Upregulation of DNMT1 mediated by HBx suppresses RASSF1A expression independent of DNA methylation

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Received July 29, 2013; Accepted September 2, 2013

DOI: 10.3892/or.2013.2848

Abstract. The hepatitis B virus (HBV) X protein (HBx) plays a key role in the molecular pathogenesis of HBV-related hepatocellular carcinoma (HCC). However, its critical gene targets remain largely unknown. RASSF1A gene (Ras-association domain family 1A, RASSF1A), a tumor-suppressor gene, is frequently found to be hypermethylated and downregulated in HCC. In the present study, we investigated whether HBx is involved in the hypermethylation and downregulation of RASSF1A and we examined the potential regulation mechanisms. RT-PCR analysis was used to determine RASSF1A and HBx expression in 9 liver cell lines and the results showed that RASSF1A expression was relatively low in HBx-positive cells. Notably, RASSF1A was downregulated in HepG2.2.15 cells, as compared to HepG2 cells. Further analysis revealed that HBx transfection suppressed RASSF1A expression and HBx knockdown induced its expression. Enforced HBx suppressed RASSF1A and meanwhile induced DNMT1 and DNMT3B expression. In addition, RASSF1A is negatively regulated by DNMT1. ChIP analysis using an antibody against DNMT1 revealed that HBx enhanced the binding of DNMT1 to the RASSF1A promoter but the inhibition of RASSF1A by HBx is DNA methylation-independent as detected by methylation-specific PCR (MSP). Further studies using MSP and bisulfite genomic sequencing (BGS) revealed that no significant methylation changes were observed for regional methylation levels of RASSF1A in DNMT1 knockdown cells, although methylation levels of specific CpG sites at the predicted binding sites for the Sp1 and USF transcription factors were reduced. Additionally, RASSF1A was downregulated in HBV-associated HCC (HBV-HCC) as detected by RT-PCR and immunohistochemistry suggesting RASSF1A expression may be related to HBx in HCC and the clinical relevance of our observations. Collectively, our data showed that HBx suppressed RASSF1A expression via DNMT1 and offered a new mechanism of RASSF1A inactive in HCC in addition to the widely known DNA methylation, enriching the epigenetic mechanism by which HBx contributes to the pathogenesis of HBV-HCC.

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Key words: hepatocellular carcinoma, hepatitis B virus X protein, Ras association domain family 1A, DNMT1, DNA methylation

Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignant tumors and a major cause of cancer-related mortality worldwide. Chronic hepatitis B virus (HBV) infection, which is the major risk factor for developing HCC in East Asia and Africa (1), is prevalent in China (2). The HBV X protein (HBx), an essential factor for HBV replication, is thought to play a key role in the molecular pathogenesis of HBV-associated HCC (HBV-HCC) (3). Previous studies revealed that HBx knocked into p21 locus caused HCC in mice (4). However, critical gene targets remain largely unknown.

RASSF1A gene (Ras-association domain family 1A, RASSF1A), localized at 3p21.3, is a tumor-suppressor gene. *De novo* methylation of the RASSF1A promoter is one of the most frequent epigenetic inactivation events detected in human cancer and leads to inactivated RASSF1A. Hypermethylation of RASSF1A was frequently found in most major types of human tumors including lung, breast, prostate, pancreas, kidney, liver, cervical, thyroid and several other types of cancer (5). Recent studies have shown that HCC is associated with hypermethylation of RASSF1A (6-8). However, it is not yet known whether these methylation abnormalities result from hepatitis B virus (HBV), a major risk factor for the development of HCC (9). Furthermore, the epigenetic involvement of HBx, the most potent carcinogenic protein in HBV, has yet to be elucidated (10,11). HBx has been implicated to play a role in the epigenetic mechanisms on gene expression (12-14). In particular, HBx has been associated with increased DNA methyltransferase (DNMT) expression levels which result in hypermethylation and downregulation of a variety of genes such as IGFBP3 (12), CDH1 (15) and p16 (16). In the present

study, we sought to investigate whether HBx is involved in the hypermethylation and downregulation of RASSF1A and the potential regulation mechanisms.

We found that enforced HBx suppressed RASSF1A and induced DNMT1 and DNMT3B expression. RASSF1A is negatively regulated by DNMT1. HBx enhanced the binding of DNMT1 to RASSF1A promoter but the inhibition of RASSF1A by HBx is DNA methylation independent. Further studies using methylation-specific PCR (MSP) and bisulfite genomic sequencing (BGS) revealed that DNMT1 may suppress RASSF1A expression by hypermethylation of special CpG sites and thereby inhibits transcriptional factor binding without changing regional methylation status. Taken together, our data suggested that HBx silenced RASSF1A by increasing DNMT1 but independent of DNA methylation. Our findings offer a new mechanism of RASSF1A inactive in HCC, enhancing the understanding of both the contribution of HBx to HCC and the pathogenesis of HBV-HCC.

Materials and methods

Cell lines and HCC patient samples. The immortalized human liver cell line L02 and HCC cell lines MHCC-97L, MHCC-97H, HepG2 and HepG2.2.15 cells were maintained in Dulbecco's Modified Eagle's Medium (Gibco-BRL, Birmingham, MI, USA) supplemented with 10% fetal bovine serum (FBS; Wisent, Quebec, Canada). The liver cell lines Chang liver and QSG-7701 and HCC cell lines SMMC-7721 and BEL-7405 cells were grown in RPMI-1640 (Gibco-BRL) containing 10% FBS. The cells were incubated at 37˚C in a humidified atmosphere with 5% CO_2.

Paired tumorous and adjacent non-tumorous liver tissues from 29 HCC patients were obtained from the First Affiliated Hospital of Nanjing Medical University. The tissue samples were immediately frozen in liquid nitrogen after resection and stored at -80˚C until use. All samples were obtained with the written consent of the patients and were analyzed anonymously. This study was performed with the approval of the Medical Ethics Committee of the Medical School of Southeast University.

Transfection. QSG-7701 and SMMC-7721 cells were transfected with 4 μg of the pcDNA4/TO-HBx construct or the control pcDNA4/TO using the FuGENE®HD transfection reagent (Roche, Mannheim, Germany). The cells were harvested 36 h after transfection. L02 cells were transfected as described above. After transfection, cells were grown and selectively cultured in 200 μg/ml Zeocin (Invitrogen Life Technologies, Carlsbad, CA, USA) for 2 months after the initial transfection. L02-HBx #8 were the isolated HBx-transfectants. Transfection of 7,721 cells with siDNMT1 or siDNMT3B constructs was performed as previously described (17,18). Small interfering RNAs (siRNAs) targeting the HBx mRNA were designed and synthesized by GenePharma (Shanghai, China). For the siRNA transfections, the cells were plated to 50% confluency and were then transfected with 50 nmol/l HBx siRNA or a negative control using the X-tremeGENE siRNA transfection reagent (Roche) according to the manufacturer's protocol. After 48 h, the cells were harvested for analysis.

Reverse-transcription (RT)-PCR and quantitative real-time polymerase chain reaction (qPCR). The total RNA was purified with TRIzol (Invitrogen Life Technologies) and first-strand cDNA was prepared using PrimeScript RT reagent kit with gDNA Eraser (Takara Bio, Inc., Dalian, China) according to the manufacturer's instructions. PCR products were electrophoresed on 2% agarose gel, stained with ethidium bromide, and visualized under UV illumination. The intensities of the specific bands were analyzed and quantified. The qPCR was carried out using the SYBR Premix Ex Taq™ (Takara Bio, Inc.) according to the manufacturer's protocol. The relative expression was evaluated by the comparative CT method. The primer sequences of each gene and PCR conditions are shown in Table I.

Bisulfite treatment and promoter methylation analysis. Genomic DNA was extracted from the cells using the phenol-chloroform method followed by bisulfite modification. BGS was performed as previously described (19). Bisulfite-treated genomic DNA from L02-Vector, L02-HBx #8, 7701-Vector, 7701-HBx, 7721-Control and 7721-siDNMT1 cells were amplified using methylated and unmethylated RASSF1A primers (20). For BGS of RASSF1A promoter region in 7721-Control and 7721-siDNMT1 cells, semi-nested PCR was carried out. The PCR products were purified with the AxyPrep gel extraction kit (Axygen, USA) and cloned into pMD19-T vectors (Takara Bio, Inc.), followed by cycle-sequencing of at least 8 clones from each cell line. Primers used for RASSF1A BGS are as previously reported (21) and the sequences are listed in Table I.

Chromatin immunoprecipitation (ChIP) and ChIP-PCR. The ChIP experiments were performed using an EZ-Magna ChIP G kit (Upstate Biotechnology, Inc., Lake Placid, NY, USA) according to the method described previously (22). The primers used for the amplification of the precipitated DNA fragments were reported previously (23).

Immunohistochemical analysis. Paraffin sections (5 mm) were stained with the anti-RASSF1A antibody by incubating the samples overnight at 4˚C. Secondary staining with biotin-conjugated anti-rabbit IgG and tertiary staining with HRP-conjugated streptavidin was performed using an ABC kit (PK6100; Vector Laboratories, Burlingame, CA, USA). Specific staining was visualized by 3,3'-diaminobenzidine (DAB) staining. The slides were then counterstained with hematoxylin. Images were captured using the Nikon (Melville, NY, USA) Eclipse TE2000-S microscope system.

Statistical analysis. The independent Student's t-test was used to compare the results, expressed as the means ± SD between any 2 pre-selected groups. A P-value <0.05 was considered to indicate statistically significant differences.

Results

HBx suppresses RASSF1A expression in HCC cell lines. RASSF1A and HBx expression in 9 liver cell lines was detected by RT-PCR. The results showed that HBx was positive in 97H, 97L and HepG2.2.15 cells that constitutively replicate HBV (Fig. 1A). Compared to immortalized human liver

Table I. Primers and annealing temperature of genes analyzed by PCR.

Gene	Primers (5' to 3')	Temperature (˚C)	Amplification size (bp)
RASSF1A	F: TCATCTGGGGCGTCGTG R: CGTTCGTGTCCCGCTCC	62	144
DNMT1	F: CCGAGTTGGTGATGGTGTGTAC R: AGGTTGATGTCTGCGTGGTAGC	61	324
DNMT3A	F: TATTGATGAGCGCACAAGAGAGC R: GGGTGTTCCAGGGTAACATTGAG	65	110
DNMT3B	F: GACTTGGTGATTGGCGGAA R: GGCCCTGTGAGCAGCAGA	64	270
HBx	F: TTCTTCGTCTGCCGTTCC R: TCGGTCGTTGACATTGCT	54	201
β-actin	F: AAAGACCTGTACGCCAACAC R: GTCATACTCCTGCTTGCTGAT	61	220
RASSF1A (BGS)	MU379: GTTTTGGTAGTTTAATGAGTTTAGGTTTTTT ML730: ACCCTCTTCCTCTAACACAATAAAACTAACC	55	381
	MU379: GTTTTGGTAGTTTAATGAGTTTAGGTTTTTT ML561: CCCCACAATCCCTACACCCAAAT	55	205
RASSF1A (M)	F: GTGTTAACGCGTTGCGTATC R: AACCCCGCGAACTAAAAACGA	56	94
RASSF1A (U)	F: TTTGGTTGGAGTGTGTTAATGTG R: CAAACCCCACAAACTAAAAACAA	58	108

RASSF1A, Ras association domain family 1A; HBx, hepatitis B virus (HBV) X protein; F, forward; R, reverse.

cell line L02, normal liver cells Chang liver and QSG-7701 non-tumor hepatocyte cells, RASSF1A expression was relatively low in HCC cell lines, especially in HBx-positive 97H, 97L and HepG2.2.15 cells. The negative correlation between HBx and RASSF1A expression suggested that the expression of RASSF1A may be related to HBx. Notably, RASSF1A was downregulated in HepG2.2.15 cells, which are derived from HepG2 cells transfected with a plasmid containing HBV DNA, as compared to HepG2 cells.

The QSG-7701 and SMMC-7721 cells were transiently transfected with the pCDNA4-HBx and pCDNA4 vectors (Fig. 1B). Results showed that RASSF1A was downregulated in HBx-transfected cells compared with the control cells (vector) (Fig. 1B). In the L02-HBx #8 cells which were stably transfected with HBx, RASSF1A was also shown to be downregulated (Fig. 1B). To further verify whether this downregulation correlated with HBx expression, we examined the expression of RASSF1A after HBx knockdown by RNAi. The results showed that HBx knockdown increased RASSF1A expression in L02-HBx #8, 97H and HepG2.2.15 cells ($P<0.05$; Fig. 1C). These data support the hypothesis that HBx downregulates RASSF1A expression.

DNMT1 mediates the suppression of RASSF1A by HBx. Hypermethylation of the RASSF1A promoter is one of the most frequent epigenetic inactivation events leading to silencing of RASSF1A expression. As DNA methylation is catalyzed by DNA methyltransferase including DNMT1, DNMT3A and DNMT3B, we investigated whether HBx regulates DNMT expression and inactivates RASSF1A via promoter hypermethylation. DNMT expression analysis in SMMC-7721 cells showed that DNMT1 and DNMT3B were all upregulated in HBx-transfected cells (Fig. 2A). RASSF1A expression was upregulated in DNMT1-knockdown SMMC-7721 cells but not in DNMT3B-knockdown SMMC-7721 cells (Fig. 2B and C), suggesting that RASSF1A was regulated by DNMT1 and the suppression of RASSF1A by HBx may be through DNMT1. Next, we investigated whether HBx induces hypermethylation of RASSF1A promoter by MSP. The results showed that HBx failed to hypermethylate RASSF1A promoter in QSG-7701 and L02 cells (Fig. 2D). ChIP analysis using an antibody against DNMT1 revealed that the binding of DNMT1 to RASSF1A promoter regions is enriched in L02-HBx cells (Fig. 2E), which suggested that without changing DNA methylation status, DNMT1 mediates the suppression of RASSF1A by HBx.

DNMT1 suppresses RASSF1A expression in a manner independent of DNA methylation. To explore how DNMT1 regulates RASSF1A, MSP and BGS were utilized to test the methylation status of RASSF1A after DNMT1 knockdown in SMMC-7721 cells. No significant changes of methylation were observed between 7721-Control and 7721-siDNMT1 cells for

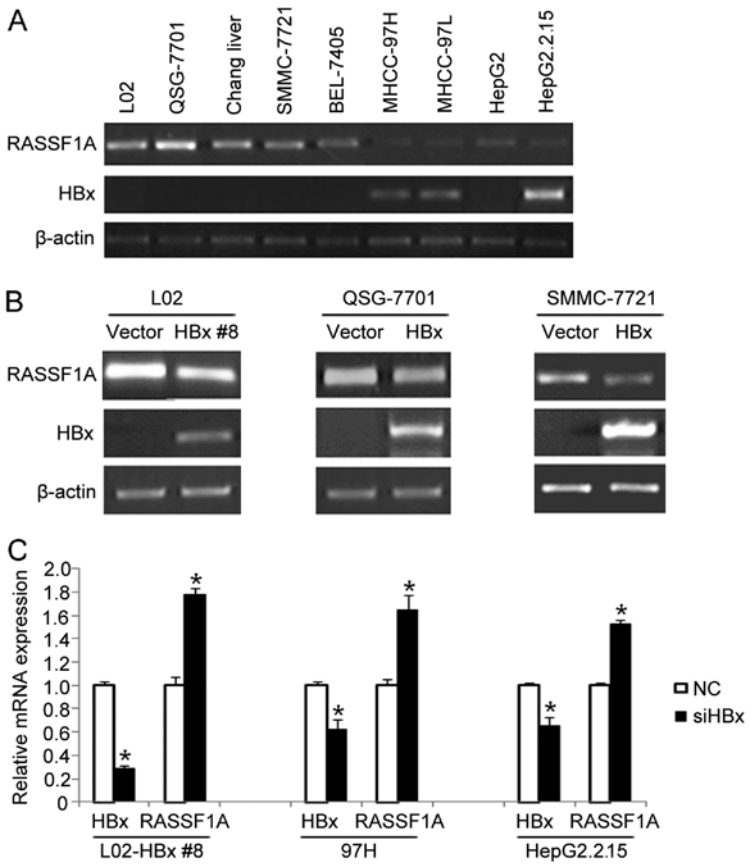

Figure 1. HBx suppresses RASSF1A expression in HCC cell lines. (A) RASSF1A and HBx expression levels were detected in 9 liver or HCC cell lines by RT-PCR. β-actin was used as a loading control. (B) RT-PCR analysis showing the expression of RASSF1A and HBx in QSG-7701 and SMMC-7721 cells transiently transfected with HBx expression constructs or control vectors and L02 cells stably transfected with HBx expression constructs. The #8 cell line is an HBx transfectant (HBx #8). β-actin was used as a loading control. (C) The HBx siRNA or the control siRNA were transfected into L02-HBx #8, 97H and HepG2.2.15 cells (NC or siHBx). The HBx and RASSF1A mRNA expression was examined by qPCR analysis (*P<0.05, independent Student's t-test). HBx, hepatitis B virus (HBV) X protein; RASSF1A, Ras association domain family 1A; HCC, hepatocellular carcinoma.

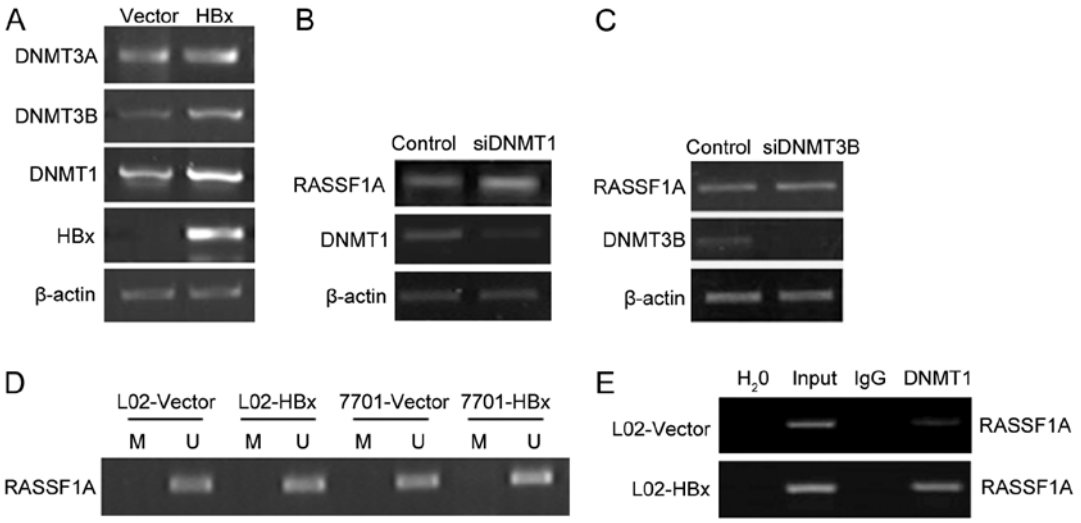

Figure 2. DNMT1 mediates the suppression of RASSF1A by HBx. (A) The levels of mRNAs encoding DNMT1, DNMT3A and DNMT3B were detected by RT-PCR in SMMC-7721 cells transiently transfected with the HBx expression construct or the control vector. β-actin was used as a loading control. HBx induced DNMT1 and DNMT3B expression. (B) RASSF1A and DNMT1 expression levels were detected by RT-PCR in SMMC-7721 cells after DNMT1 was knocked down by RNAi (siDNMT1) and control cells. β-actin was used as a loading control. (C) RASSF1A and DNMT3B expression levels were detected by RT-PCR in SMMC-7721 cells after DNMT3B was knocked down by RNAi (siDNMT3B) and control cells. β-actin was used as a loading control. (D) The methylation status of RASSF1A promoter was analyzed by methylation-specific PCR (MSP) in QSG-7701 cells transiently transfected with HBx and control constructs and HBx stably transfected L02 cells. M, methylation-specific primer amplification; U, unmethylation-specific amplification. (E) ChIP assay was performed in L02-Vector and L02-HBx cells using a DNMT1 specific antibody, followed by PCR amplification of individual fragments representing RASSF1A promoter regions. Chromatin (defined as 'input') and DNA products immunoprecipitated by IgG were used as positive and negative controls, respectively. H_2O was used as a blank control. RASSF1A, Ras association domain family 1A; HBx, hepatitis B virus (HBV) X protein. ChIP, chromatin immunoprecipitation.

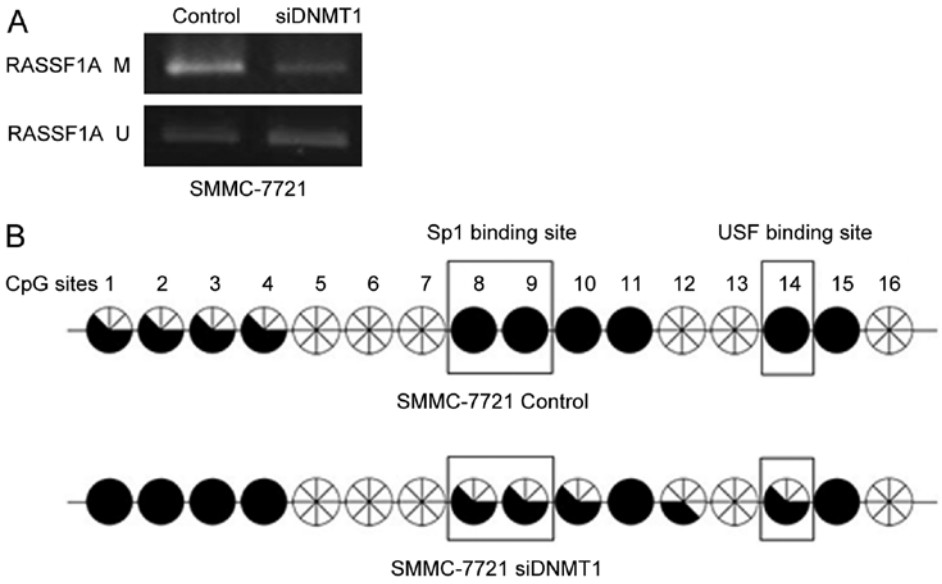

Figure 3. DNMT1 suppresses RASSF1A expression in a manner independent of DNA methylation. (A) The methylation status of the RASSF1A promoter was detected in SMMC-7721-siDNMT1 and SMMC-7721-Control cells by methylation-specific PCR. M, methylation-specific primer amplification; U, unmethylation-specific amplification. (B) Mapping of the methylation status of individual CpG sites in the RASSF1A promoter by bisulfite genomic sequencing in SMMC-7721-siDNMT1 and SMMC-7721-Control cells. The regions spanning the CpG island with 16 CpG sites were analyzed. Black represents the methylated CpG site; white represents the unmethylated CpG site; each circle represents bisulfite sequencing of one CpG site for 8 random clones from SMMC-7721-siDNMT1 and SMMC-7721-Control cells. The boxed regions show the schematic of transcription factor binding sites. RASSF1A, Ras association domain family 1A.

the methylation status of RASSF1A promoter region as evaluated by MSP (Fig. 3A).

The methylation levels of 16 CpG sites of the RASSF1A gene were examined using BGS (Fig. 3B). There were no significant changes in methylation levels in 7721-Control and 7721-siDNMT1 cells ($P>0.05$; Fig. 3B), the methylation ratio of the 16 CpG sites in the RASSF1A promoter was 53.13 and 55.47% in 7721-Control and 7721-siDNMT1 cells, respectively. Of note, methylation levels of CpG sites at the predicted binding sites for the Sp1 and USF transcription factors were altered in 7721-Control and 7721-siDNMT1 cells ($P<0.05$; Fig. 3B). Both for the Sp1 and USF transcription factors binding sites, the methylation ratio of the CpG sites was 100 and 62.5% in 7721-Control and 7721-siDNMT1 cells, respectively. Collectively, these data suggest that DNMT1-induced silencing of RASSF1A in SMMC-7721 cells is independent of DNMT1 DNA methyltransferase activity.

RASSF1A is downregulated in HBV-related HCC. RASSF1A expression in HBV-HCC was analyzed by RT-PCR analysis (29 cases). Seven representative results are shown in Fig. 4A. Compared to the paired corresponding non-tumor tissues, 48% of HCC cases showed a downregulation of RASSF1A at the mRNA level (Fig. 4B). Expression of RASSF1A was further examined by immunohistochemistry (IHC). The results showed that the RASSF1A protein was mainly localized in the nucleus. The protein levels were reduced in HCC cells when compared to the adjacent non-cancerous hepatocytes (Fig. 4C). Further evaluation of the relationship between HBx and RASSF1A mRNA levels in HCC samples revealed the negative correlation between RASSF1A and HBx expression in some HCC patients. RASSF1A expression was relatively low in HBx-positive tumor tissues, as shown in 8 representative cases (Fig. 4D).

Discussion

Ras association domain family 1 isoform A (RASSF1A) is a recently discovered tumor-suppressor gene. RASSF1A knockout mouse were prone to spontaneous tumorigenesis at advanced age (24), and RASSF1A is apparently involved in several growth regulatory and apoptotic pathways. Ectopic expression of RASSF1A in cancer cell lines, which lack endogenous RASSF1A transcripts, resulted in reduced growth of the cells *in vitro* and in nude mice (21,25-30). In addition, hypermethylation and inactivation of RASSF1A during tumor development was observed in a variety of different tumor types, including HBV-related HCC (6-8). However, whether HBV or HBx was involved in the downregulation of RASSF1A and the regulation mechanisms during HCC carcinogenesis remain unclear. Herein, we report that the major HBV oncoprotein, HBx, suppresses RASSF1A expression. RASSF1A is negatively regulated by DNMT1 and HBx induces DNMT1 expression. Further analysis revealed that HBx promoted recruitment of DNMT1 to RASSF1A promoter regions and may suppress RASSF1A expression by upregulating DNMT1.

HBx promoted recruitment of DNMT1 to RASSF1A promoter regions without changing the methylation status of RASSF1A DNA. Further studies on the regulation mechanisms of RASSF1A by DNMT1 revealed that DNMT1 RNAi influenced methylation of special CpG sites rather than regional methylation status. DNMT1 was previously shown to possess transcriptional suppression abilities, independent of catalyzing DNA methylation, that were partially mediated through a direct interaction with the histone deacetylases, HDAC1 and HDAC2, and other co-repressors (31-33). Thus, HBx-mediated DNMT1 may suppress RASSF1A expression by recruitment of other histone modification proteins and

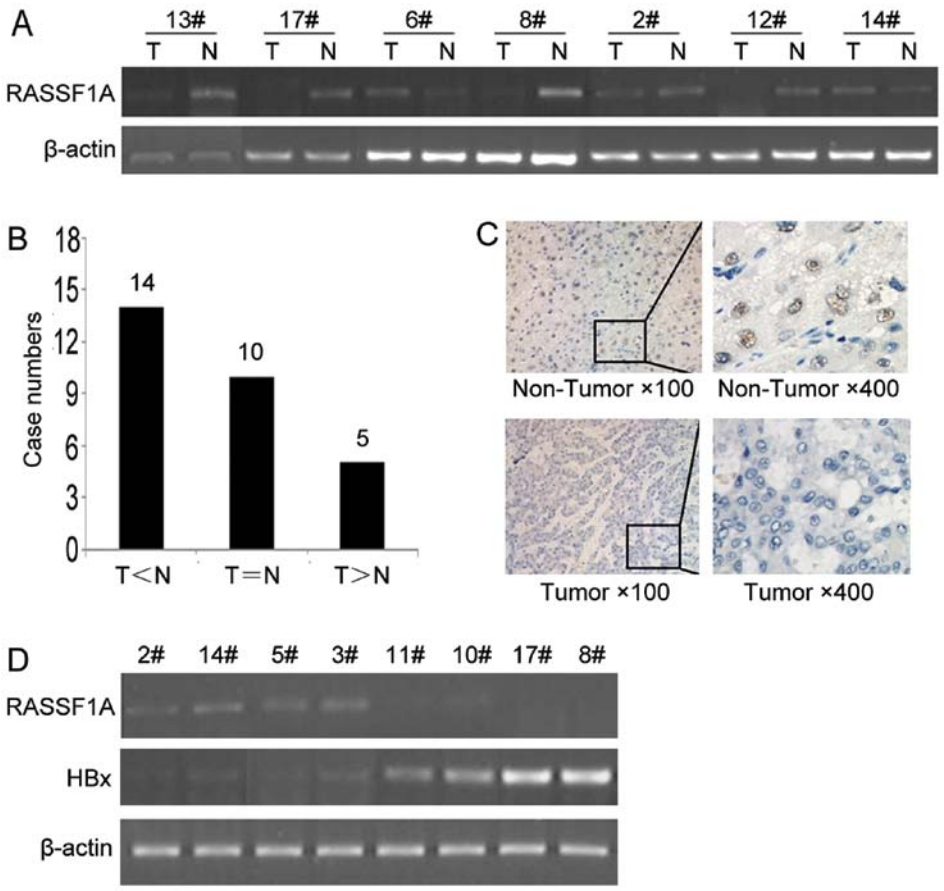

Figure 4. RASSF1A expression is downregulated in HBV-related HCC. (A) RASSF1A mRNA expression in 7 representative HCC (T) and their matched non-tumor tissues (N) detected by RT-PCR analysis (n=29). β-actin was used as a loading control. (B) Analysis of the relative expression of RASSF1A in the 29 paired HCC and matched non-tumor tissues. The numbers 14, 10 and 5 are case numbers; 2-folds was used as cutoff. (C) Immunohistochemical staining of RASSF1A protein in a representative HCC sample and its matched non-tumor tissue specimen. RASSF1A protein was localized mainly in the nucleus. RASSF1A was expressed at lower levels in HCC samples compared to their matched non-tumor tissues. (D) The mRNA levels of RASSF1A and HBx (detected by RT-PCR) in 8 HCC samples showed a negative correlation between RASSF1A and HBx expression. β-actin was used as a loading control. RASSF1A, Ras association domain family 1A; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HBx, hepatitis B virus X protein.

affects chromatin structure. Further studies will reveal the precise regulation mechanisms of RASSF1A by HBx through upregulating DNMT1.

BGS analysis showed that DNMT1 affected methylation status of specific transcription factor binding sites such as Sp1 and USF. The binding of Sp1 is DNA methylation insensitive (34,35). Methylation of Sp1 binding sites is associated with impaired binding of Sp1 at the RASSF1A promoter and the inactivation transcription of RASSF1A in proliferating human mammary epithelial cells (36). HBx-DNMT1-induced methylation of Sp1 binding sites may inhibit binding of Sp1 at the RASSF1A promoter, which results in the suppression of RASSF1A expression. This is consistent with previous reports that HBx specifically suppressed insulin-like growth factor-3 expression through DNMT3A1 and DNMT3A2 and by inhibiting Sp1 binding via recruiting methyl CpG binding protein 2 to the newly methylated Sp1 binding element (12). Upstream Stimulatory Factor (USF) is a family of helix-loop-helix transcription factors. Strong activation of transcription was observed both *in vitro* and *in vivo* following binding of USF to specific sites in gene promoters (37-39). Furthermore, CpG methylation can impair USF interaction with core motifs and subsequently alter gene expression, as for the metallothionein-I gene which is silenced in mouse lymphosarcoma (40). Therefore, HBx-DNMT1 may also suppress RASSF1A expression by inducing methylation of USF binding sites and subsequently inhibition of binding of USF at RASSF1A promoter.

In conclusion, we found that HBx enhances recruitment of DNMT1 to RASSF1A promoter regions and, hence, suppresses RASSF1A expression. DNMT1 could suppress RASSF1A expression independently of its regional DNA methylation status. Our studies offer another mechanism for the downregulation of RASSF1A in HBV-HCC other than the widely known DNA methylation, thereby providing insight into the epigenetic mechanism by which HBx contributes to the pathogenesis of HBV-HCC.

Acknowledgements

This study was supported by the National Natural Science Foundation of China, nos. 30470950 and 91229107, and in part by the Innovative Scientific Research Projects for College Graduates of Jiangsu Province, no. CXZZ_0137. The authors thank Professor Guan Xinyuan at the University of Hong Kong for providing the HBx constructs.

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