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miR-146 promotes HBV replication and expression by targeting ZEB2

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ABSTRACT

Hepatitis B virus (HBV) is associated with the development of a wide spectrum of liver diseases. The involvement of miRNAs in HBV replication is being gradually identified. Among these miRNAs, miR-146a expression was found to be positively correlated with HBV replication levels. However, the regulatory relationship between miR-146a and HBV replication is still unclear. In the present study, miR-146a was upregulated in HBV-expressing HepG2.2.15 cells compared with HepG2 cells. Overexpression of miR-146a or knockdown of Zinc finger E-box-binding homeobox 2 (ZEB2) promoted HBV replication and expression, while downregulation of miR-146a or overexpression of ZEB2 suppressed HBV replication and expression. In addition, miR-146a was demonstrated to directly target ZEB2. Furthermore, ZEB2 silencing abated anti-miR-146a-induced inhibition on HBV replication and expression. These findings suggested that miR-146a promoted HBV replication by targeting ZEB2, providing a new antiviral strategy for HBV infection.

1. Introduction

Hepatitis B virus (HBV), a small, partially double-stranded and enveloped DNA virus, is associated with increased risk for a variety of liver diseases, such as hepatitis, liver cirrhosis, and hepatocellular carcinoma (HCC) [1]. HBV infection represents a major global public health issue, with more than 350 million infectious cases and approximately 1 million deaths annually due to HBV-related liver pathologies [2]. Although interferon-α (IFN-α) and nucleotide analogues (NA) are currently available to minimize HBV replication, the therapeutic efficacy is far from being satisfactory on account of side effects and drug-resistant virus mutations [3,4]. Therefore, it is urgently required to elucidate the molecular mechanism underlying HBV replication and identify novel and effective therapeutic targets for HBV therapy.

microRNAs (miRNAs) are a large family of highly conserved, endogenous, small non-coding RNAs with 19–22 nt in length that mediate mRNA degradation or translational repression by partial or complete complementarity with the binding sites in the 3'-untranslated region of target mRNA [5]. It has been proposed that miRNAs play a crucial role in diverse biological events including cell differentiation, apoptosis, metabolism, and virus infection [6,7]. Moreover, a handful of studies suggest that miRNAs function as key regulators in host-virus interaction and participate in the regulation of HBV infection, replication and HBV-related diseases [8,9]. Serum miRNA-125b strengthened HBV replication ability *in vitro* and was capable to discriminate grades of liver necroinflammation in patients with chronic hepatitis B (CHB) infection [10]. miR-26b suppressed HBV gene expression and replication by attenuating HBV enhancer/promoter activities via regulating cysteine- and histidine-rich domain containing 1 (CHORDC1) [11]. Other miRNAs, such as miR-141, miR-501 and miR-125a-5p were also identified to be involved in the regulation of HBV replication [12–14]. miR-146a, located on human chromosome 5q34, was reported to be linked to human cancers [15,16] and immune responses [17,18]. A previous study revealed that miR-146a was upregulated in stable HBV-expressing HepG2.2.15 cells and positively correlated with HBV replication levels [19]. However, the effect and molecular mechanism of miR-146a in HBV pathogenesis are still largely unknown.

In the present study, we attempted to explore the involvement of miR-146a in HBV replication and its possible molecular basis. Our data indicated that miR-146a promoted HBV replication by targeting Zinc finger E-box-binding homeobox 2 (ZEB2), contributing to a better understanding of miRNAs in HBV replication and suggesting a potential application of miR-146a in HBV treatment.

Abbreviations: HBV, hepatitis B virus; HCC, hepatocellular carcinoma; ZEB2, Zinc finger E-box-binding homeobox 2; IFN-α, interferon-α; NA, nucleotide analogues; miRNAs, microRNAs; CHB, chronic hepatitis B; CHORDC1, cysteine- and histidine-rich domain containing 1; FBS, fetal bovine serum; DMEM, Dulbecco's modified Eagle's medium; ELISA, enzyme-linked immunosorbent assay; VEGF, vascular endothelial growth factor; CFH, complement factor H; NFIA, nuclear factor IA

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2. Materials and methods

2.1. Cell culture and transfection

Human HCC cell lines HepG2 and HepG2.2.15 were purchased from American type culture collection (ATCC, Manassas, VA, USA). All cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% heat-inactivated fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin at 37 °C in a humidified atmosphere with 5% CO_2. HepG2.2.15, derived from HepG2 cell line, possesses HBV genome and is able to generate HBV particle. G418 (Sigma-Aldrich St. Louis, MO, USA) was added into culture medium to maintain stable HBV replication in HepG2.2.15 cells.

miR-146a mimic (miR-146), miRNA scrambled control (miR-control), miR-146a inhibitor (anti-miR-146), miRNA inhibitor control (anti-miR-control), ZEB2-overexpressing plasmid (pcDNA-ZEB2), pcDNA3.1 empty vector (pcDNA3.1), siRNA against ZEB2 (si-ZEB2), and siRNA scrambled control (si-control) were obtained from Genechem, Inc. (Shanghai, China). Plasmids or oligonucleotides were transfected into HepG2.2.15 cells at the specified concentrations using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Cells were collected for further analysis 48 h posttransfection.

2.2. Quantitative real-time PCR for mRNA/miRNA

Total RNA from cultured cells was isolated using TRIzol reagent (Invitrogen). A total of 2 μg RNA was reversely transcribed into the first-strand cDNA with random primers and High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA, USA). qPCR reactions were performed by the TaqMan MicroRNA Assays (Applied Biosystems) and SYBR Premix Ex TaqTM kit (TaKaRa, Shiga, Japan) on the Applied Biosystems 7900 Sequence Detection system (Applied Biosystems, Foster City, CA, USA), respectively. The relative expressions of miR-146a and ZEB2 were calculated using the $2^{-\triangle\triangle Ct}$ method and normalized to U6 and GAPDH, respectively. The primer sequence used for PCR were: miR-146a, 5'-CCGATGTGTATCCTCAGCT TTG-3' (forward), 5´-GCTGAAGAACTGAATTTCAGAGGTC-3' (reverse); ZEB2, 5'-CGCTTGACATCACTGAAG GA-3' (forward), 5'-CTTGCCACAC TCTGTGCATT-3' (reverse); U6, 5'-GCTTCGGCAGCACATATACTAA AAT-3' (forward), 5'-CGCTTCACGAATTTGCGTGTCAT-3' (reverse); GAPDH, 5'-TATGATGATATCAAGAGGGTAGT-3' (forward), 5'-TGTATC CAAACTCATTGTCATAC-3' (reverse).

2.3. Western blot

Total proteins from HepG2.2.15 cells were isolated with RIPA reagents (Thermo Scientific, Rockford, IL, USA). Equivalent amounts of proteins were subjected to 12% SDS-PAGE and transferred to PVDF membrane. After being blocked with 10% nonfat milk for 1 h, the membranes were then incubated overnight with the primary antibody against ZEB2 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) or β-actin (Sigma-Aldrich) at 4 °C overnight, followed by immunoblotted with horseradish peroxidase (HRP)-linked secondary antibody (Santa Cruz Biotechnology) for 1 h. The protein signals were detected with SuperSignal West Pico Chemiluminescent Substrate kit (Pierce, Rockford, IL, USA).

2.4. Luciferase reporter assay

The predicted miR-146a binding sites in the wild-type 3'UTR of ZEB2 were amplified by PCR and inserted into the pGL3 firefly luciferase reporter plasmid (Promega Corp., Madison, WI, USA) to generate WT-ZEB2-3'UTR. The mutant 3'UTR of ZEB2 was produced using the Quick-Change Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA, USA) and cloned into the pGL3 vector (Promega Corp.), named as MUT-ZEB2-3'UTR. HEK293 cells were seeded into 96-well plates for 24 h incubation, followed by cotransfected with 30 ng WT-ZEB2-3'UTR or WT-ZEB2-3'UTR and 100 nM miR-146a mimic or miR-control using Lipofectamine 2000 reagent (Invitrogen). A renilla luciferase reporter vector pRL-TK was also cotransfected as an internal control. At 48 h posttransfection, the activities of firefly luciferase were measured with dual-luciferase reporter assay system (Promega Corp.) by normalizing to renilla luciferase activity.

2.5. Southern blot analysis

HBV DNA replicative intermediates in HBV core particles from transfected HepG2.2.15 cells were extracted by HBV DNA isolation kit (GenMed Scientifics, Arlington, MA, USA), followed by southern blot detection as described previously [20].

2.6. HBV replication analysis

Cell culture medium was harvested 48 h posttransfection, followed by centrifugation to remove debris. HBV DNA was extracted from culture supernatant using Column Viral DNAout kit (TIANDZ, China) and quantified by HBV PCR assay reagent II (Qiagen, Valencia, CA, USA) according to the manufacturer's instruction. HBV specific primers were 5'-CCTAGTAGTCAGTTATGTCAAC-3' (forward) and 5'-TCTATAAGCT GGAGTGCGA-3' (reverse). The concentrations of HBV surface antigen (HBsAg) and HBV e-antigen (HBeAg) in the supernatant were determined using an enzyme-linked immunosorbent assay (ELISA) kits (Shanghai KeHua Biotech, Shanghai, China).

2.7. Statistical analysis

All data were presented as mean ± standard deviation (SD) from at least three independent experiments and analyzed by SPSS 19.0 (SPSS, Inc., Chicago, IL, USA). For analysis of differences between two or more groups, Student's *t* test or one-way analysis of variance was applied. *P* value $< .05$ was considered to be statistically significant.

3. Results

3.1. miR-146a promoted HBV replication and expression

To explore the role of miR-146a in HBV replication and expression, the expression of miR-146a in HCC cell line HepG2 and HBV stable replication cell line HepG2.2.15 was initially evaluated by qRT-PCR. As shown in Fig. 1A, miR-146a expression was markedly higher in HepG2.2.15 cells than that in HepG2 cells. Subsequently, gain- and loss-of-function experiments were performed in HepG2.2.15 cells by transfecting with miR-146a, anti-miR-146a, or respective controls. qRT-PCR analysis confirmed the up-regulation of miR-146a after introduction with miR-146 mimic, and the down-regulation of miR-146a after treatment with anti-miR-146a in HepG2.2.15 cells (Fig. 1B). The effect of miR-146a in HBV replication and expression was investigated by measuring HBV DNA replication and HBV replicative intermediates, as well as HBsAg and HBeAg expression levels. qPCR and southern blot analysis results exhibited that ectopic expression of miR-146a dramatically increased the HBV DNA copies (Fig. 1C) and HBV DNA replicative intermediates (Fig. 1D) in HepG2.2.15 cells compared with miR-control group. On the contrary, anti-miR-146a transfection suppressed HBV DNA copies and HBV DNA replicative intermediates in HepG2.2.15 cells (Fig. 1C and D). ELISA analysis revealed that miR-146a overexpression led to a marked increase in HBsAg (Fig. 1E) and HBeAg (Fig. 1F) levels compared with miR-control group in HepG2.2.15 cells. However, secretion levels of HBsAg and HBeAg were dramatically decreased in anti-miR-146a-transfected cells. Taken together, all these results demonstrated that miR-146a promoted HBV replication and expression in HepG2.2.15 cells.