TAGCTTATCAGACTG ATG-3' and reverse, 5'-ACATCGAGT GTAGCATA-3'; U6 RT 5'-AACGCTTCACGAATTTGCGT-3'; U6 forward, 5'-CTC GCTTCGGCAGCACA-3' and reverse, 5'-AACGCTTCACGA ATTTGCGT-3'.

Antibodies. An anti-HBx antigen mouse monoclonal antibody [3F6-G10] (cat. no. ab235) was purchased from Abcam (Cambridge, UK). A phosphatase and tensin homolog (PTEN) rabbit monoclonal antibody (138G6; cat. no. 9559S) was purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). A phosphorylated (p)-protein kinase B (Akt) 1/2/3 (Ser 473)-R antibody (a rabbit polyclonal IgG; 200 µg/ml; cat. no. sc-7985-R) and mouse monoclonal Akt antibody (BD111; 50 µg/0.5 ml; cat. no. sc-56878) were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). Rabbit polyclonal anti-matrix metalloproteinase (MMP)-2 (cat. no. ab37150) and anti-MMP-9 (cat. no. ab38898) antibodies were purchased from Abcam. Mouse monoclonal anti-β-actin antibody (cat. no. TA310155) was purchased from OriGene Technologies, Inc. (Beijing, China). All the primary antibodies were diluted at 1:1,000. Goat anti-rabbit IgG-horseradish peroxidase (HRP; cat. no. sc-2004) and goat anti-mouse IgG-HRP (cat.

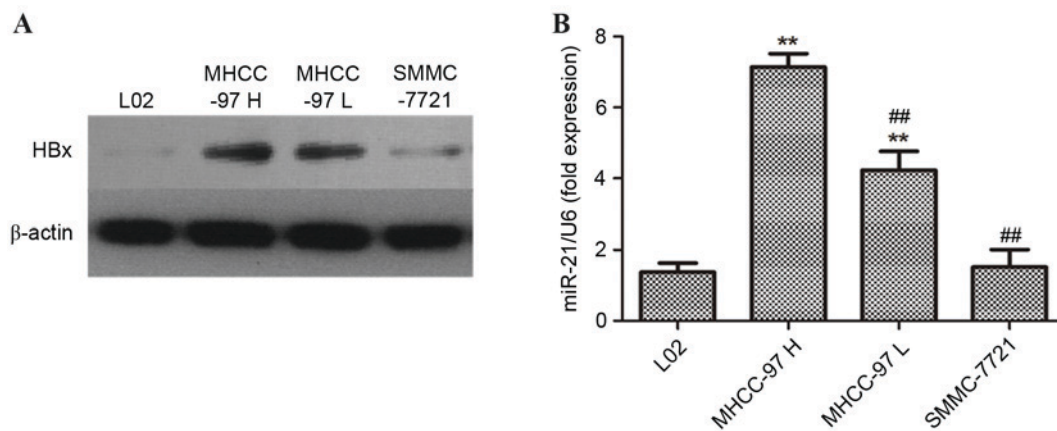


Figure 1. Levels of HBx are positively correlated with the expression of miR-21. The highly metastatic MHCC-97H and the low metastatic MHCC-97L and SMMC-7721 human liver cancer cell lines were assessed, and the L02 immortalized human liver cell line was used as the control. (A) HBx protein expression levels were determined by western blot analysis. β -actin served as an internal control. (B) miR-21 levels were detected by reverse transcription-quantitative polymerase chain reaction. Data are presented as the mean \pm standard deviation. ** P <0.01 vs. L02 cells; ## P <0.01 vs. MHCC-97H cells. HBx, hepatitis B virus X protein; miR, microRNA.

no. sc-2005) secondary antibodies were purchased from Santa Cruz Biotechnology, Inc.

Western blot analysis. The liver cancer cells were seeded into 48-well plates (5×10^5 cells) for 8 h, prior to treatment with miR-21 inhibitors or miR-21 mimics for 24 or 48 h. The cell lysates were prepared using radioimmunoprecipitation assay buffer (50 mmol/l Tris, 1% NP-40, 150 mmol/l NaCl, 1 mmol EDTA, 0.1% SDS, 0.25% sodium deoxycholate detergent). The lysates were separated by 10-12% SDS-PAGE and subsequently transferred onto a nitrocellulose membrane (Beijing BioDee Biotechnology, Co., Ltd., Beijing, China) at 400 mA for 1 h. The membrane was blocked with Tris-buffered saline with 0.1% Tween-20 (TBST) supplemented with 5% bovine serum albumin (BSA; Beyotime Institute of Biotechnology, Inc., Haimen, China) for 30 min at room temperature prior to incubating the membrane with specific antibodies in TBST containing 2% BSA at 4°C overnight. The membrane was washed three times with TBST and subsequently incubated with the corresponding secondary antibodies for 1 h at room temperature. The bands were detected in a dark room using chemiluminescence techniques. β -actin was used as an internal reference. The experiments were repeated twice.

Transwell assay. A Transwell assay was used to detect the invasion and metastasis of liver cancer cells. Corning® Transwell® polycarbonate membrane cell culture inserts (cat. no. CLS3421) were obtained from Sigma-Aldrich; Merck Millipore (Darmstadt, Germany). The BD Matrigel™ (BD Biosciences, Franklin Lakes, NJ, USA) was stored at 4°C overnight to keep it in a liquid state. Serum-free medium (300 μ l) and the Matrigel™ (50 μ l) were mixed and placed into the insert, which was incubated at 37°C for 4-5 h. The liver cancer cells (5×10^5) were loaded into the insert and treated with the miR-21 mimics or miR-21 inhibitors for 48 h at 37°C. The insert was taken off and the cells were washed twice with PBS buffer. The cells were subsequently fixed with 5% glutaraldehyde and stained with crystal violet (0.1%). The cells were washed twice with PBS, and counted under a light microscope.

Statistical analysis. Statistical analysis was performed using analysis of variance followed by Tukey's honest significant difference post hoc test using SPSS 20.0 software (IBM SPSS, Armonk, NY, USA). The data are presented as the mean \pm standard deviation. P <0.05 was considered to indicate a statistically significant difference.

Results

HBx levels are positively associated with the expression of miR-21. In order to detect whether the levels of HBx were associated with the invasion of liver cancer cells, HBx levels were detected in three liver cancer cell lines with different metastatic potentials, including MHCC-97H (a highly metastatic human hepatocellular carcinoma cell line), and MHCC-97L and SMMC-7721 (low metastatic human hepatocellular carcinoma cell lines). The L02 immortalized human liver cell line was used as the control. As presented in Fig. 1A, the western blotting results demonstrated that protein expression of HBx in MHCC-97H cells was markedly increased compared with in MHCC-97L and SMMC-7721 cells. miR-21 is the most commonly over-expressed miRNA in cancer and it is a recognized oncogene. mRNA expression levels of miR-21 in MHCC-97H, MCH-97L, SMMC-7721 and L02 cells were additionally measured. As presented in Fig. 1B, the results demonstrated that the expression levels of miR-21 were significantly increased in MHCC-97H and MHCC-97L cells compared with L02 cells. In addition, the miR-21 levels in MHCC-97H cells were significantly increased than those observed in MHCC-97L and SMMC-7721 cells (** P <0.01). Collectively, the results indicated that the levels of HBx may be positively associated with the expression of miR-21, and that they may contribute to the invasion of liver cancer cells.

Interference with endogenous HBx in liver cancer cells is accompanied by a corresponding decrease in miR-21 expression. To further investigate the association between HBx and miR-21, three pairs of siRNAs specific to HBx were designed and synthesized. The three pairs of HBx-siRNAs

and the scrambled siRNA were used for interference with endogenous HBx in human liver cancer cells. Following transfection with HBx-siRNA for 48 h, HBx-siRNA1 was most effective at downregulating HBx in liver cancer cells (Fig. 2A). The relative expression of miR-21 was detected in MHCC-97H and MHCC-97L cells, which were transfected with HBx-siRNA for 48 h. Notably, the results demonstrated that interference with endogenous HBx expression may significantly decreased the levels of miR-21 in MHCC-97H (Fig. 2B) and MHCC-97L (Fig. 2C).

Interference with the expression of HBx inhibits the proliferation of MHCC-97H cells. The present study revealed that interference with the levels of HBx decreased the expression of miR-21 in MHCC-97H and MHCC-97L cells. Thus, it was of interest to investigate whether knockdown of endogenous HBx would affect the proliferation of MHCC-97H cells. The MHCC-97H cells were transfected with HBx-siRNA1, HBx-siRNA2 and HBx-siRNA3 for 48 h and cell viability was determined by MTT assay. As presented in Fig. 3A, proliferation of MHCC-97H cells was significantly suppressed following transfection with siRNA specific to HBx when compared with scrambled siRNA (* $P < 0.05$ and ** $P < 0.01$), indicating that knockdown of HBx levels in MHCC-97H cells may inhibit liver cancer cell proliferation.

Transfection with miR-21 inhibitor or mimics regulates the cell proliferation of MHCC-97H cells. The results so far revealed that HBx expression regulated the levels of miR-21 in liver cancer cells. To further confirm the role of miR-21 in the proliferation of MHCC-97H cells, the miR-21 mimics and miR-21 inhibitor were transfected into MHCC-97H cells and cell viability was determined by MTT assay. As presented in Fig. 3B, transfection with miR-21 mimics had no marked effects on the proliferation of MHCC-97H cells. However, in MHCC-97H cells transfected with miR-21 inhibitor for 48, 72 and 96 h, cell viability was significantly decreased compared with miR-21 mimics-transfected cells (** $P < 0.01$).

Invasion and metastasis of MHCC-97H cells is regulated by miR-21 and HBx. To further determine the effects of miR-21 on the invasion and metastasis of MHCC-97H cells, a Transwell assay was performed by transfecting with miR-21 mimics and miR-21 inhibitors. As presented in Fig. 4A and B, following transfection with inhibitors, the number of migratory cells were significantly decreased in MHCC-97H cells compared with the untreated group (** $P < 0.01$). In addition, miR-21 mimics were used to transfect the MHCC-97H cells for 48 h, and the results demonstrated that the number of migratory cells significantly increased when compared with untreated cells (* $P < 0.05$). The role of HBx in the invasion and metastasis of MHCC-97H cells was additionally investigated. Briefly, the MHCC-97H cells were transfected with three pairs of siRNA specific to HBx for 48 h, and a Transwell assay was performed. As presented in Fig. 4C, the migration and invasion of MHCC-97H cells was significantly suppressed in HBx-knockdown cells compared with scrambled siRNA-transfected cells (* $P < 0.05$ and ** $P < 0.01$). Collectively, these results revealed that downregulation of HBx and miR-21 inhibitors may contribute to the inhibition of invasion and metastasis in human liver cancer cells.

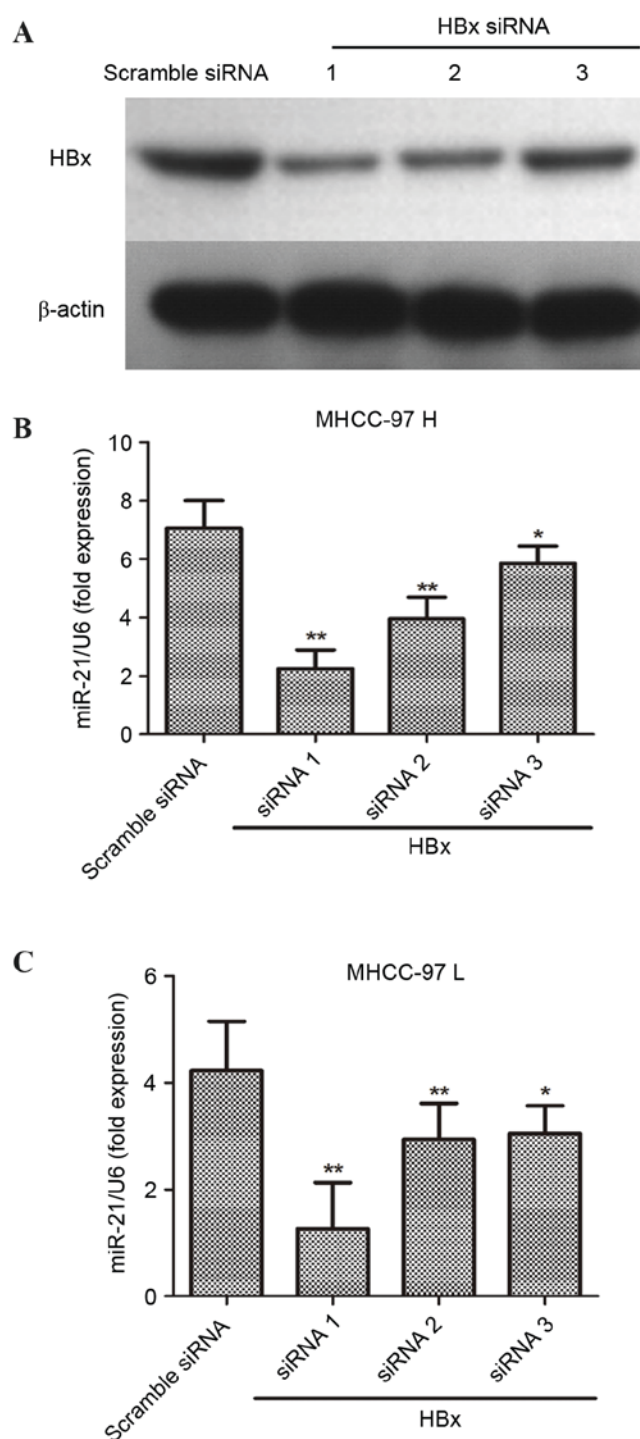


Figure 2. Interference with endogenous HBx in liver cancer cells is accompanied by a corresponding decrease in miR-21 expression. Scramble siRNA served as the negative control. (A) Representative western blot images of protein expression levels of endogenous HBx. β -actin served as an internal control. miR-21 expression in the (B) MHCC-97H and (C) MHCC-97L HBx-transfected liver cancer cell lines. Data are presented as the mean \pm standard deviation. * $P < 0.05$ and ** $P < 0.01$ vs. scramble siRNA-transfected cells. HBx, hepatitis B virus X protein; miR, microRNA; siRNA, small interfering RNA.

HBx and miR-21 regulate the PTEN/PI3K/Akt signaling pathway. It has been reported that PTEN may be a direct target of miR-21 and that overexpression of miR-21 may lead to increased p-Akt signaling by directly targeting PTEN (26). The present study investigated whether HBx regulates miR-21

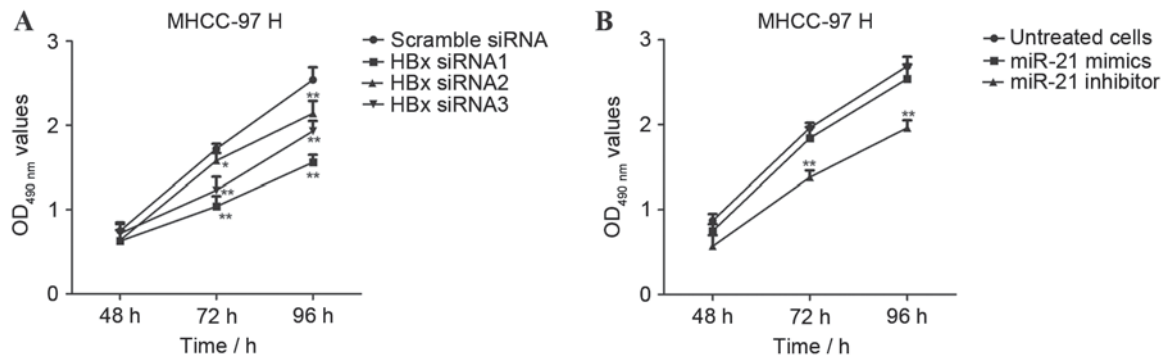


Figure 3. Inhibition of HBx and miR-21 decreases cell viability of MHCC-97H cells. (A) Interference with the expression of HBx inhibited proliferation of MHCC-97H cells, as determined by MTT assay. * $P < 0.05$ and ** $P < 0.01$ vs. scramble siRNA-transfected cells. (B) Transfection with miR-21 inhibitor or mimics regulated cell proliferation of MHCC-97H cells. ** $P < 0.01$ vs. untreated cells. Data are presented as the mean \pm standard deviation. HBx, hepatitis B virus X protein; miR, microRNA; siRNA, small interfering RNA; OD, optical density.

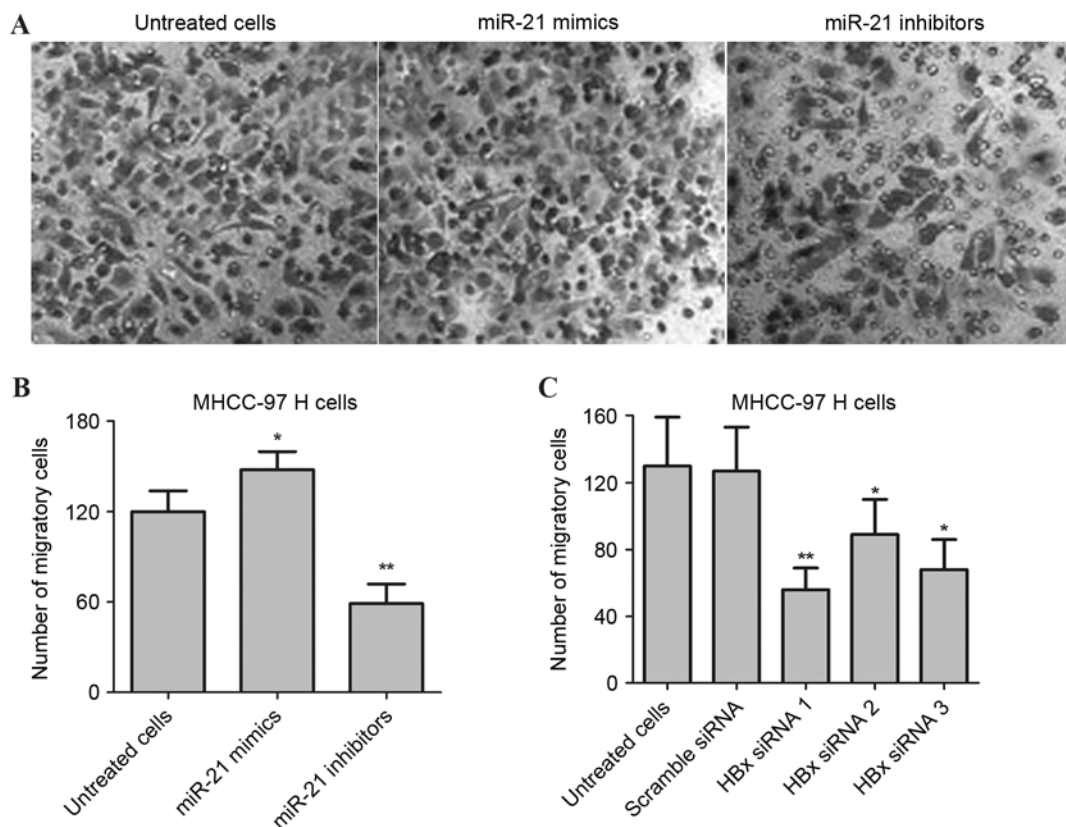


Figure 4. Invasion and metastasis of MHCC-97H cells is regulated by miR-21 and HBx. (A) Representative microscope images and (B) quantification of invasion ability in untreated, miR-21 mimic and miR-21 inhibitor cells, as determined by Transwell assay. * $P < 0.05$ and ** $P < 0.01$ vs. untreated cells. Magnification, $\times 200$. (C) Quantification of invasion ability in MHCC-97H cells transfected with HBx-siRNA1, HBx-siRNA2 and HBx-siRNA3 for 48 h. * $P < 0.05$ and ** $P < 0.01$ vs. scramble siRNA-transfected cells. Data are presented as the mean \pm standard deviation. miR, microRNA; HBx, hepatitis B virus X protein; siRNA, small interfering RNA.

via the PTEN/PI3K/Akt signaling pathway. The miR-21 inhibitors were used to transfect MHCC-97H cells for 24 h. The level of PTEN was upregulated, which was accompanied by a decrease in the levels of PI3K and p-Akt, as well as down-regulation of MMP-2 levels (Fig. 5A). Notably, the expression of MMP-9 was not altered in miR-21 inhibitor-treated cells. Western blot analysis further confirmed that transfection with HBx-siRNA led to an upregulation of PTEN; however, the levels of p-Akt and MMP-2 were reduced when compared with

cells transfected with scramble siRNA (Fig. 5B). Collectively, these results indicated that downregulation of miR-21 and HBx may suppress the activity of MMP-2 via the PTEN/PI3K/Akt signaling pathway.

Discussion

In China, the HBV infection is one of the primary causes of HCC. In the HBV genome, the association between the

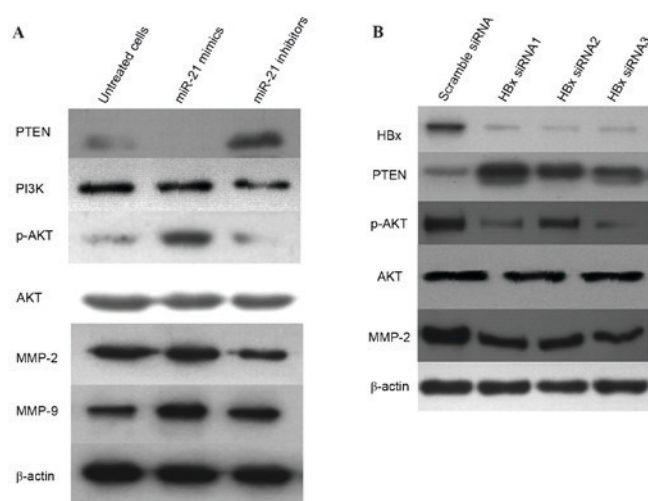


Figure 5. HBx and miR-21 regulate the PTEN/PI3K/Akt signaling pathway. (A) MHCC-97H cells were transfected with miR-21 mimics and miR-21 inhibitors for 24 h, and western blotting was performed to determine protein expression levels of PTEN, PI3K, p-Akt, Akt, MMP-2 and MMP-9. (B) MHCC-97H cells were transfected with HBx-siRNA1, HBx-siRNA2 or HBx-siRNA3 for 48 h. Protein expression levels of HBx, PTEN, p-Akt, Akt and MMP-2 were determined by western blot analysis. β -actin served as an internal control. HBx, hepatitis B virus X protein; miR, microRNA; PTEN, phosphatase and tensin homolog; PI3K, phosphoinositide 3-kinase; p, phosphorylated; Akt, protein kinase B; MMP, matrix metalloproteinase; siRNA, small interfering RNA.

X-gene and HCC has been a key area of interest. A recent study using HBx-expressing transgenic mice under authentic promoter control demonstrated a high rate of HCC development (86%) (27). The HBV gene integrates into the genome of the host cell, which may interfere with normal cell cycle and apoptosis, and thus may be an important factor in the induction of malignant transformation (28). However, the exact mechanisms underlying HBV carcinogenesis have not been fully elucidated. Previous studies have revealed that the molecular mechanism of HBx in the carcinogenesis of HCC is complex and may involve multiple mechanisms (29).

In the present study, three human HCC cell lines with different metastatic potentials were selected. MHCC-97H is a human liver cancer cell line with high metastatic potential, whereas MHCC-97L and SMMC-7721 are human liver cell lines with low metastatic potentials. In order to detect the role of HBx in the progression and metastasis of human HCC cells, protein expression levels of HBx in MHCC-97H, MHCC-97L and SMMC-7721 cells were detected by western blot analysis. The L02 immortalized liver cell line was used as negative control. The results demonstrated that MHCC-97H cells had increased levels of HBx compared with MHCC-97L and SMMC-7721 cells, indicating that HBx may be involved in the metastasis of liver cancer cells.

In addition, increased levels of HBx were associated with increased expression of miR-21 in the highly metastatic MHCC-97H liver cancer cell line. However, interference with HBx decreased the levels of endogenous HBx, which was accompanied by a corresponding downregulation of miR-21. MHCC-97H cells were treated with miR-21 inhibitors, which decreased the migration and invasion of MHCC-97H cells compared with untreated cells. This was consistent with the

results of MHCC-97H cells with knocking down of HBx by HBx-siRNA.

PTEN is considered the target gene of miR-21 and exerts its biological function as a tumor-suppressor (30,31). In the present study, interference with endogenous HBx was demonstrated to contribute to upregulation and activation of PTEN, which led to a decrease in p-Akt and downregulation of MMP-2. This was consistent with the results of cells treated with miR-21 inhibitors. Thus, interference with endogenous HBx may suppress the activity of MMP-2 via the PTEN/PI3K/Akt signaling pathway to inhibit the invasion and metastasis of MHCC-97H cells, mediated by downregulation of miR-21 levels. The present study provided novel information, and potentially an effective target, for future HCC therapies.

Acknowledgements

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