Arch Virol (2013) 158:1773–1781
DOI 10.1007/s00705-013-1655-1

BRIEF REPORT

HBx and HBs regulate RhoC expression by upregulating transcription factor Ets-1

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Received: 9 December 2012 / Accepted: 1 February 2013 / Published online: 9 March 2013

Abstract The available evidence suggests that HBV proteins play an important role in the development of hepatocellular carcinoma (HCC). RhoC, a member of the Rho subfamily of the Ras superfamily of homologous genes, had been implicated in tumorigenesis and tumor progression. In a previous study, we demonstrated that HBx and HBs could up-regulate RhoC expression by enhancing its promoter activity. However, the specific mechanisms remain unclear. Here, we demonstrate that overexpression of Ets-1 results in upregulation of RhoC promoter activity and mRNA and protein levels. Expression of transcription factor Ets-1 was significantly higher in HepG2.2.15 cells than that in HepG2 cells. Meanwhile, infection of HepG2 cells with an HBV-adenovirus recombinant virus led to up-regulation of Ets-1. Of the four HBV proteins, HBx and HBs, could increase expression of Ets-1, which consequently contributed to the upregulation of RhoC. These findings might provide a novel insight into HBV-induced HCC metastasis.

Keywords HCC · HBV · HBx · HBs · Ets-1 · RhoC · Promoter

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Introduction

Hepatocellular carcinoma (HCC) is the most common malignancy and the third leading cause of cancer mortality worldwide [1, 2]. Chronic hepatitis B virus infection is the most prominent cause, which accounts for 55 % of cases worldwide and 80 % or more of those in the eastern Pacific region and sub-Saharan Africa [3–5]. However, the mechanism by which HBV contributes to the development of HCC remains unclear. Now, increasing evidence suggests that HBx and HBs protein play a pathogenic role in HCC. Especially, HBx is regarded as a promiscuous trans-activator of viral and cellular promoters and enhancers [6].

RhoC is a small (21-25 kDa) G-protein in the Ras superfamily of guanosine triphosphatases (GTPases) with intrinsic GTPase activity [7]. Activation of Rho protein leads to the assembly of the actin-myosin contractile filaments into focal adhesion complexes, which finally leads to cell polarity and facilitates motility [8]. Increasing evidence suggests that RhoC expression is associated with highly aggressive cancers where it promotes metastasis [9–13]. Consistently, genomic analysis has revealed that RhoC expression levels during HCC development correlate significantly with tumor vascular invasion, the number of tumor nodes, and differentiation status [14]. However, how RhoC is regulated by HBV in the process of HCC development remains to be explained.

Recently, Bellovin et al. [15] found that RhoC transcription could be regulated by transcription factor Ets-1 during epithelial-mesenchymal transition (EMT) in colon carcinoma. Also, Spangler et al. showed that Ets-1 is an important modulator of tumor progression in melanoma [16]. Analysis of the RhoC promoter revealed multiple putative binding sites for the Ets-1 transcription factor

(GGAA/T) [17]. Interestingly, several studies showed that Ets-1 is significantly overexpressed in HCC tissue compared with noncancerous liver tissue [18, 19]. Therefore, we investigated whether the HBV/HBx/HBs/Ets-1/RhoC signaling pathway is one of the mechanisms that contribute to invasion of HCC.

Our previous study revealed that HBx and HBs could upregulate RhoC expression by enhancing its promoter activity [20]. In this study, we found that HBx and HBs can upregulate Ets-1 mRNA expression, which led to enhancement of RhoC promoter activity and mRNA and protein expression. Moreover, gene silencing of HBx and HBs in HepG2.2.15 cells resulted in significant downregulation of Ets-1 and RhoC. Our study might provide a novel mechanism of HBV-related HCC invasion and possible molecular targets for therapy of HCC malignant metastasis.

Materials and methods

Materials

Human hepatocarcinoma cell lines HepG2 and HepG2.2.15 were purchased from ATCC (American Type Culture Collection, USA) and Shanghai Second Military Medical University, respectively, and kept by our laboratory. The pRL-TK plasmid and the dual-luciferase reporter assay system were purchased from Promega (USA). The pGL3-Basic plasmid and the transfection reagent Lipofectamine 2000™ were bought from Invitrogen (USA). Ad-HBV adenovirus and its control Adeasy-GFP adenovirus were constructed in our laboratory.

Construction of plasmids

The sequences of the genomic regions flanking of the RhoC gene promoter were obtained from the Eukaryotic Promoter Database (http://epd.vital-it.ch/) and Regulatory Element Database (http://rulai.cshl.edu/cgi-bin/TRED/tred.cgi?process=home). The RhoC promoter construct pGL3-RhoC-P was generated by PCR using HepG2 cell genomic DNA as a template. The primer sequences were as follows: F, 5′-TGAGAGCTCCCTAGGAACAAGAGGGTG-3′; R, 5′-ATA CTCGAGTCGGACCTCAGGAGGCCA-3′ (*Sac*I and *Xho*I restriction endonuclease sites are underlined). pCMV-Sport6-HBx pCMV-Sport6-HBs pCMV-Sport6-HBc and pCMV-Sport6-HBp were constructed by our laboratory, and their correct expression was confirmed in HepG2 cells (data not shown). pcDNA3.1 and pcDNA-Ets-1 were donated by Dr. Youquan Pu (Chongqing Medical University, China).

RNA interference

siRNAs were designed to target various regions of Ets-1 and were synthesized by Invitrogen (si-NC as control). Their sequences were as follows: Ets-1-siRNA1, 5′-GC UUCGACUCAGAGGACUATT-3′; Ets-1-siRNA2, 5′-CC AGCUAUGGCAGUUUCUUTT-3′; Ets-1-siRNA3, 5′-G CAGUUUCUUCUGGAAUUATT-3′. shRNA for HBs and HBx was generated in our laboratory [20]. HepG2.2.15 cells were seeded at a density of 60 % and transfected with Ets-1 siRNAs and control siRNA (si-NC) using Lipofectamine™ 2000 (Invitrogen). Forty-eight hours after transfection, the total RNAs and proteins were extracted, and RT-PCR, qRT-PCR and western blot were performed to check the expression levels of Ets-1 and RhoC.

Cell culture

HepG2 and HepG2.2.15 cells were cultured in MEM (Hyclone) with 10 % fetal bovine serum (GIBCO), penicillin (100 units/mL), streptomycin (100 μg/mL), and glutamine (5 mmol/L) at 37 °C in a 5 % CO_2 atmosphere.

Transient transfection

Cells were seeded into 6-wells plate at approximately 60 % confluence and grown overnight. To investigate the regulatory effect of the four major HBV proteins on transcription factor Ets-1, HepG2 cells were transfected with 4 μg of pCMV-Sport6-HBx, pCMV-Sport6-HBc, pCMV-Sport6-HBp or pCMV-Sport6-HBs (with pCMV-Sport6 as a negative control) using Lipofectamine™ 2000 (Invitrogen). In addition, HepG2.2.15 cells were transfected with 4 μg of HBx or HBs shRNA (with pGensil-scramble as a negative control). To investigate the regulatory effect of transcription factor Ets-1 on the RhoC promoter, cells were co-transfected with 0.3 μg of a RhoC promoter luciferase reporter construct (pGL3-RhoC-P) and 0.5 μg of the expression plasmid pcDNA-Ets-1, which contains the gene for the transcription factor Ets-1 (the plasmid pcDNA3.1 was used as a negative control). To investigate the regulatory effect of transcription factor Ets-1 on RhoC mRNA expression, cells were transfected with 4 μg of the expression plasmid pcDNA-Ets-1, which contains the gene for transcription factor Ets-1 (with the pcDNA3.1 plasmid as a negative control).

Luciferase assays

The activity of the RhoC promoter was measured using the Dual-Luciferase Reporter Assay system. Cells were collected, lysed with 1 × passive lysis buffer (PLB), and then gently shaken for 15 min at room temperature.

Luminescence was measured using a Turner TD20/20 luminometer. Fifty microliters of Luciferase Assay Reagent II and 20 µl of PLB lysate were added successively to the tubes, and firefly luciferase activity was then measured. The reaction was stopped by adding 50 µl of Stop & Glo Reagent, which activated the *Renilla* luciferase. The corresponding promoter activity was expressed as the ratio of the firefly luciferase activity to the *Renilla* luciferase activity. Each experiment was repeated at least three times.

Total RNA isolation, RT-PCR and quantitative real-time PCR

Forty-eight hours after transfection, total RNA was isolated using TRIzol Reagent (Invitrogen, USA), and 1 µg of total RNA was used to synthesize cDNA using a PrimeScript RT Reagent Kit (Takara, Japan) following the manufacturer's instructions. The amplification was performed using GoTaq Green Master Mix (Promega) for 28 cycles of initial denaturation at 94 °C for 3 min, denaturation at 94 °C for 30 s, annealing at 59 °C for 30 s, extension at 72 °C for 45 s and a final extension at 72 °C for 5 min. The PCR products were analyzed on a 1 % agarose gel using a GELDOC 2000 system (Bio-Rad). The primers used were as follows: for β-actin, F, 5′-GTGGATCAGCAAGCAG GAGT-3′; R, 5′-TGTGTGGACTTGGGAGAGGA-3′; for Ets-1, F, 5′-ACGATAGTTGTGATCGCCTC-3′; R, 5′-TT GAATTCCCAGCCATCTCC-3′; for HBx, F, 5′-CCCGTC TGTGCCTTCTCATC-3′; R, 5′-CCCAACTCCTCCCAGT CTTT-3′; for HBs, F, 5′-ATGGAGAACATCACATCA GG-3′; R, 5′-GCAATGTATACCCAGAGACAAAA-3. For quantitative real-time PCR, total RNAs from transfected cells were prepared as described above. One microliter of cDNA (diluted tenfold), 10 µl of SYBR green mix and 1 µl of forward and reverse primer (10 µM) in a total of 20 µl were applied. The following PCR conditions were used: one cycle of 90 °C for 10 min and 40 cycles of 94 °C for 15 s, followed by the primer-specific annealing temperature for 40 s, and 72 °C for 60 s. The primers used were as follows: for RhoC, F, 5′-TCCTCATGTGCTT CTCCATC-3′; R, 5′-TCCTGCTTCATCTTGGCCAG-3′; for β-actin, F, 5′-CCTTCTACAATGAGCTGCGT-3′; R, 5′-CCTGGATAGCAACGTACATG-3′. Relative changes in gene expression were calculated using the $2^{-\Delta\Delta CT}$ method [21].

Western blot analysis

For western blot analysis, HepG2 cells were infected with Adeasy-HBV (Adeasy-GFP adenovirus as negative control). HepG2.2.15 cells were transfected with 8 µg of shRNA of HBx and HBs. Forty-eight hours after transfection, cells were collected and lysed in protein lysis buffer. A BCA Protein Assay Kit (Beyotime, China) was used to determine the protein concentration. Equal amounts of total protein were separated by 12 % SDS-PAGE and then transferred to a PVDF membrane. The membrane was blocked in 5 % nonfat dry milk in TBST (Tris-HCl-buffered saline supplemented with 0.5 % Tween 20) for 3 h followed by primary antibody (MoAb anti-ETS1, diluted 1:500; MoAb β-actin, diluted 1:6,000; MoAb RhoC, diluted 1:600) overnight. The membrane was then incubated with secondary antibody conjugated with HRP (diluted 1:6,000) and visualized using an ECL™ chemiluminescence detection system (Pierce, USA).

Statistical analysis

All statistical analysis was carried out using Student's *t*-test. The mean ± SD is reported. Significance was defined as $p < 0.05$.

Results

Analysis of transcription factor binding sites in RhoC promoter sequences

In order to search for transcription factor binding sites in RhoC promoter sequences, the programs TFSEARCH (http://www.cbrc.jp/research/db/TFSEARCH.html) and Gene-Regulation (http://www.gene-regulation.com/pub/programs.html#alibaba2) were used. This resulted in the identification of multiple putative binding sites for Ets-1 (GGAA/T) (Fig. 1). The *in silico* analysis indicated that transcription factor Ets-1 may be involved in regulating the expression of RhoC.

The expression of RhoC is up-regulated by transcription factor Ets-1

To investigate whether transcription factor Ets-1 regulates RhoC expression in hepatoma cells, HepG2 cells were cotransfected with pcDNA-Ets-1 (expression plasmid of transcription factor Ets-1) and pGL3-RhoC-P (RhoC

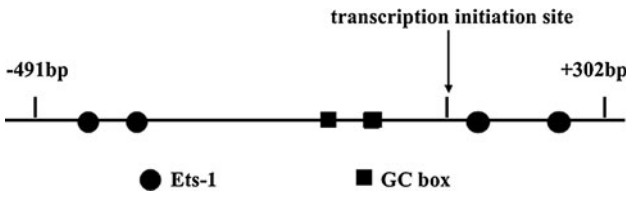

Fig. 1 Transcription factor Ets-1 binding sites in the RhoC promoter region. Multiple putative binding sites for the transcription factor Ets-1 were found in the RhoC promoter using computer software

promoter luciferase reporter construct), and RhoC promoter activity was measured using a luciferase reporter system. We found that the promoter activity of RhoC could be significantly enhanced by Ets-1 (Fig. 2A). Furthermore, using real-time PCR and western blot analysis, we found that overexpression of Ets-1 led to obvious upregulation of RhoC compared to its control (Fig. 2B, C). In parallel, three different si-RNAs were used to confirm our conclusion, and this resulted in a decrease in expression of RhoC together with Ets-1, at both the mRNA and protein level (Fig. 2D, E, F). These data suggest that the expression of RhoC is positively regulated by transcription factor Ets-1.

The expression of Ets-1 is significantly upregulated by HBV

Our previous data showed that HBV can upregulate RhoC promoter activity [20], therefore, we speculated whether there was some kind of relationship between HBV and transcription factor Ets-1. To investigate the effect of HBV on transcription factor Ets-1, the differences in expression levels of Ets-1 between HepG2 and HepG2.2.15 (stable HBV-expressing cells) cells was measured by both RT-PCR and western blot analysis. As expected, expression of transcription factor Ets-1 was significantly higher in

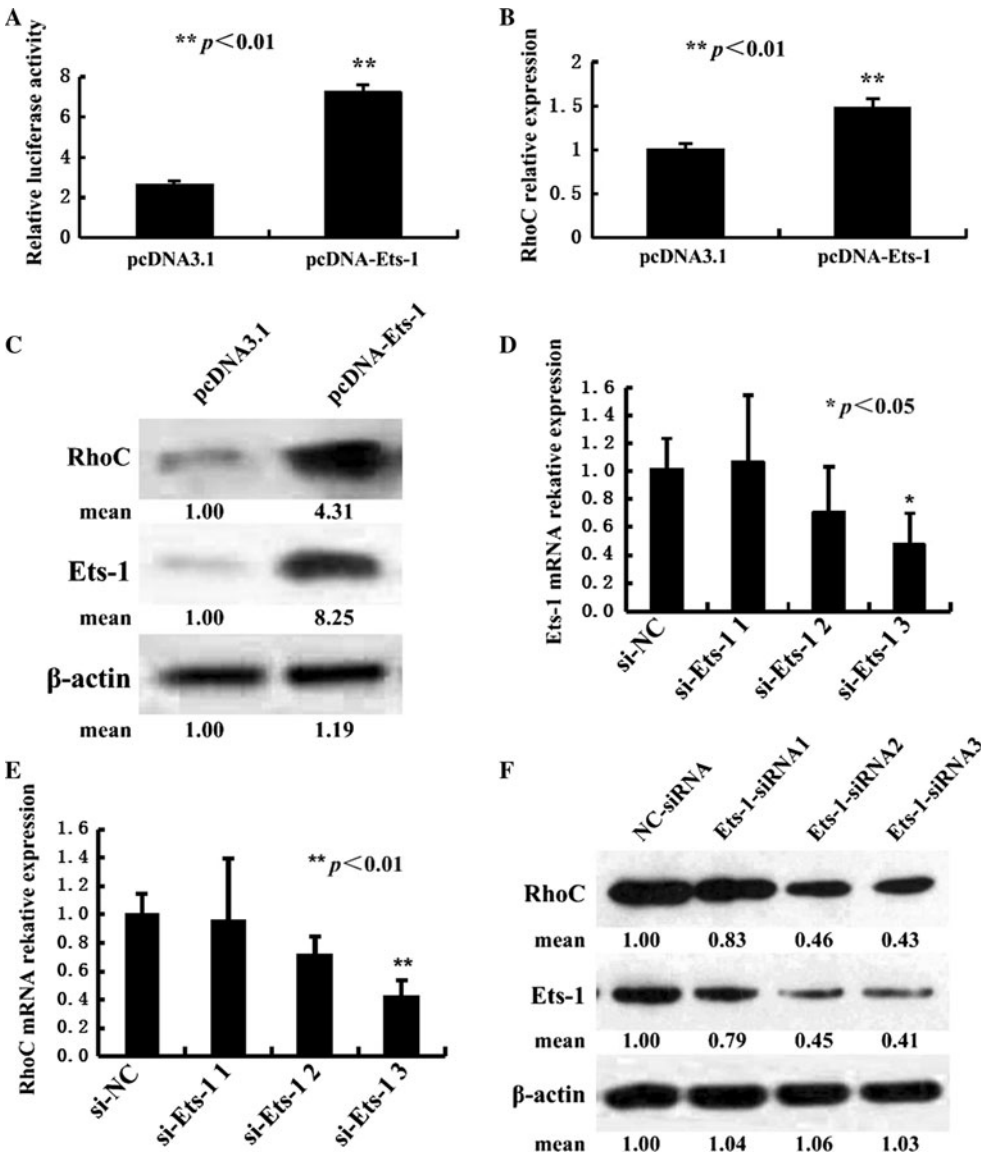

Fig. 2 Expression of RhoC could be up-regulated by Ets-1. (**A**) HepG2 cells and SMMC-7721 cells were co-transfected with a RhoC promoter luciferase reporter plasmid (pGL3-RhoC-P) and expression plasmid containing the gene for transcription factor Ets-1 (pcDNA-Ets-1). The relative activity of the RhoC promoter was measured using a luciferase reporter system. (**B**) After HepG2 cells were transfected with pcDNA-Ets-1, the relative expression of RhoC mRNA was measured by real-time PCR. The data are shown as mean ± SD,*$p < 0.01$ compared to the control (pcDNA3.1). (**C**) After transfection of SMMC7721 cells with pcDNA-Ets-1, a western blot was performed to analyze the expression of RhoC protein. (**D**) Three different siRNA were used to block Ets-1 expression, and qRT-PCR was then carried out to check the effective interference. (**E**) Differences in expression of RhoC caused by the Ets-1 block were measured by qRT-PCR. (**F**) Differences in expression of Ets-1 and RhoC were shown by western blot

HepG2.2.15 cells than that in HepG2 cells (Fig. 3A, B). An HBV-adenovirus recombinant (Ad-HBV) was used to confirm our finding. Effective infection with HBV was confirmed by measuring the fluorescence signal (Fig. 3C) and the expression of HBsAg and HBeAg was measured by ELISA (Fig. 3D). RT-PCR, qRT-PCR and western blot were also performed to analyze the differential expression of Ets-1. As shown in Fig. 3E, F and G, the level of expression of Ets-1 was higher in Ad-HBV-infected HepG2 cells than that in the GFP-adenovirus-infected control. Taken together, these results suggest that transcription factor Ets-1 can be upregulated by HBV.

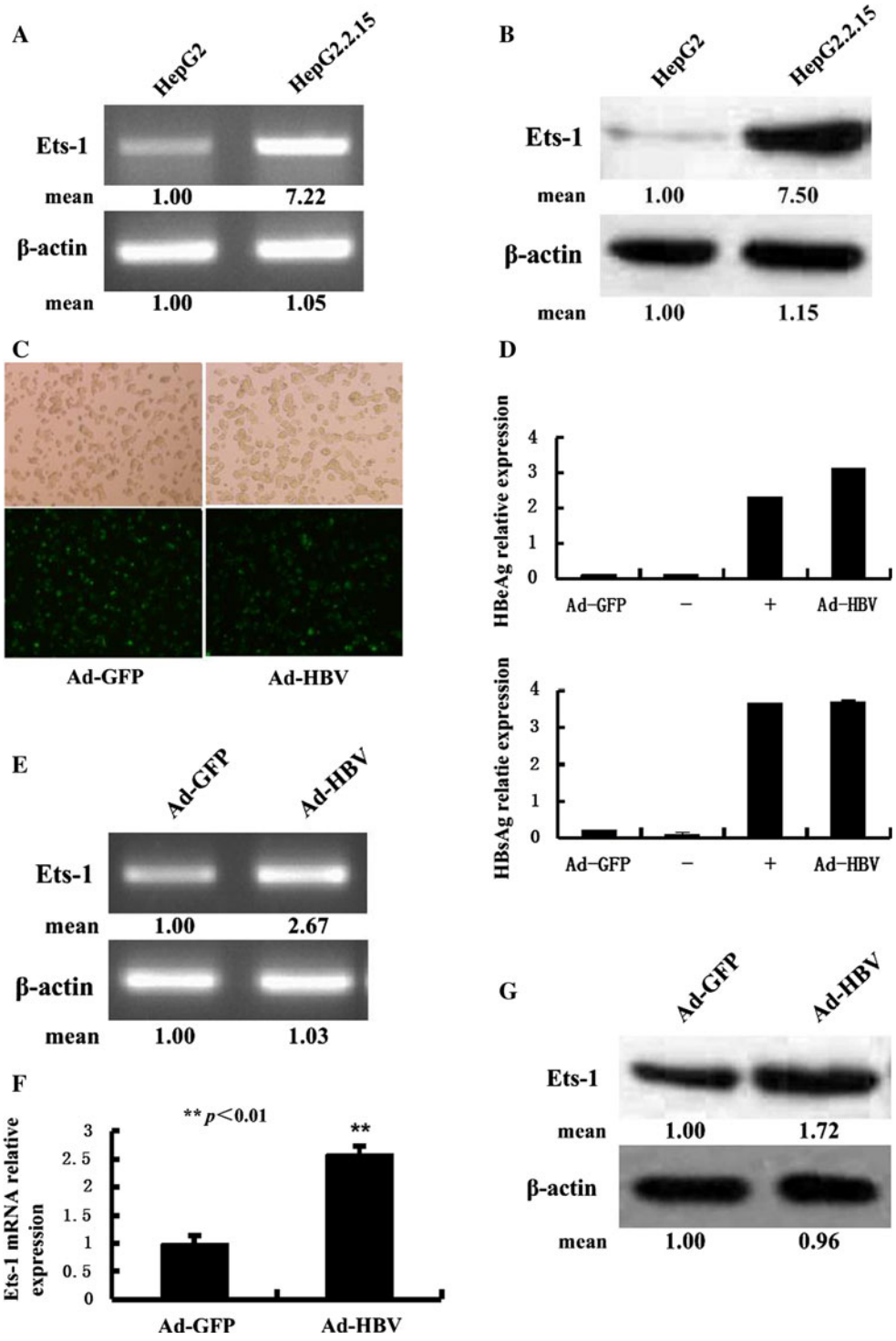

Fig. 3 Expression of Ets-1 is positively modulated by HBV. (**A**) RT-PCR analysis was performed to analyze Est-1 mRNA expression in HepG2.2.15 and HepG2 cells. β-actin was used as an internal quantitative control. (**B**) Western blot analysis was performed to analyze Est-1 protein expression in HepG2.2.15 and HepG2 cells. β-actin was used as an internal quantitative control. (**C**) Efficiency of Ad-HBV infection shown by fluorescence. (**D**) Expression of HBV measured by ELISA. "−" and "+" represent negative and positive controls, respectively. (**E**) (**F**) (**G**) Expression of Ets-1 measured by RT-PCR, qRT-PCR and western blot. β-actin was used as an internal quantitative control

HBx and HBs play a major role in increasing Ets-1 expression

HBV has four open reading frames encoding four major proteins, HBs, HBc, HBp and HBx. To reveal which viral protein contributes to upregulation of Ets-1, SMMC7721 cells were transfected with expression plasmids containing genes for four HBV proteins (pCMV-Sport6-HBx, pCMV-Sport6-HBc, pCMV-Sport6-HBp and pCMV -Sport6-HBs). RT-PCR and qRT-PCR were performed to detect expression of Ets-1, and it was found that overexpression of HBx and HBs resulted in increased expression of Ets-1 (Fig. 4A, B). Furthermore, to confirm this result, HepG2.2.15 cells were transiently transfected with shRNAs of HBx and HBs. shRNA2 of HBx and shRNA2 of HBs, which have a stronger inhibitory function than shRNA1 were used for western blot. After confirming the efficiency of transfection and interference by fluorescence and RT-PCR (Fig. 4C, D), RT-PCR, qRT-PCR and western blot were performed to analyze the differential expression of transcription factor Ets-1. It was found that the expression of Ets-1 mRNA and protein was reduced when HBx and HBs were inhibited (Fig. 4D, E, F). Also, the expression of RhoC protein was inhibited when HBx and HBs were knocked down by shRNAs (Fig. 4F). Taken together, these data indicate that Ets-1 can be modulated by HBx and HBs.

Discussion

The leading cause of death in cancer patients is not the primary tumor but its metastasis [22], as is the case with HCC. The metastatic cascade is a complex and multistage process involving modulation of cell phenotype, cell migration, and dynamic homeotypic and heterotypic cell-cell interactions [23]. Migration and invasion of tumor cells is also promoted by the loss of interaction of E-cadherin with the cytoskeleton and subsequent changes in the activities of Rho family GTPases [9, 24]. Loss or weakening of cell-cell junctions is required for the migration of epithelial cells. Interestingly, Wang et al. have suggested that suppression of RhoC expression results in inhibition of invasion and migration *in vitro*, and knockdown of the RhoC expression in an HCC metastatic mouse model resulted in inhibition of metastasis *in vivo* [12]. Several migration-related genes that contribute to the metastasis process can be modulated by HBx, such as membrane-type matrix metalloproteinase 1 (MT1-MMP) [25], MMP-9 [26], heat shock protein 90 (HSP90) [27], and RhoC [20].

Almost all HBV-associated HCCs studied so far harbor chromosomally integrated HBV-DNA [28]. Integrated HBV-DNA could encode two types of transcriptional activators: the already well-studied HBx [29] and the PreS2 activators LHBs (large hepatitis B virus surface protein) and MHBs^t (C-terminally truncated middle-size surface proteins) [30, 31]. The PreS2 domain binds PKC alpha/beta and triggers PKC-dependent activation of the c-Raf-1/MAP2-kinase signal transduction cascade, resulting in an activation of transcription factors such as AP-1 and NF-kB [32]. HBx protein causes transcriptional activation by its interaction with nuclear transcription factors and modulation of cytoplasmic signal transduction pathways, including the Ras, Raf, c-jun, MAPK, NF-κB, Jak-Stat,FAK, and protein kinase C pathways, as well as Src-dependent and phosphatiylinositol-3 kinase signaling cascades [33]. Importantly, emerging evidence suggests that HBx functions at the transcription level. For example, HBx protein induces expression of transcription factor AP-1 by activation of extracellular signal-regulated and c-Jun N-terminal mitogen-activated protein kinases [33]. HBx also augments the DNA binding activity of the phosphorylated form of Sp1 in HepG2 cells [34]. HBx and Mst may contribute to the pathogenicity of chronic hepatitis B and have been suggested to promote hepatocyte transformation via upregulation of cellular proto-oncogenes. Therefore, we hypothesized that the regulation of the RhoC promoter by HBx and HBs is mediated by co-activation with other transcription factors. In this study, we demonstrated that transcription factor Ets-1 can be upregulated by HBV. Both HBx and HBs proteins play a major role in this process (possibly functioning as transcriptional activators). However, evidence has indicated that the DNA-binding activity of many transcription factors is regulated at the level of phosphorylation, and some transcription factors can be modulated both at the total protein level and by phosphorylation, such as SP1 [34], Ets-1 [35, 36]. Our study revealed that transcription factors can be regulated not only at the epigenetic level but can also be directly modulated by pathogenic factors at the expression level.

Why was Ets-1 selected as the subject of this study? Though there were some other transcription factors found in the promoter region, no obvious difference was found by promoter relative activity assay (data not shown), i.e. SP1, NF-κB, YY1, etc. Ets-1 plays an important role in angiogenesis [37], and its expression varies positively with different types of cancers with metastatic potential, including prostate [38], gall bladder [39], breast [40], lung [41] and esophageal cancers [42]. Ghosh et al. reported that the Ets-1 transcription factor contributes to VEGF-mediated regulation of expression and function of different MMPs in SKOV-3 cells [35]. Ito et al. [19] studied the clinical significance of Ets-1 in human hepatocellular carcinoma (HCC) by using immunohistochemical staining methods. Expression of Ets-1 was scarcely detected in normal liver but was markedly enhanced in noncancerous lesions adjacent to HCC lesions [19]. Ozaki et al. [18] suggested that Ets-1 is upregulated and

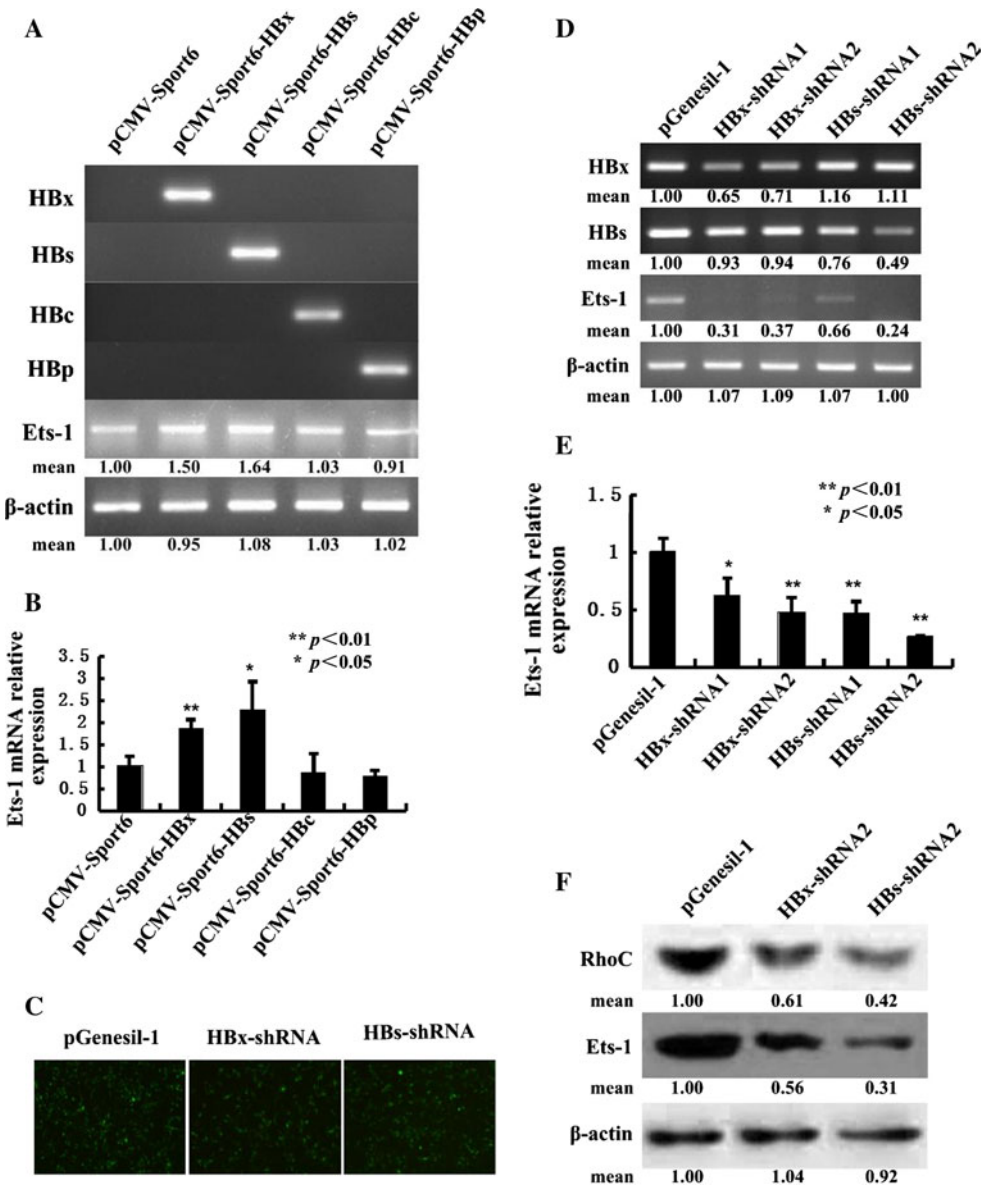

Fig. 4 Upregulation of Ets-1 expression by HBx and HBs (**A**) (**B**) SMMC-7721 cells were transfected with pCMV-sport6-HBx, pCMV-sport6-HBs, pCMV-sport6-HBc and pCMV-sport6-HBp, the expression of four HBV proteins was measured by RT-PCR, and differences in expression of Ets-1 were detected by RT-PCR and qRT-PCR. (C) HepG2.2.15 cells were transfected with shRNAs of HBx and HBs, and the transfection efficiency was measured using GFP fluorescence. (**D**) (**E**) The interference efficiency and differential expression of transcription factor Ets-1, analyzed by RT-PCR and qRT-PCR. (**F**) Forty-eight hours after interference of HBx and HBs in HepG2.2.15 cells, expression of Ets-1 and RhoC was detected by western blot. β-actin was used as an internal quantitative control

involved in the overexpression of MMP-7 in human HCC and may contribute to the progression of HCC. Despite the fact that our research has indicated that Ets-1, together with HBx and HBs, can enhance RhoC promoter activity, a realistic combination of transcription factors and promoter fragments should be studied by EMSA *in vitro* and ChIP *in vivo*. For example, anti-Ets-1 antibody should be used to immunoprecipitate whole genomic DNA fragments, and then PCR analysis should be used to identify RhoC promoter DNA. Also, the mechanism of how HBx and HBs interact with Ets-1 should be further studied.

Recently, Spangler et al. [16] indicated that the loss of E-cadherin activates the transcriptional regulator Ets-1 and consequently leads to the induction of RhoC expression, which stabilizes c-Jun in melanoma. The link between RhoC and c-Jun seems to be indirect via the cytoskeleton, while c-Jun is a member of the AP-1 transcription factor family and a key player in the processes of cell proliferation and tumor development. Therefore, we presume that the E-cadherin/Ets-1/RhoC/c-Jun pathway in melanoma may be suitable for HCC, where E-cadherin is first regulated by HBx and/or HBs.

We have found that HBV proteins, especially HBx and HBs, can regulate Ets-1 expression, which is an important transcription factor of the RhoC promoter. Upregulation of Ets-1 increases the expression of RhoC, which is a key factor in HCC metastasis. This study revealed a novel model for HBV-induced HCC metastasis, and the mechanism of HBx/HBs-induced E-cadherin change in HCC will be further investigated.

Acknowledgment This work was supported by Nature Science Foundation of China (30771924) and Natural Science Foundation Project of CQ CSTC (2010BB5359).

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