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HBx-elevated SIRT2 promotes HBV replication and hepatocarcinogenesis

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ABSTRACT

Sirtuin 2 (SIRT2) is a class III histone deacetylase that has been implicated to promote HCC development. However, the functional role of SIRT2 in HBV is still unclear. In this study, we found that HBV could upregulate SIRT2 expression. Additionally, HBx could activate SIRT2 promoter to upregulate the mRNA and protein level of SIRT2. Furthermore, we found that SIRT2 could facilitate HBV transcription and replication. Finally, we demonstrated that upregulation of SIRT2 by HBx promoted hepatocarcinogenesis. In summary, our findings revealed a novel function of SIRT2 in HBV and HBV-mediated HCC. First, SIRT2 could promote HBV replication. And then HBx-elevated SIRT2 could enhance the transformation of HBV-mediated HCC. Those findings highlight the potential role of SIRT2 in HBV and HBV-mediated HCC by interaction with HBx.

1. Introduction

Silent information regulator 2 (SIR2) are the highly conserved NAD^+ −dependent histone deacetylases which has been known to promote life span and mediate gene silencing in yeast [1]. In human, there are seven members in this family (SIRT1−SIRT7) which belong to class III histone deacetylases (HDACs) [2]. As one of this family, SIRT2 participated in a wide range of biological processes including genome maintenance [3], cell proliferation [4] and transformation [5]. Although SIRT2 is primarily a cytoplasmic protein [6], the shuttling of SIRT2 from the cytosol to the nucleus has been reported [7]. Growing evidences have supported the nuclear functions of SIRT2. For instance, nuclear SIRT2 is responsible for deacetylation of H4K16 [7] and methylation of H4K20 [8] during cell cycle. The above studies indicated the potential role of SIRT2 in nucleus.

In recent years, the role of SIR2 in virus has received much attentions. Zhang C et al. have found that SIRT1 can mediate hepatic steatosis during hepatitis C virus (HCV) infection [9]. As for hepatitis B virus (HBV), we have reported that SIRT1 facilitate HBV replication by targeting HBV core promoter through regulating transcription factor AP-1 [10] and the SIRT1 inhibitor, nicotinamide, exhibited an inhibitory effect on HBV promoters [11]. It has reported that the failure of virological remission during HBV infection is a significant risk factor for HCC [12]. In previous study, we have showed that SIRT2 can promote the epithelial to mesenchymal transition (EMT) in HCC [13]. However, the role of SIRT2 and the molecular mechanism in HBV and HBV-induced HCC are largely unknown.

In this study, we aim to evaluate the biological function of SIRT2 in HBV and HBV-induced HCC. We revealed that HBV or HBx could upregulate the expression of SIRT2 by targeting its promoter. Meanwhile, the positive functional role of SIRT2 in HBV replication and HBV-induced HCC has been elucidated in this study. Those findings highlight the potential role of SIRT2 in HBV and HBV-induced HCC and provide a new prosperity of HBV treatment.

2. Materials and methods

2.1. Antibodies, plasmids

Rabbit anti-SIRT2 monoclonal antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA); Mouse anti-GAPDH monoclonal antibody was obtained from Zhongshan Golden Brige Biotechnology (ZSGB-Bio, China).

SIRT2 expression vector was purchased from Addgene (Cambridge, MA). SIRT2 short hairpin RNAs (shSIRT2-1 and shSIRT2-2) were kindly provided by Dr. D.Y. Jin (The University of Hong

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Kong, Hong Kong, China). HBx-Flag expression plasmid was stored in our laboratory. HBV expression plasmid pCH9/3091 was kindly provided by Prof. Lin Lan (The Third Military Medical University, Chongqing, China). As previous described [14], HBV expression plasmid with HBx mutation (HBx MUT) was constructed based on pCH9/3091 (as the wild-type HBV, HBV WT) by inserting a stop codon at the beginning of the HBx gene.

2.2. Cell culture

Huh-7 cell line was purchased from the HSRRB (Osaka, Japan) and HepG2 cell line was acquired from ATCC (USA). Huh-7 and HepG2 cells were cultured in Dulbecco's modified Eagle medium (DMEM) (Corning, New York, USA) with 10% fetal bovine serum (FBS). HepAD38 cell line was purchased from the Shanghai Second Military Medical University and cultured in DMEM with 10% FBS, and 400 μg/ml of G418 (Merck, Germany). All the cells were cultured in a humidified incubator at 37 °C with 5% CO2.

2.3. Western blot

The cells were lysed by RIPA lysis buffer which contains protease inhibitor (Roche, Mannheim, Germany). The protein concentration was determined by BCA (Roche, Mannheim, Germany) and the lysates containing 30 μg of total protein was separated by SDS-PAGE. Then the protein was transferred to nitrocellulose membrane (GE Healthcare, Buckinghamshire, UK). After blocked in 5% nonfat milk, the membrane was incubated with primary antibody (Anti-SIRT2 protein 1:3000; Anti-GAPDH 1:10,000) overnight at 4 °C. The corresponding HRP-conjugated secondary antibody was incubated at room temperature for 2 h. The signals were visualized by ECL Western blot reagents (Millipore, Massachusetts, USA). GAPDH was used as a loading control.

2.4. Real-time PCR

The total RNA was extracted by TRNzol (TIANGEN, Beijing, China) methods. IScript™ cDNA Synthesis Kit was acquired from Bio-Rad (Bio-Rad, California, USA). Relative expression level of SIRT2, HBV total RNA and pregenome RNA (pgRNA) were detected by Fast Start Universal SYBR Green Master. β-actin mRNA was used as an endogenous control. The fold changes of various genes were calculated by using the $2^{-\triangle\triangle CT}$ method. The sequences of primers are as follows: SIRT2: forward, CTCTCTAGCCGTTCCCACAT, reverse, GGACTACGAGTCCCAGAAGG. HBV total RNA: forward, 5′-ACCGACCTTGAGGCATACTT-3′, reverse, 5′- GCCTA-CAGCCTCCTAGTACA-3′. HBV pgRNA: forward, 5′- GCCTTA-GAGTCTCCTGAGCA-3′, reverse, 5′- GAGGGAGTTCTTCTTCTAGG-3'. β-actin: forward, 5′-CTCTTCCAGCCTTCCTTCCT-3′, reverse, 5′-AGCACTGTGTTGGCGTACAG-3′.

HBV replicative intermediates were prepared as described previously [10]. The absolute quantification of the HBV replicative intermediates was determined by using Fast Start Universal SYBR Green Master (Roche, Mannheim, Germany). The sequences of HBV replicative intermediates primer: forward, 5′-CCTAGTAGTCAG TTATGTCAAC-3′, reverse, 5′-TCTATAAGCTGGAGGAGTGCGA-3'.

2.5. Luciferase reporter assay

The luciferase report vectors (pGL3-Basic SIRT2 promoter) were cotransfected with HBx-Flag or vector into HepG2 or Huh-7 cells and the relative luciferase activity was measured by dual luciferase reporter assay (Promega, USA). The transfection efficiency was normalized by cotransfecting with pRL-TK.

2.6. Southern blot

The HBV DNA replicative intermediates were separated by agarose gel and then denatured by alkali solution for 30 min. After transferred to the nylon membrane, the DNA was fixed by UV cross-linking. The membrane containing interest DNA was hybridized with digoxigenin-labeled DNA probe at 42 °C overnight. And then, the membrane was washed by SSC/0.1% SDS solution. Following blocked at 37 °C for 30 min in blocking solution, the membrane incubated with anti-digoxin secondary antibody at 37 °C for 30 min. The signal was collected by X-ray film.

2.7. Transwell migration and invasion assays

Cell metastasis ability was assessed by the transwell migration and invasion assay. 80,000 cells and 100,000 cells were seeded for the migration and invasion assay, respectively. After fixed by methanol, the cells migrated to the underside of the membrane were stained with 0.1% crystal violet and were enumerated for 10 microscope fields.

2.8. Enzyme-linked immunosorbent assay (ELISA)

The secretion level of HBsAg and HBeAg in supernatant were determined by ELISA assay (KHB, China) according to the manufacturer's instructions.

2.9. Statistical analysis

Results are expressed as mean ± SD. The data between two groups were compared by the Student's t-test. A difference was considered significant when $P < 0.05$. All statistical analysis was performed by the SPSS 19.0 software.

3. Results

3.1. HBV upregulated SIRT2 expression

We have previously reported that SIRT2 has been in implicated in epithelial-mesenchymal transition of hepatocellular carcinoma [13]. However, the relation between SIRT2 and HBV replication has not been investigated. The mRNA and protein levels of SIRT2 was examined in HepAD38 cell lines which is an HBV stably transfected cell line constitutively producing HBV under the control of tetracycline (Fig. 1A and B) and human hepatoma Huh-7 cells transiently transfected with HBV expressing plasmid pCH9/3091 (containing a 1.1-unit length HBV genome driven by a cytomegalovirus promoter) (Fig. 1C and D). Both mRNA and protein levels of SIRT2 were upregulated in HBV-expressing cells relative to control cells.

3.2. HBx enhanced SIRT2 expression in HCC cell lines

To determine whether viral protein HBx is responsible for SIRT2 upregulation, HepG2 or Huh-7 cells were transfected with HBV 1.1-mer replicons in which the expression of X gene was abrogated: HBV-HBx mutant. As expected, HBx mutation abolished the SIRT2 upregulation induced by wild type HBV replicons expression in both two cell lines (Fig. 2A and B), which suggesting the potential role of HBx in SIRT2 expression.

To further confirm the relationship between HBx and SIRT2, HBx-Flag or vector plasmid were transfected into HepG2 or Huh-7 cells and the expression level of SIRT2 were determined by Real-time PCR and Western blot. Both mRNA and protein levels of SIRT2 in HepG2 or Huh-7 were significantly elevated when transfected with HBx expression plasmid (Fig. 2C and D). HBx is closely related

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