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HBx-Dependent Activation of Twist Mediates STAT3 Control of Epithelium-Mesenchymal Transition of Liver Cells

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ABSTRACT

This study investigated the molecular mechanisms of liver cells with HBx expression on epithelium–mesenchymal transition (EMT) change using Western blot analysis and Transwell assay to assess EMT-related protein expression and cell mobility. Luciferase reporter assay and chromatin immunoprecipitation (ChIP) assay were used to test the Twist promoter containing different STAT3 binding loci. Electrophoretic mobility band-shift assay (EMSA) was used to detect Twist activity. Results showed that HBx expression affected the EMT-related protein expression and the cell mobility of liver cancer cells (MHCC97) and liver cells (HL-7702) in vitro or in vivo. These proteins exhibited reversed expression to a certain extent after Twist inhibition. In addition, the wound-healing capability and the mobility of HL-7702/HBx cells were lower than those treated with control-siRNA. The expressions of p-STAT3 and Twist were positively correlated with HBx expression. The second STAT-3 binding sequence in the Twist promoter region of the HL-7702/HBx cells was the first locus. Twist activity in the HL-7702/HBx2 cells was higher than that in HL-7702 cells. Moreover, the activity decreased when the cells were treated with HBx-siRNA to inhibit HBx expression, or with STAT3 inhibitor to reduce STAT3 activation. Therefore, Twist is essential for the regulation of the mobility of liver cells with HBx expression. HBx activates the Twist promoter by activating STAT3 and promotes EMT occurrence in liver cells. J. Cell. Biochem. 114: 1097–1104, 2013. © 2012 Wiley Periodicals, Inc.

KEY WORDS: HBX; LIVER CELL; EPITHELIUM-MESENCHYMAL TRANSITION; TWIST; STAT3

Epithelium-mesenchymal transition (EMT) is the phenomenon in which epithelial cells change phenotype to exhibit epithelial-like features (down-regulated expression of E-cadherin/catenin complex), concomitant expression of mesenchymal markers (N-cadherin and vimentin), cytoskeletal rearrangement, and enhanced cell locomotivity [Thiery, 2003]. EMT and the production of cancer cells by primary tumors are the key steps for the completion of metastasis. Hepatocellular carcinoma (HCC) is one of the most common malignant tumors worldwide with high fatality rates. Recurrence and metastasis are the major factors influencing prognosis. Previous studies showed that HCC invasion is closely related to EMT [Lee et al., 2006b; Hao et al., 2012].

Worldwide, it is estimated that more than 50% of HCC cases are attributed to chronic and persistent hepatitis B virus (HBV) infections [Bosch et al., 2004]. As an oncogenic virus, HBV leads to HCC both directly and indirectly [Neuveut et al., 2010]. Further studies revealed that, of the four HBV genes, HBV X (HBx) is the most common viral open reading frame that integrates into the host genome. The HBx, a 17 kDa protein, is of significant interest because it plays an important role in hepatocarcinogenesis. Scholars investigated the promotion of HCC cell metastasis by HBx through EMT induction and initially explored its molecular mechanisms. Lee found that STAT 5b activation results in EMT of HCC cells [Lee et al., 2006a]. Yang revealed that activated c-Src contributes to the

Abbreviations used: EMT, epithelium–mesenchymal transition; HCC, hepatocellular carcinoma; HBV, hepatitis B virus; HBx, HBV x protein; p-STAT3, phosphorylated signal transducer and activator 3; H&E, hematoxylin and eosin; EMSA, electrophoretic mobility band-shift assay; RLA, relative luciferase activity; ChIP, chromatin immunoprecipitation.

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regulation of cytoskeletal rearrangement following HBx gene transfection into SMMC-7721 cells [Yang et al., 2009]. In our previous study on clinical liver cancer samples, HBx expression was found to be closely related to the higher expression of Twist and N-cadherin, as well as the lower expression of E-cadherin. However, despite these extensive studies, the accurate and reasonable mechanism of how HBx promotes EMT remains unclear. In this study, we further examined the HBx expression and EMT status of different liver cancer cell lines, and used the HL-7702 cell line that derived from normal human liver to investigate whether HBx affects the EMT of liver cells through Twist, as well as the molecular mechanisms involved in this process. The present study provides new evidence that Twist is an important target molecule for the inhibition of invasion and metastasis of liver cancer with HBV infection.

MATERIALS AND METHODS

CELL LINES

Human HCC cell lines including Huh-7, HepG2, BEL7404, and SMMC7721 were maintained in our laboratory, human HCC cell lines with highly metastatic potential, MHCC97H and MHCC97L, were purchased from Liver Cancer Institute of Fudan University (Shanghai, China) [Tian et al., 1999]. Human liver cell lines HL-7702, HL-7702/con, HL-7702/HBx1, and HL-7702/HBx2 have been obtained as previously described [Chen et al., 2008].

WESTERN BLOT

Approximately 40 μg protein of cell extract was ran in 10% SDS–PAGE gels and electroblotted to PVDF or nitrocellulose membranes. The cells were visualized using different primary antibodies, including mouse anti-HBx antibody (Merck Millipore, Billerica, MA); rabbit primary antibodies against human E-cadherin, N-cadherin, β-catenin, vimentin, signal transducer and activator 3 (STAT3) (C-20), p-STAT3 (Tyr705), and β-tublin, goat antibody against Twist and HRP-labeled secondary antibodies (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). The cells were subsequently detected using enhanced chemiluminescence. The relative amount of each protein band was quantified as a ratio to the β-tublin band indicated underneath each gel using the densitometric scanning software Quantity One (BIO-RAD).

GENE SILENCING WITH SIRNA

(1) HBx RNAi vectors including pGPU6/Hygro-HBx-328(GCATACTTCAAAGACTGTGTG), -295(GGACTTTCAGCAATGTCAACG), -371(GGGAGGAGATTAGGTTAAAGG), -414(GCATAAATTGGTGTG TTCACT), and a control were constructed using the pGPU6/Hygro-siRNA gene silencing system (GenePharma Inc., Shanghai, China). HL-7702/HBx2 cells were transfected by these vectors using Lipofectamine2000 (Life Technologies, Carlsbad, CA). HL-7702/HBx-siRNA cell clones were then selected by hygromycin and denoted as HL-7702/HBx-siRNA1–4. The MHCC97H/HBx-siRNA (with HBx inhibition expression) and MHCC97H/con cell clone were also selected using this method. (2) Cells (2×10^5) were seeded in six-well plates, and then transfected with Twist-siRNA1–3 (including sc-38604a, sc-38604b, and sc-38604c) and control–siRNA (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) in serum-free DMEM medium using Lipofectamine2000 after overnight incubation. The cells were immersed in trypsin and resuspended in serum-free DMEM medium for wound-healing and cell migration assay.

WOUND-HEALING ASSAY

Cells were plated on the coverslips placed in six-well culture plates in growth medium and grown to confluence. A wound was made by scrapping with a sterilized 200 μl pipette tip in the middle of the cell monolayer. Cells were then incubated for 24 h and pictures of a defined wound spot were made with computer-aided phase contrast microscope (Olympus, Japan) at $t = 0$ and 24 h. The area of the wound in the microscopic pictures was measured using ImageJ software (NIH) at different time points. The percentage of wound-healing after 24 h was calculated in relative to the total wound area at $t = 0$ h of the same wound spot.

CELL MIGRATION ASSAY

The 5×10^4 cells in 500 μl of serum-free DMEM were seeded on the top chamber of the Transwell insert (Falcon 3097, BD Medical, Franklin Lakes, NJ) following the protocol indicated by the manufacturer. Migrated cells were counted using an inverted microscope at ×100 magnification.

LUNG METASTASIS EXPERIMENTS

Male BALB/c nude mice (six weeks old) were purchased from the Chinese Academy of Sciences (Shanghai, China) and were given human care. The experimental protocol was approved by the Medical Experimental Animal Care Committee of Fujian Medical University. MHCC97H, MHCC97H/con, and MHCC97H/HBx-siRNA cells (1×10^7 in a 0.2 ml volume of normal saline) were inoculated subcutaneously into the right flank of the nude mice ($n = 8$). The mice were sacrificed at the end of the sixth week. The lungs were excised, fixed, cut into 4 μm consecutive sections, and then stained with H&E. Each metastatic focus in the lung was identified as it appeared on the same site on consecutive sections. Finally, all determined foci were counted for the histological evaluation of metastasis [Wang et al., 2008].

LUCIFERASE REPORTER ASSAY

The full-length human Twist promoter and its subsequent truncated promoters were generated by nested PCR using genomic DNA isolated from HL-7702 cells. The sequence of all primers used was the same as that in Cheng's study [Cheng et al., 2008]. PCR products were digested with *Kpn*I/*Xho*I and subcloned into pGL3-luciferase vector (Promega Corporation, Madison, WI). The final constructs were confirmed by DNA sequencing. Luciferase activity was measured using a Luciferase Dual Assay Kit (Promega Corporation, Madison, WI) according to the instructions of the manufacturer. Firefly luciferase activity was normalized to that of Renilla luciferase. Luciferase activity was expressed as relative light units or fold change.

CHROMATIN IMMUNOPRECIPITATION (CHIP) ASSAY

ChIP assay was performed essentially as previously described with modifications [Cheng et al., 2008]. Briefly, soluble chromatins were

prepared from a total of 2×10^7 HL-7702, HL-7702/con, HL-7702/HBx2, HL-7702/HBx-siRNA3 or HL-7702/HBx2 with inhibited p-STAT3 cells, respectively. The chromatin solution was diluted with ChIP dilution buffer, then the precleared chromatin solution was divided and utilized in immunoprecipitation assays with either an anti-STAT3 antibody or an anti-IgG antibody. Following multiple washes, the antibody–protein–DNA complex was eluted from the beads. After reversal cross-link incubation at 67°C, protein and RNA were removed by incubation with proteinase K and RNase A at 42°C for 3 h. Purified DNA was subjected to PCR with primers specific for the STAT3-binding sites upstream of the transcriptional start site. The sequences of the PCR primers used are: proximal forward, 5′-GCCAGGTCGTTTTTGAATGG-3′, and reverse, 5′-CGTGCAGGCG-GAAAGTTTGG-3′; distal forward, 5′-TGCCTTTCCCATGGACTGGG-3′, and reverse, 5′-GAGTTCCAAAGGCCAAACCG-3′. The proximal and distal PCR products contain the three proximal and two distal STAT3-binding sites, respectively.

ELECTROPHORETIC MOBILITY BAND-SHIFT ASSAY (EMSA)

The nuclear proteins extracted from HL-7702, HL-7702/con, HL-7702/HBx2, HL-7702/HBx-siRNA3 or HL-7702/HBx2 with inhibited p-STAT3 cells were treated with 0.5 μM STAT3 inhibitor JSI-124 (Merck, Darmstadt, Germany) for 24 h. Approximately 7 μg of nuclear protein was incubated with biotin-labeled Twist and double-stranded oligonucleotides (5′-CTGTGGCCGGCAGGTGAACCCT-CAGCCA-3′). The incubation was performed in a binding buffer for 20 min at room temperature using the EMSA Kit (Viagene Biotech Inc., Tampa, FL) and following the instructions of the manufacturer. The activated Twist was then electroblotted to a nylon membrane and visualized after 6.5% PAGE.

STATISTICAL ANALYSIS

Data from different groups were compared by one-way ANOVA or unpaired two-tailed Student's *t*-test using SPSS 11.0. Statistical significance was accepted at $P < 0.05$.

RESULTS

HBX-SIRNA INHIBITS EMT-RELATED PROTEIN EXPRESSION AND MHCC97H CELL METASTASIS

BEL7404, SMMC7721, HepG2, MHCC97L, and MHCC97H cell lines were examined to ascertain the relationship between HBx and EMT-related proteins in cancer cells. Higher expression of Twist and N-cadherin and lower E-cadherin expression were found in MHCC97H cells (higher HBx expression) compared with those in MHCC97L cells (lower HBx expression) ($P < 0.05$). No obvious changes on the expression of EMT-related molecules were observed in other cell lines (BEL7404, SMMC7721, and HepG2) without HBx expression (Fig. 1A,B). The metastatic potential of these five liver cancer cell lines was detected using Transwell assay. The migrated cells of

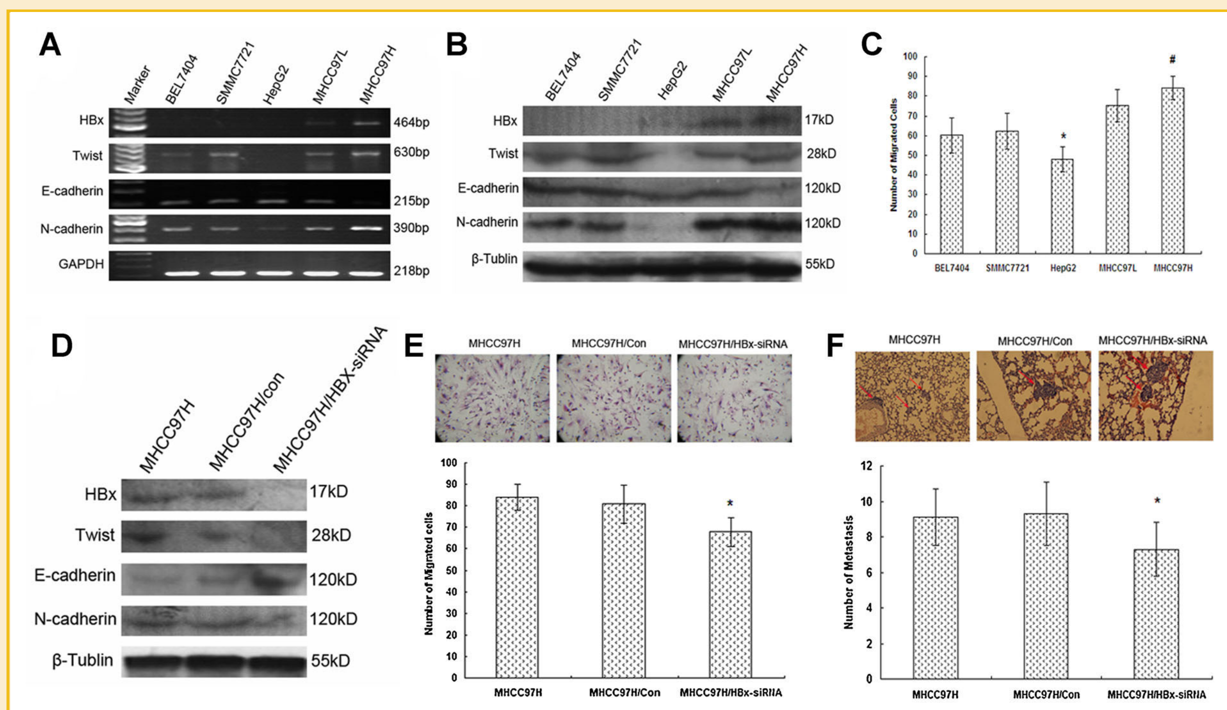

Fig. 1. Effect of HBx-siRNA on EMT-related protein expression and metastasis of MHCC97H cells. A: Representative gel images from three independent studies for mRNA expression of HBx, Twist, E-cadherin, N-cadherin, and GAPDH in five cancer cell lines. B: Representative immunoblots from three independent studies for HBx, Twist, E-cadherin, N-cadherin, and β-tublin protein expressions in five cancer cell lines. C: Cells that migrated to the bottom chamber of the Transwell insert were counted (mean ± SD, n = 5). $^*P < 0.05$ versus other cell lines; $^{\#}P < 0.05$ versus other cell lines, except MHCC97L. D: Representative immunoblots from three independent studies for HBx, Twist, E-cadherin, N-cadherin, and β-tublin in MHCC97H, MHCC97H/con, and MHCC97H/HBx-siRNA cells. E: Representative images of cells that migrated to the bottom of the Transwell culture insert (H&E staining × 100). Group data represent the mean ± SD (n = 5). $^*P < 0.05$ versus control groups. F: Representative images of metastasis in lungs of the nude mice (H&E staining × 100, lung metastasis). Group data represent the mean ± SD (n = 8). $^*P < 0.05$ versus control groups.

MHCC97H were 84.0 ± 6.0/field, a value higher than that of MHCC97L (75.2 ± 8.3/field) and other cell lines ($P < 0.05$) (Fig. 1C). These results indicated that HBx can improve the metastastic potential of liver cancer cells through up-regulation of Twist and change in EMT-related protein expression. Moreover, the down-regulated expression of HBx, Twist, and N-cadherin and the up-regulated expression of E-cadherin were more obvious in MHCC97H/HBx-siRNA cells compared with those in MHCC97H and MHCC97H/con cells (Fig. 1D). The number of migrated cells of MHCC97H/HBx-siRNA (67.8 ± 6.8/field) was significantly less than that of MHCC97H (84.0 ± 6.0/field) and MHCC97H/con (80.8 ± 9.0/field) ($P < 0.05$; Fig. 1E). The mean number of lung metastasis from MHCC97H/HBx-siRNA cells in all eight nude mice was 7.3 ± 1.5, a value less than that of MHCC97H (9.1 ± 1.6) and MHCC97H/con cells (9.3 ± 1.8) ($P < 0.05$; Fig. 1F). These results suggested that the down-regulated expression of HBx inhibits the metastasis of MHCC97H cells in vitro and in vivo.

EMT-RELATED PROTEIN CHANGES OF HL-7702/HBX BASED ON INHIBITION OF TWIST EXPRESSION

The HL-7702 liver cell line was selected as the study object to further clarify the effects of HBx on EMT in human normal liver cells and to ascertain the role of Twist in the process. Figure 2A shows that the expressions of Twist, N-cadherin, and vimentin were higher, whereas that of E-cadherin and β-catenin were lower in HL-7702/HBx1 and HL-7702/HBx2 cells compared with those observed in the control cells. Twist-siRNAs were used to inhibit the Twist expression of HL-7702/HBx2 cells, wherein Twist-siRNA3 (sc-38604c) was the most effective. Compared with the control cells, E-cadherin and β-catenin expressions visibly increased, whereas N-cadherin and vimentin expressions decreased in HL-7702/HBx2 cells treated with Twist-siRNAs (Fig. 2B). These changes further revealed that Twist may be involved in the effect of HBx on the expression of EMT-related proteins.

MOBILITY DECREASE OF HL-7702/HBX LIVER CELLS BASED ON INHIBITION OF TWIST EXPRESSION

The effect of Twist inhibition on cell morphology and mobility of HL-7702/HBx2 was further observed using Twist-siRNA. Figure 3A shows that HL-7702/HBx2 cells treated with Twist-siRNA3 exhibited shorter and fewer pseudopodia compared with those treated with control-siRNA. In addition, the number of migrated HL-7702/HBx cells treated with Twist-siRNA3 was obviously lower than that treated with control-siRNA ($P < 0.05$). However, the difference between HL-7702 and HL-7702/con cells treated with Twist-siRNA and control-siRNA was not significant ($P > 0.05$). Figure 3B shows that wound closure proceeded more slowly in HL-7702/HBx2 cells treated with Twist-siRNA than in those treated with control-siRNA after the scratch test was carried out for 24 h. The difference of wound-healing percentage between HL-7702/HBx2 cells with Twist-siRNA (51.2 ± 4.5%) and with control-siRNA (73.6 ± 2.9%) was significant ($P < 0.01$). These results indicated that Twist plays a more important role in regulating the mobility of HL-7702/HBx cells than HL-7702 cells. Moreover, Twist possibly influences the mobility of HL-7702/HBx cells through HBx interaction.

HBX IN LIVER CELLS INFLUENCE TWIST EXPRESSION THROUGH STAT3 ACTIVATION

HBx does not have a direct effect on DNA, but it influences DNA expression through interaction between proteins [Zhang et al., 2006]. The transcription factor involved is considered to clarify the

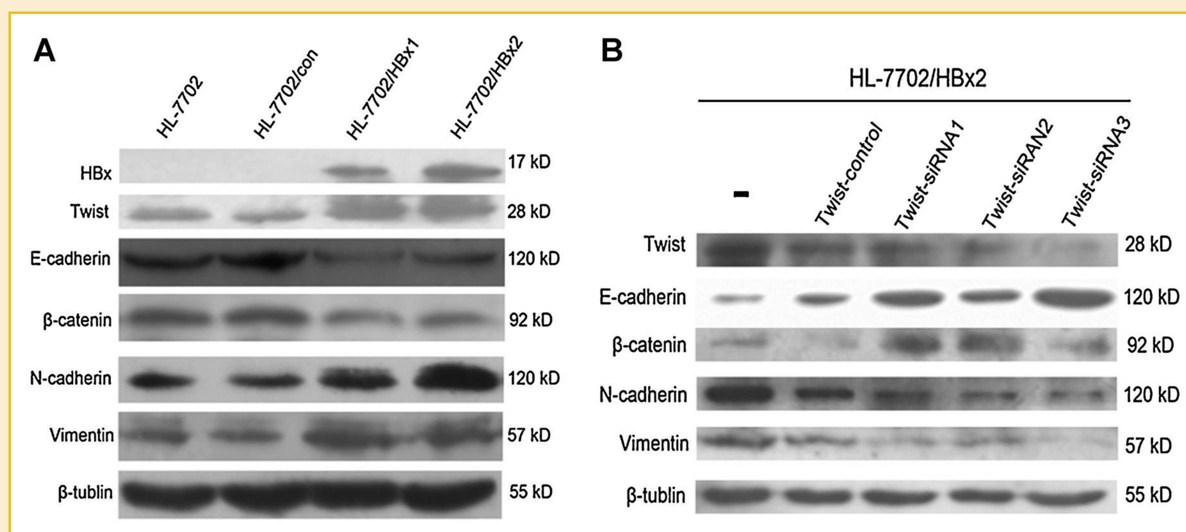

Fig. 2. EMT-related protein changes of HL-7702/HBx cells based on Twist expression inhibition. A: Representative immunoblots from three independent studies for HBx, Twist, epithelial markers (E-cadherin and β-catenin), mesenchymal markers (N-cadherin and vimentin), and β-tublin in HL-7702, HL-7702/con, HL-7702/HBx1, and HL-7702/HBx2 cells. B: Representative immunoblots from three independent studies for Twist, E-cadherin, β-catenin, N-cadherin, vimentin, and β-tublin in HL-7702/HBx2 cells after transfection using Twist-siRNAs and control-siRNA.

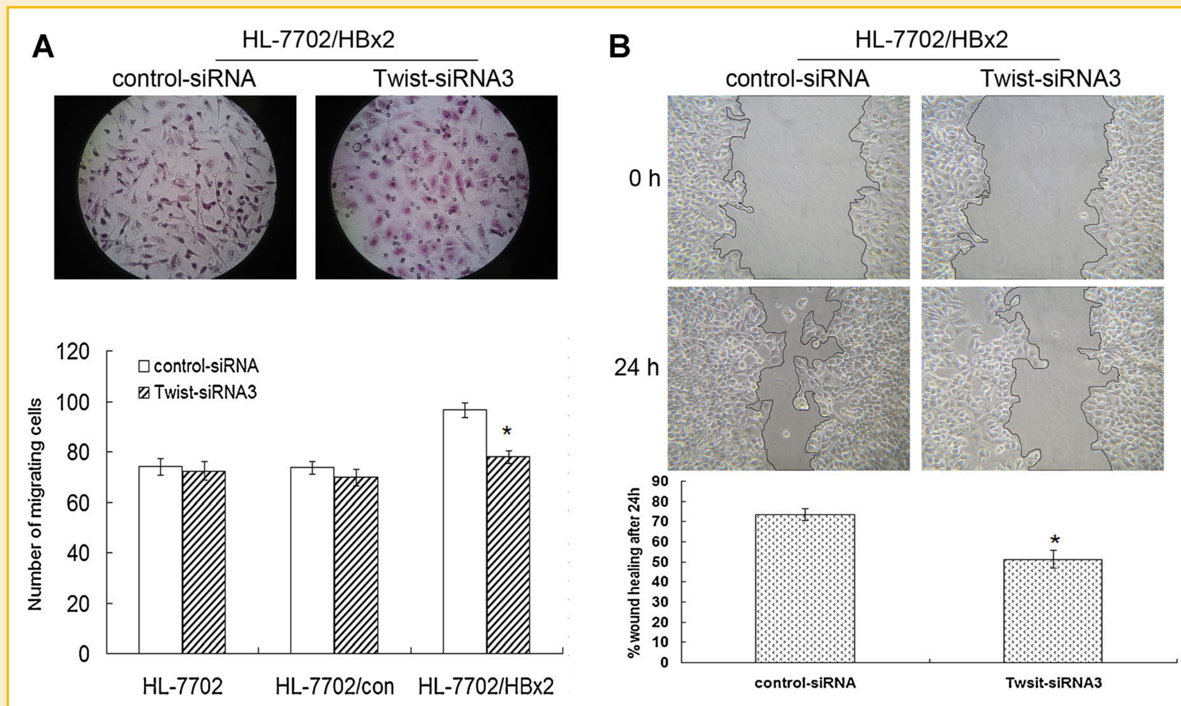

Fig. 3. Mobility decrease of HL-7702/HBx liver cells based on inhibition of Twist expression. A: Representative field of cell morphology (×100) and number of migrated HL-7702/HBx2 cells treated with Twist-siRNA and control-siRNA (mean ± SD, n = 5). $^{*}P < 0.05$ versus treated with control-siRNA. B: Representative field (×100) of wound-healing for HL-7702/HBx2 cells treated with Twist-siRNA and control-siRNA after scratched for 24 h. The graph shows the percentage of wound-healing (mean ± SD, n = 3). $^{*}P < 0.01$ versus treated with control-siRNA.

mechanism of EMT induced by HBx. Among the transcription factors, Twist is crucial in regulating cell EMT [Yang et al., 2004; Lee et al., 2006a]. In their study on breast cancer, Cheng found that a binding locus of STAT exists in the Twist promoter [Cheng et al., 2008]. Therefore, HBx in the liver cells may act on Twist promoter after STAT3 activation to influence Twist expression. Figure 4A shows that the expressions of HBx, p-STAT3, and Twist in HL-7702/HBx2 cells were higher than those in the control cells (HL-7702 and HL-7702/con). The p-STAT3 and Twist levels of HL-7702/HBx2 cells treated with different HBx-siRNAs can be reversed along with the decreased HBx expression in varying degrees. Compared with HL-7702/HBx2 cells, the expressions of HBx, p-STAT3, and Twist in HL-7702/HBx-siRNA3 and HL-7702/HBx-siRNA4 cells were significantly inhibited. These findings indicated that the expression of p-STAT3 is positively correlated with HBx expression and the up-regulation of Twist in HL-7702/HBx cells may be attributed to STAT3 activation.

EMSA assays were performed to determine whether STAT3 can bind with the Twist promoter in vitro. Figure 4B shows that Twist activity in HL-7702/HBx2 cells increased more compared with that in HL-7702 and HL-7702/con cells. However, Twist activity in HL-7702/HBx2 cells decreased when treated with HBx-siRNA (HL-7702/HBx-siRNA3 cells) or STAT3 inhibitor (HL-7702/HBx2 with JSI-124). In summary, our data provided evidence that HBx expression in HL-7702 liver cells can activate Twist gene promoter via STAT3 phosphorylation.

Different lengths of human Twist promoter were cloned into the pGL3-luciferase vector based on the putative human Twist promoter genomic structure that contained five putative sites conforming to the STAT3-binding sequences TT(N4–6)AA to investigate further the molecular mechanism of HBx in liver cells that possibly acts on Twist promoter via STAT3 activation [Haura et al., 2005; Cheng et al., 2008]. Figure 4C shows the PCR amplification results, with the length of Twist promoters presented in decreasing order. Figure 4D demonstrates that the relative luciferase activity (RLA) of HL-7702/HBx cells increased significantly when transfected by pTwist-188Luc, pTwist-452Luc, pTwist-746Luc, and pTwist-970Luc reporter plasmid. Moreover, RLA decreased when HL-7702/HBx cells were treated with STAT3 inhibitor (JSI-124) before transfection of the plasmids. The difference from the control group was statistically significant, indicating that HBx can activate Twist promoter by activating STAT3 (p-STAT3). The Twist promoter region close to the second STAT3 binding sequence (TTCCTATAA, −108 to −116 bp) was the first locus, where the activation of STAT3 was caused by HBx-promoted Twist expression, whereas the first and third sites did not facilitate the promoter activity. In addition, the RLA of HL-7702/HBx cells transfected by pTwist-970Luc reporter plasmid showed the highest value. Therefore, the fifth STAT3 binding sequence on pTwist-970Luc may be another important locus for promoting Twist expression.

As shown in Figure 4E, ChIP analysis of nuclei derived from HL-7702/HBx cells revealed two dominant bands, 171 and 248 bp,

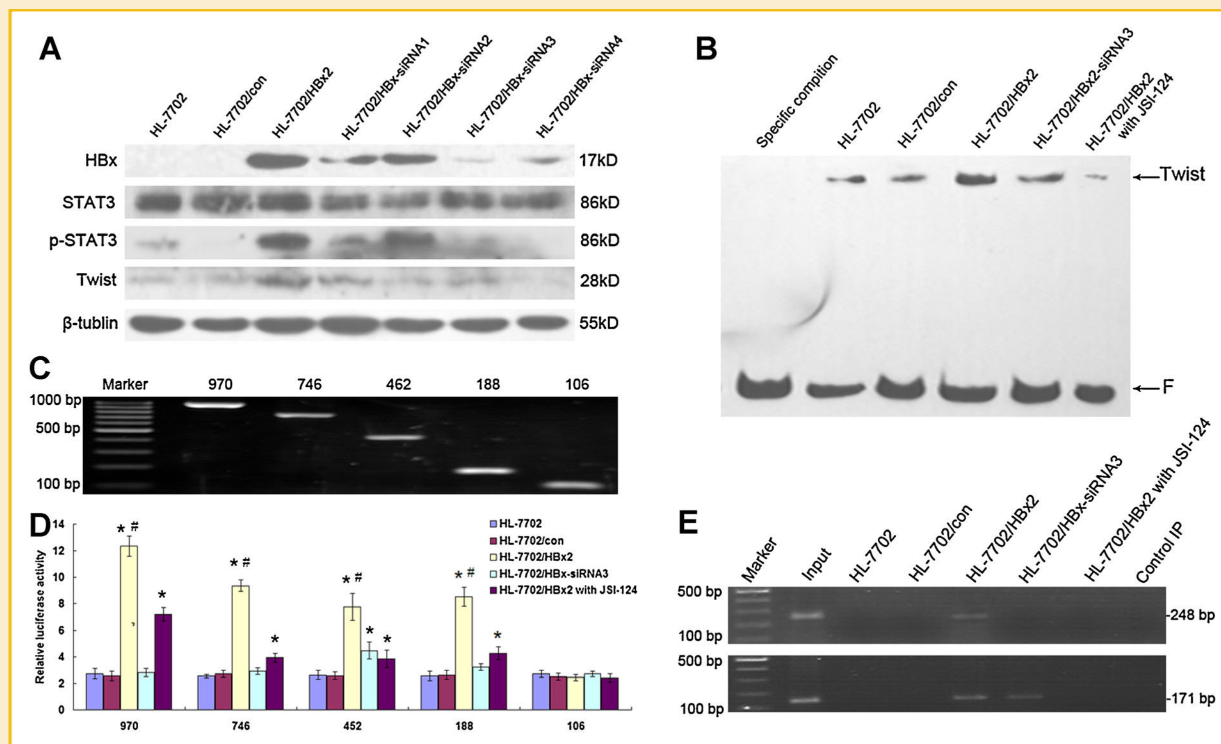

Fig. 4. HBx in liver cells influence Twist expression through STAT3 activation. A: Representative immunoblots from three independent studies for HBx, Twist, STAT, p-STAT, and β-tublin in HL-7702, HL-7702/con, HL-7702/HBx2, and HL-7702/HBx-siRNA1 in four cells. B: EMSA assay showed that activated Twist electroblotted to the nylon membrane was visualized by specific HRP-labeled probe after 6.5% PAGE for nuclear protein (F zone indicates non-binded free probe). C: PCR showed the fragments of Twist promoter with different lengths (106, 188, 452, 746, and 970 bp) but with a common 3′ end, which were used to establish luciferase reporter vectors. D: Relative luciferase activity of HL-7702, HL-7702/con, HL-7702/HBx2, HL-7702/HBx-siRNA3 or HL-7702/HBx2 with JSI-124 cells transfected by vectors of Twist promoter series (mean ± SD, n = 3). $^{*}P < 0.05$ versus HL-7702 or HL-7702/con cells. E: PCR analysis of the protein–chromatin immunoprecipitates showed the fragments of STAT3 binding to the Twist promoter in HL-7702, HL-7702/con, HL-7702/HBx2, HL-7702/HBx-siRNA3 or HL-7702/HBx2 with JSI-124 cells. Reaction controls included immunoprecipitations performed by using a nonspecific IgG monoclonal antibody (Cont IP) and PCR was performed by using whole cell genomic DNA (input). A representative example of three independent experiments was shown.

which containing possible STAT3 binding sites of Twist promoter DNA. Only 171 bp band was showed in HL-7702/HBx-siRNA3 cells and no bands were evident in the other cells and the control IgG immunoprecipitates. These results further provide evidence that the proximal region contains the main STAT3-binding site in the Twist promoter, where STAT3 activation was caused by HBx expression.

DISCUSSION

The occurrence of HBx is essential in the development of HCC with HBV infection. For example, HBx can promote invasion and metastasis of liver cancer by changing the proteolytic enzymes secreted by liver cancer cells [Chan et al., 2004]. Recent studies suggested that EMT change may be also an important influence factor: HBx promotes the activation of STAT5b to induce EMT, thereby facilitating the invasion of liver cancer cells [Lee et al., 2006a]; The cytoskeleton actin and cadherin complexes are damaged and cell adherence by Src dependence is destroyed with the presence of HBx [Lara-Pezzi et al., 2001].

EMT is a key event in the tumor invasion process whereby epithelial cell layers lose polarity and cell–cell contacts, and undergo dramatic remodeling of the cytoskeleton [Yang et al., 2004]. The hallmark of EMT are loss of E-cadherin expression (a central component of cell–cell adhesion junctions in the maintenance of cell polarity and environment [Hirohashi, 1998]) and cadherin transformation (increased N-cadherin expression [Hazan et al., 2004; Yang et al., 2007]). The transcription factors, including Twist, Snail, and Slug, were reported the important regulatory factors involved in tumor-induced EMT [Lopez et al., 2009]. Particularly, as a molecule with a helix–loop–helix structure, Twist draw more attention in the study of early metastasis of cancer [Yang et al., 2004]. Inhibition of gene expression by Twist activation is realized by the targeted integration of the conserved E-box sequence (–CANNTG–) of promoters (e.g., E-cadherin) [Lopez et al., 2009]. In addition, vimentin and β-catenin, the important associated molecules with Twist, also participate in the EMT change. Vimentin (an intermediate sized protein) is the key characteristic of mesenchymal cell phenotype [Leonard et al., 2008] and, moreover, an increase in vimentin/E-cadherin ratio has been considered to be the first indicator of Twist-induced EMT [Kang and Massague,

2004]. β-Catenin is also critical for the maintenance of EMT associated cancer [Li and Zhou, 2011]. Interestingly, we found in this study that HBx expression affected the EMT-related protein expression and the cell mobility of liver cells in vitro and in vivo, and these indicators, including E-cadherin, β-catenin, N-cadherin, and vimentin, exhibited reversed expression after HBx or Twist inhibition. These findings significantly indicated that HBx can promote the occurrence of EMT by activating Twist, thereby causing diminished adherence between cells and easy mobility. Unlike other researches on the relationship between HCC and Twist [Lee et al., 2006b; Matsuo et al., 2009], this study took into account the factor of HBV infection, and provided a new explanation of the effects of HBx on liver cancer cell invasion and metastasis.

Clarifying the molecular mechanism of HBx in the promotion of invasion and metastasis of liver cancer cells is essential. The detailed mechanism of the influence of HBx on Twist expression has not been reported to date; thus, a preliminary exploration was conducted in this study. HBx protein is expressed in the cytoplasm, so it can combine with a transfer factor to activate transcription factors. The latter migrates to the cell nucleus to bind the promoter *cis*-element of target molecules and influence its expression [Zhang et al., 2006]. The transcription factors activated by HBx include STAT [Waris et al., 2001], NF-κB [Waris et al., 2001], AP-1 [Tanaka et al., 2006], CREB [Cougot et al., 2007], C/EBP [Yue et al., 2011], HIF-1α [Yoo et al., 2004], etc. Twist promoters contain several transcription factor-binding *cis*-elements, such as STAT [Chan et al., 2004] and HIF-1α-binding locus [Sun et al., 2009]. The study of HIF-1α requires anoxic conditions, so we presumed that the effect of HBx on Twist expression by activating STAT3 is an important pathway revealed by in vitro experiments without anoxic conditions. The results confirmed our hypothesis. The p-STAT3 expression, Twist expression and activity increased after HBx transduction into the HL-7702 cells. Different p-STAT3 expressions with different degrees resulted together with the inhibition of HBx. A similar result was found in the activation test of Twist promoters, particularly on their effect on STAT3-binding locus on the Twist promoter region, which was observed after the second binding locus from the starting point. HBx, which activates STAT3 and upregulates Twist expression, is an important factor that promotes liver cell EMT.

Further investigations are necessary to ascertain whether other signal molecules are involved in the regulation of Twist protein by HBx. In conclusion, Twist is essential in regulating the mobility of liver cells with HBx expression. In addition, Twist as a target molecule for inhibiting invasion and metastasis of liver cancer with HBV infection, presents potential application value.

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