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Suppression of hepatitis B virus replication by microRNA-199a-3p and microRNA-210

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ABSTRACT

Accumulating evidence suggests that microRNAs (miRNAs) control the replication of both RNA and DNA viruses. In order to determine whether host-encoded miRNAs affect hepatitis B virus (HBV) replication, antisense oligonucleotides (ASOs) of 328 identified human miRNAs were transfected into HepG2 2.2.15 cells, respectively. ELISA and MTS assay were used to measure the expression level of HBV S protein (HBsAg), HBV e antigen (HBeAg) and cell proliferation. Compared to experimental controls, miR-199a-3p and miR-210 efficiently reduced HBsAg expression without affecting HepG2 2.2.15 cell proliferation. Quantification of HBV DNA by real-time PCR showed that both miRNAs suppressed viral replication. Bioinformatics analysis indicated a putative binding site for miR-199a-3p in the HBsAg coding region and a putative binding site for miR-210 in the HBV pre-S1 region. The direct effect of miRNAs on the target region in HBV transcripts was validated by a fluorescent reporter assay, and the suppression of HBs gene expression by both miRNAs was measured by real-time PCR and Western blot. These results suggest that up-regulation of miR-199a-3p and miR-210 in HepG2 2.2.15 cells compared to HepG2 cells may play a role in regulating HBV replication and maintenance of a suitable level of virion production in persistent infection by targeting crucial HBV genes.

1. Introduction

MiRNAs are short 19- to 24-nucleotide (nt) endogenous RNA species that are expressed in almost all eukaryotes examined to date. MiRNAs are recognized as key regulators of gene expression through the miRNA-guided RNA silencing pathway (Ambros, 2004; Bartel, 2004; He and Hannon, 2004), and have diverse functions, including a role in the regulation of cellular differentiation, proliferation and apoptosis (Hwang and Mendell, 2006). MiRNAs are generated from miRNA precursors (pre-miRNAs) by the RNase III, Dicer. One strand of the resulting short double-stranded RNA guides the RNA-induced silencing complex to its target mRNA (Zhang et al., 2004). In the case of perfect sequence complementarity, miRNA-armed RISC (RNA-induced silencing complex) can lead to mRNA degradation. In the case of imperfect sequence complementarity, inhibition of mRNA translation occurs (Mourelatos et al., 2002).

Increasing evidence suggests that the miRNA pathway also plays a key role in the regulation of replication and gene expression in viruses (Voinnet, 2005; Berkhout and Jeang, 2007). Jopling et al. (2005) reported that the liver-specific miR-122 could enhance the replication of hepatitis C viruses. Impaired translation of target sequences from primate foamy virus type 1 (PFV-1) by miR-32 has also been found (Lecellier et al., 2005). The interaction of HIV with host T cells may also be modulated by miRNAs, as indicated by the fact that cellular miRNAs potently inhibit HIV-1 production in resting primary CD4+ T cells which contributes to HIV-1 latency (Huang et al., 2007). Endogenous levels of miRNA-mediated silencing effectors negatively regulate HIV-1 gene expression and affect HIV-1 latency and/or maintenance in infected patients (Chable-Bessia et al., 2009). Since RNA silencing serves as an innate antiviral mechanism in plants, fungi and animals, an infecting virus can also trigger changes in cellular miRNA or produce viral miRNA (vmiRNA) to resist cellular anti-virus activities. Experimental evidence, including computational approaches, suggest that many viruses can also encode miRNA, including the Epstein–Barr virus, the herpes simplex virus and so on (Umbach et al., 2008; Pfeffer et al., 2004). Some viruses that are attacked by cellular miRNAs may even affect miRNA abundance via synthesis of viral proteins or RNAs (de Vries

Abbreviations: miRNA, microRNA; HBV, hepatitis B virus; ASO, antisense oligonucleotide; HBsAg, HBV S antigen; HBeAg, HBV e antigen; ELISA, enzyme linked immunosorbent assay; MTS, 3-(4,5-dimethylthiazol-2yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium salt; vmiRNA, viral miRNA; HBVPS1, pre-S1 region of HBV; EGFP, enhanced green fluorescent protein; RFP, red fluorescent protein; ORF, open reading frame.

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and Berkhout, 2008). The HIV-1 Tat protein has been reported to suppress RNAi by inhibiting Dicer activity. HIV-1 TAR hairpin is also capable of saturating TAR RNA-binding protein (TRBP), thereby suppressing RNAi (Bennasser and Jeang, 2006; Bennasser et al., 2006). Although evidence regarding miRNA-mediated cross-talk in viral infections is just beginning to emerge, studies on the role of miRNA on virus proliferation and expression may contribute to better understanding host-pathogen interactions, viral tropism, latency and oncogenesis, and to developing new biomarkers and therapeutics.

HBV is a hepatotropic, partially double-stranded DNA virus. Its genome contains four overlapping open reading frames that codes for hepatitis B virus surface antigen, hepatitis B virus core protein, viral reverse DNA polymerase and X protein (HBx) (Tiollais et al., 1985). Chronic HBV infection is a major cause of liver fibrosis, which eventually leads to cirrhosis and hepatocellular carcinoma. However, the mechanisms of persistent HBV infection as well as the interactions between the host and HBV are not completely understood.

Currently, complex regulatory networks involving miRNAs have not been assessed comprehensively in HBV replication. During interactions between virus and host cells, viruses encode their own miRNAs, which can target cellular genes or viral genes. Cellular miRNAs can also regulate viral replication and pathogenesis. Using a computational approach, Jin et al. (2007) found that HBV putatively encodes one candidate – vmiRNA, which targets viral mRNA and regulates its own gene expression, but not that of cellular genes. To elucidate the role of cellular miRNAs in regulating HBV replication, we have studied the effects of human miRNAs on HBV gene expression. Here we report that human miRNAs can suppress HBV replication by targeting HBs genes. The identification of miRNA-mediated inhibition of HBV replication may provide new clues for the development of anti-HBV strategies.

2. Materials and methods

2.1. Cell culture

HepG2 2.2.15 and HEK 293 cell lines were propagated and maintained in Dulbecco's modified Eagle's medium (DMEM) or MEM-α medium (GIBCO) supplemented with 10% fetal bovine serum (MIN HAI Bio-Engineering, China), 20 mM HEPES, 2 mM glutamine and antibiotics, in a humidified atmosphere at 37 °C with 5% CO_2.

The miRNA 2′-O-methyl (2′-OMe) antisense oligonucleotides (ASO) synthesized by IDT (Coralville, IA) used in the study are completely complementary to human mature miRNA sequences that can be found in the miRNA Register (Griffiths-Jones et al., 2006). The cells were trypsinized and plated at 1×10^4 cells per well in 96-well plates the day before transfection. Cells (triplicate cultures) were transfected with 50 nM miRNA inhibitors or 5 ng/μL miRNA expression vectors using Lipofectamine 2000 (Invitrogen) following the Manufacturer's protocol. The medium was removed at 48 h post-transfection, and the cells were replenished with fresh medium. Twenty-four hours later, the cell culture supernatant was collected for detection of HBsAg, HBeAg and HBV DNA. Each experiment in this study was performed at least three times.

2.2. Quantitative real-time polymerase chain reaction

Quantitation of miRNAs was carried out using SYBR Green-based real-time RT-PCR detection. For RT reactions, 10 ng of RNA used in each reaction (20 μl) was mixed with the RT primer (1 μl). After the RT reaction, 1 μl of cDNA was used for the PCR reaction using SYBR® Premix Ex Taq™ (TakaRa, Otsu, Shiga, Japan). The PCR reaction was conducted at 94 °C for 3 min, followed by 40 cycles of 94 °C for 30 s, 50 °C for 30 s and 72 °C for 30 s in an ABI 7500 Real-time PCR system. The real-time PCR results were analyzed and expressed as relative miRNA expressions of CT (Threshold Cycle) value, which was then converted to fold changes (Chen et al., 2005). U6 was used for normalization.

For HBV DNA copy detection, supernatants of HepG2 2.2.15 were harvested and DNA was extracted with a commercial kit (QIAGEN, Germany) following the Manufacturer's instructions. Real-time PCR was used to quantify HBV DNA according to a diagnostic kit for quantification of Hepatitis B virus DNA (Da An Gene, Guangzhou, China) in an ABI-Prism 7300 System.

2.3. MTS assay

HepG2 2.2.15 cells were seeded in 96-well plates at a density of 10,000 cells per well and then transfected with oligonucleotides or miRNA overexpression plasmids. At 72 h after transfection, the cells in 100 μl culture medium were incubated with 20 μl of the combined MTS/PMS at a final concentration of 333 μg/ml MTS and 25 μM PMS (phenazine methosulfate) (Promega) solution at 37 °C for 4 h in a humidified, 5% CO_2 atmosphere. The absorbance at 490 nm was read using a μQuant Universal Microplate Spectrophotometer (Biotek, Germany).

2.4. ELISA for detection HBsAg and HBeAg

Seventy-two hours after transfection, the supernatants of HepG2 2.2.15 were harvested and diluted 1:5 in phosphate-buffered saline. The diluted supernatants were used to determine the concentrations of HBsAg and HBeAg using ELISA test kits (InTec Products, Xiamen, China) following the manufacturer's protocol. Absorbance was determined in a microtiter plate reader (μQuant, Biotek, Germany) with dual-wavelength measurement (450/630 nm).

2.5. Plasmid construction

To construct a plasmid expressing miRNA precursors, we amplified DNA fragments carrying miRNA precursors from the HEK 293 genome using PCR primers: pri-miR-199a-3p sense 5′-GGAGATCTTGAGCCCAGAAGCCACGATC-3′, and pri-miR-199a-3p antisense 5′-CGGAATTCGCCACCCTCTTAGATGCC-3′; pri-miR-210 sense 5′-GGAGATCTGACCAGGTCATTTGCATAC-3′, and pri-miR-210 antisense 5′-GGGAATTCGATATGACCACACCTGTG-3′. The amplified fragments of pri-miR-199a-3p and pri-miR-210 were cloned into pcDNA3B (deleting the BglII site and inserting a BglII site at multiple cloning sites) at BglII and EcoRI sites.

Fragments containing the predicted miR-199a-3p and miR-210 binding sites in HBV transcripts were amplified by PCR using primers: HBsAg sense 5′-CGGAATTCTTCCTCTTCATCCTGCTG-CT-3′, and HBsAg antisense 5′-CAGTCCTCGAGACATAGAGGTT-CCTTGAGCAG-3′; HBPS1 sense 5′-CGGAATTCAGGCATTATTTACAT-ACTCT-3′, and HBPS1 antisense 5′-CAGTCCTCGAGCCCGCTCCT-ACCTGATTT-3′. The amplified fragment was cloned downstream of EGFP CDS (coding sequence) with stop codons in pcDNA3.1(+)/EGFP at EcoRI and XhoI sites. The resultant constructs were named pcDNA3/EGFP-HBsAg and pcDNA3/EGFP-HBPS1, respectively. Similarly, fragments containing mutated miR-199a-3p and miR-210 binding sites were introduced into pcDNA3.1(+)/EGFP at the same sites. The constructed vectors were named pcDNA3/EGFP-HBsAg-mut and pcDNA3/EGFP-HBPS1-mut, respectively.

2.6. Fluorescent reporter assay

HepG2 2.2.15 and HEK 293 cells were seeded in 48-well plates the day before transfection. Cells were first transfected with

oligonucleotides or miRNA overexpression plasmids, and then with reporter vectors on the following day. The RFP expression vector pDsRed2-N1 (Clontech) was used for normalization. Proteins were harvested 48 h later. The intensity of EGFP and RFP fluorescence was detected using a Fluorescence Spectrophotometer F-4500 (HITACHI, Japan).

2.7. Western blot

Total proteins from HepG2 2.2.15 cells transfected with either oligonucleotides or plasmids were extracted using RIPA buffer (10 mM Tris–HCl, pH 7.4, 1% Triton X-100, 0.1% SDS, 1% NP-40, 1 mM $MgCl_2$) at 72 h post-transfection, and protein expression was analyzed by Western blot. β-Actin served as a loading control. Total protein extracts were separated on 12% SDS-PAGE gels and transferred to nitrocellulose membranes. The level of HBsAg expression was evaluated using the rabbit polyclonal anti-HBsAg antibody (Saier Biotech, Tianjin, China) which is immunogen affinity purified. As a loading control, the β-actin expression level was measured using rabbit polyclonal anti-β-actin antibody (Sigma–Aldrich). Bands were quantified with Labworks 4.0 software.

2.8. Statistical analysis

Statistical significance was determined using a two-tail homoskedastic Student's *t* test. For all data analyzed, a significance threshold of $P < 0.05$ was assumed. In all figures, values were expressed as mean ± standard deviation (SD), and statistical significance was indicated by a single ($P < 0.05$) asterisk. The data generated *in vitro* were representative of at least three separate experiments conducted in triplicate.

3. Results

3.1. HBsAg expression and poliferation of HBV were ehanced by antisense oligomers of miR-199a-3p and miR-210

To determine whether human miRNAs participate in the regulation of HBV replication, we used the HepG2 2.2.15 cell line which was derived from a clone of HepG2 cells stably transfected with a dimer of the complete HBV genome as a model system. HepG2 2.2.15 cells constitutively express hepatitis B surface (HBsAg), e (HBeAg) and core (HBcAg) antigens, also supporting full HBV replication (Sells et al., 1987). Because inhibition of endogenous miRNAs using antisense oligonucleotides is an effective technique for the characterization of miRNA function, here we used a library of miRNA 2′-OMe ASOs in functional screening assays to find miRNAs that affect HBV antigen expression. 2′-OMe ASOs could result in a marked reduction of corresponding miRNA levels (Chan et al., 2005). Monitoring miRNA expression using real-time PCR was served to validate the efficiency of miRNA ASOs, as shown in Fig. 1A. A total of 328 identified antisense oligomers of human miRNAs were transfected into HepG2 2.2.15 cells, respectively. The antisense oligo of LacZ was used as control. Transfection efficiency was over 90%, tested using a Cy5-oligomer (data not shown). At 72 h post-transfection, the levels of HBsAg and HBeAg in the cultures' supernatant were assessed by using the ELISA assay, and cell proliferation was measured using MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium), a soluble alternative to MTT. The results indicated that most miRNA ASOs tested did not affect expression of HBV antigens. In comparison to controls, transfection of 50 nM ASOs of miR-184, miR-185, miR-196a, miR-199a-3p, miR-210 and miR-217 caused a 1.4- to 1.6-fold increase in HBsAg expression, which was not due to the promotion of cell proliferation (Fig. 1B and Supplemental Fig. 1A). Transfection of miR-370 ASO down-regulated HBsAg expression (Supplemental Fig. 1A), however, antisense oligomers of each miRNA had no effect on HBeAg expression.

Since miRNAs are known to have diverse functions through regulating multiple target gene expressions, these significant miRNAs might either directly target HBV transcripts to inhibit HBsAg mRNA expression or regulate cellular genes involved in HBV proliferation and antigen expression. Using bioinformatics analysis of ViTa database (Hsu et al., 2007), miR-199a-3p and miR-210 were found to have putative binding sites in the HBV pre-S1/pre-S2/S region. Here we selected miR-199a-3p and miR-210 to perform studies in order to elucidate the direct antiviral activity of human miRNA.

Because miR-199a-3p and miR-210 ASOs had the same effect on HBsAg expression, our next experiment was to test their synergy. Both miR-199a-3p and miR-210 ASOs were cotransfected into HepG2 2.2.15 cells. The HBsAg expression level in cotransfected HepG2 2.2.15 cells showed no significant difference compared to single ASO-transfected cells which indicated that combining the two antisense oligomers had no additive effect on HBsAg expression (Supplemental Fig. 1B).

To further validate the effect of miR-199a-3p and miR-210 ASOs on HBV proliferation, we also detected HBV DNA copies using real-time PCR, which is a measurement of the amount of virus present in the supernatant of transfected HepG2 2.2.15 cells. These results show that antisense oligomers of miR-199a-3p and miR-210 can increase the generation of HBV (Fig. 1C).

3.2. HBsAg expression and proliferation of HBV were suppressed by overexpression of miR-199a-3p and miR-210

To overexpress these miRNAs, we constructed plasmids expressing the miRNA precursors by inserting the fragments containing precursor sequences of miRNAs amplified by PCR into pcDNA3 vector (pcDNA3/pri-199a-3p and pcDNA3/pri-210). Real-time PCR was used to detect the miRNA levels in cells after overexpression by plasmids. Compared to vector control, ectopic expression plasmids of miR-199a-3p and miR-210 resulted in a 3-fold increase in miR-199a-3p expression and a 2-fold increase in miR-210 expression (Fig. 1D). To determine whether overexpression of miRNAs can regulate the generation of HBV and antigen expression, we transfected these two miRNA expression plasmids into HepG2 2.2.15 cells. ELISA and real-time PCR assays showed that ectopic expression of miR-199a-3p and miR-210 resulted in a 30–40% reduction of HBsAg expression and suppression of HBV replication but had no effect on cell proliferation (Fig. 1E and F). In addition, combining transfection of plasmids expressing pri (primary)-miR-199a and pri-miR-210 was also performed, but the cumulative effect of both miRNAs on HBsAg expression was not observed (Supplemental Fig. 1C), indicating their antiviral mechanism might be similar, so that single miR-199a or miR-210 may be sufficient to play the role in suppression of HBV replication.

3.3. MiR-199a-3p and miR-210 target the transcripts of HBV genes

Identification of functional target genes of miR-199a-3p and miR-210 will be necessary in order to illuminate the mechanism of their regulation of HBV proliferation. We used ViTa (Hsu et al., 2007) to identify viral targets of these human miRNAs. The results show that the HBsAg encoded region carries a putative miR-199a-3p binding site, while the pre-S1 region of HBV (HBVPS1) carries a putative miR-210 binding site (Table 1). The alignments of miR-199a-3p with HBsAg and miR-210 with HBPS1 are shown in Fig. 2A. Because miR-199a-3p and miR-210 both have only one putative binding site in the HBV pre-S1/pre-S2/S region, we separately amplified 165 bp and 306 bp fragments of

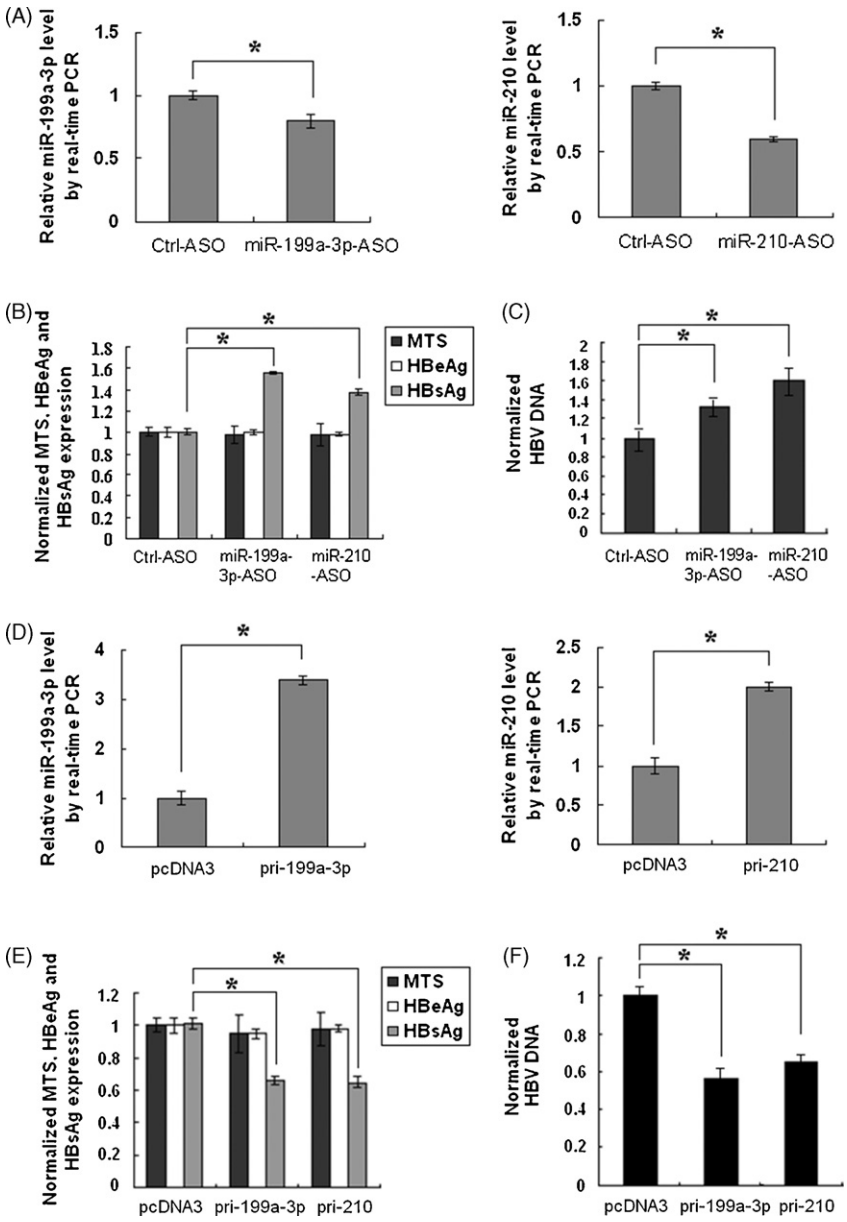

Fig. 1. MiR-199a-3p and miR-210 efficiently suppress HBsAg expression and HBV replication. (A) HepG2 2.2.15 cells were transfected with miRNA ASOs or control oligo. The expression levels of miR-199a-3p and miR-210 were assessed by real-time RT-PCR. Expression levels were normalized to that of the small nuclear RNA U6. (B) ASO of miR-199a-3p and miR-210 increased HBsAg expression. HBsAg and HBeAg levels from the culture supernatant harvested from HepG2 2.2.15 cells transfected with miRNA ASOs or control oligo were detected by ELISA. In addition, cell activity was tested by MTS. (C) ASO of miR-199a-3p and miR-210 increased HBV replication. HBV DNA levels were analyzed by real-time PCR. The HBV DNA levels in the control group were set to 1. (D) HepG2 2.2.15 cells were transfected with miRNA overexpression vector or control vector, and the expression levels of miR-199a-3p and miR-210 were assessed by real-time RT-PCR. Expression levels were normalized to that of the small nuclear RNA U6. (E, F) Overexpression of miR-199a-3p and miR-210 reduced HBV replication and HBsAg expression. HepG2 2.2.15 cells were transfected with miRNA overexpression vector or control vector. At 72 h post-transfection, culture supernatants were harvested and analyzed by ELISA (E) and real-time PCR (F). The expression level of HBsAg and HBV DNA in the control group was set to 1. The data represent results from three independent experiments performed in triplicate. $^*P < 0.05$.

pre-S1 and HBsAg encoded regions from the HBV genome containing only one putative binding site of miR-199a-3p or miR-210, and cloned them downstream of an enhanced green fluorescent protein (EGFP) CDS with stop codon in pcDNA3.1(+)/EGFP as a reporter. At the same time, mutated vectors bearing a triple mutation in the seed sequence were also constructed (Fig. 2B). HepG2 2.2.15 cells were subsequently transfected with the reporter vector, as well as with miRNA ASO or control oligomer. The RFP expression vector pDsRed2-N1 (Clontech) was also transfected in HepG2 2.2.15 cells for normalization. EGFP and RFP activity was measured at 48 h post-transfection. Results are represented as normalized ratios of EGFP to RFP. As shown in Fig. 2C and D, the intensity of EGFP fluorescence in the miRNA-suppressed groups was significantly greater than in the control groups. Furthermore, ectopic expression of miR-199a-3p or miR-210 in HepG2 2.2.15 cells transfected with miRNA expression vectors was sufficient to reduce the level of EGFP (Fig. 2E and F). Reporter expression was unrelated to the lev-

Table 1
Target genes of miR-199a-3p and miR-210.

Human miRNAs	Target genes
hsa-miR-199a-3p	(1) HBV S protein (2) HBV polymerase
hsa-miR-210	(1) Pre-S1 region of HBV large S protein (pre-S1/pre-S2/S) (2) HBV polymerase

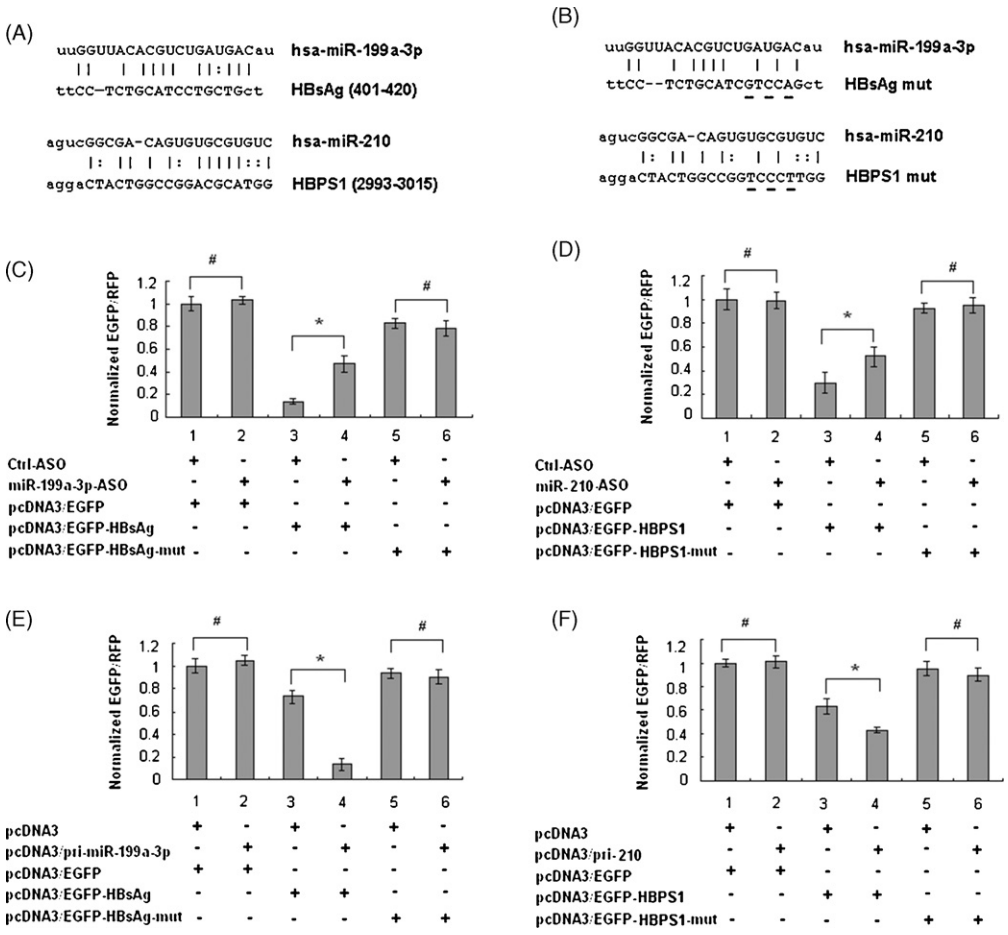

Fig. 2. HBsAg is a target of miR-199a-3p and HBSP2 is a target of miR-210 by EGFP reporter experiments. (A, B) The complementary sequences of miR-199a-3p and miR-210 candidate target 'seed sequence' (A) or a mutated form (B), as indicated. Mutated nucleotides are underlined. The complementary sequences of HBV genome to miR-199a-3p and miR-210 were obtained from publicly available algorithms. (C, D) HepG2 2.2.15 cells were cotransfected with an EGFP reporter plasmid (pcDNA3/EGFP-HBsAg, pcDNA3/EGFP-HBSP1, pcDNA3/EGFP-HBsAg-mut, pcDNA3/EGFP-HBSP1-mut) and a pDsRed1-N1 plasmid, together with miRNA ASO or control ASO. EGFP expression of sensor plasmids bearing the target gene sequence was inhibited by endogenous miRNA in HepG2 2.2.15 cells. The inhibition was reduced by the introduction of miRNA ASO, whereas expression of EGFP with the HBsAg mutant and HBSP1 mutant showed no difference between the miRNA ASO group and the control group. The fluorescence value in the control group was set to 1. The histogram shows normalized mean values (±SD) of fluorescence from three independent experiments. (E, F) HepG2 2.2.15 cells were cotransfected with an EGFP reporter plasmid and pDsRed1-N1 plasmid, together with either a miRNA overexpression vector or control vector. Forty-eight hours after transfection, EGFP and RFP activities were measured with an F-4500 fluorescence spectrophotometer. Overexpression of miR-199a-3p and miR-210 by miR-199a-3p and mi-210 expression vector in HepG2 2.2.15 cells inhibited the expression of EGFP with HBsAg coding sequence or HBSP1 sequence, whereas expression of EGFP with mutant sequence showed no difference between the miRNA ectopic expression group and the control group. The fluorescence intensity in control group was set to 1, and the normalized mean ± SD of fluorescent intensities of three independent experiments are shown in the histograms. $^{*}P < 0.05$; $^{\#}P > 0.05$.

els of miR-199a-3p and miR-210 when the miRNA seed sequence binding site was mutated. In order to further confirm the direct effects of the two miRNAs on their candidate targets, HEK 293 cells were cotransfected with EGFP 'sensor' plasmids and corresponding miRNA ASOs or precursor miRNAs. The same results were obtained as with HepG2 2.2.15 cells (data not shown). Thus, all our observations indicate that miR-199a-3p and miR-210 directly target the transcripts of HBV genes to suppress HBsAg expression.

Since miRNAs may suppress the expression of target genes through translational repression or through degradation of a target's transcript, we used real-time PCR to assess whether the HBsAg mRNA level is regulated by miR-199a-3p and miR-210. In contrast to the controls, overexpression of miR-199a-3p or miR-210 in HepG2 2.2.15 cells resulted in a significant reduction in HBsAg mRNA levels, whereas a knockdown of miR-199a-3p or miR-210 by antisense oligomer increased HBsAg mRNA levels (Fig. 3A). Western blot assay showed that miR-210 and miR-199a-3p could also inhibit translations of the HBsAg protein (Fig. 3B). These results indicated that miR-199a-3p and miR-210 could reduce HBsAg expression through both translation inhibition and RNA degradation. Meanwhile, HBsAg mRNA and protein levels in cotransfected HepG2 2.2.15 cells showed no significant difference compared to single-ASO or single-overexpression plasmid transfected cells (Fig. 3A and B), which is consistent with previous results.

3.4. MiR-199a-3p and miR-210 were upregulated in HepG2 2.2.15 cells compared to HepG2 cells

MiR-199a-3p and miR-210 negatively regulated virus replication by targeting the HBV pre-S1/pre-S2/S region in HepG2 2.2.15 cells. The next question addressed was whether HBV replication could modulate the endogenous expression of miR-199a-3p and miR-210 in cells. Therefore, we compare the expression level of miR-199a-3p and miR-210 in HepG2 2.2.15 cells with its parent cell line HepG2 cells using real-time PCR. Stem-loop RT followed by real-time PCR has been reported to be a sensitive and specific approach for the quantification of miRNAs (Chen et al., 2005).

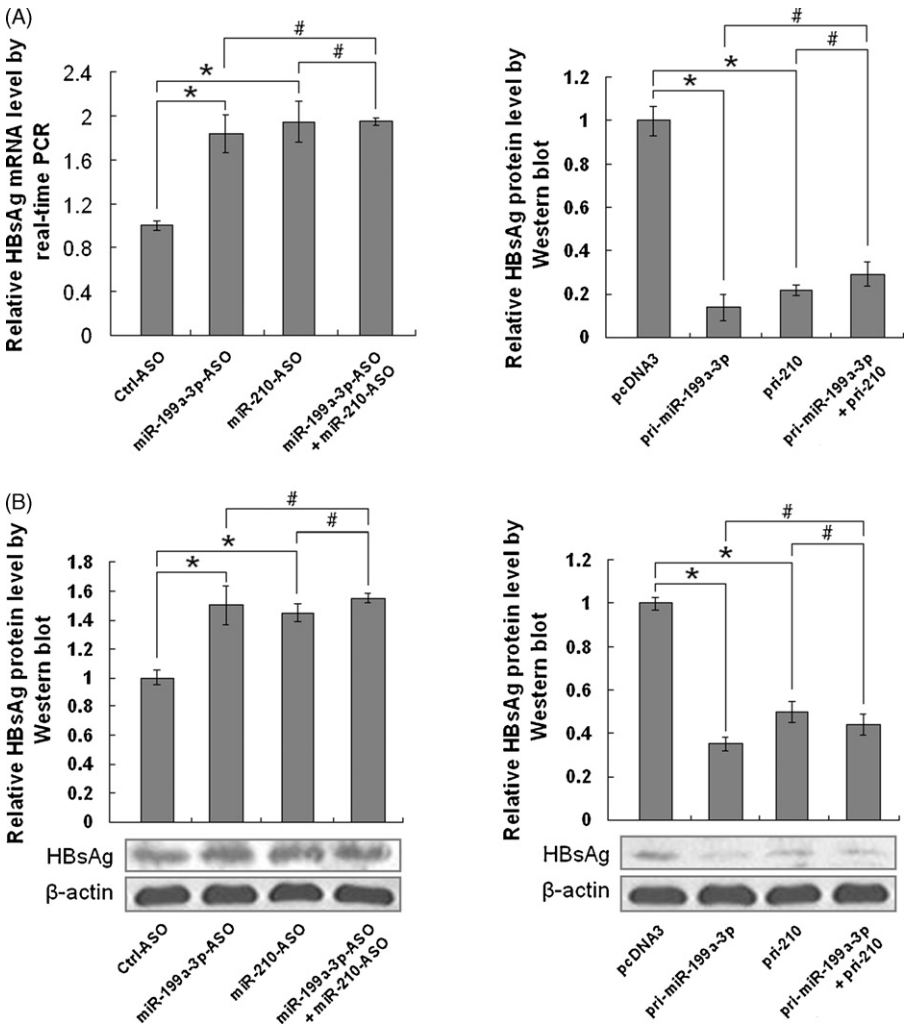

Fig. 3. Overexpression of miR-199a-3p and miR-210 reduced the level of mRNAs and proteins of HBs gene. (A) HepG2 2.2.15 cells were transfected with miRNA precursors and ASOs, respectively. At 48 h post-transfection, the mRNA level of HBsAg was determined by real-time PCR. Expression levels were normalized to that of β-actin. The data represents the mean value(±SD), and the control group was set to 1. (B) The HBsAg protein level of transfected HepG2 2.2.15 cells was detected by Western blot. The upper charts show the quantification results for Western blot. Data are shown as the mean values (±SD) of three independent experiments. $^{*}P < 0.05$; $^{\#}P > 0.05$.

Genomic DNA contamination was detected by performing a "no-RT" control, and no bands were found in agarose gel analysis, indicating the fine quality of our RNA samples (data not shown). The result showed a 9-fold increase of miR-199a-3p and miR-210 in HepG2 2.2.15 cells compared to HepG2 cells (Fig. 4). MiR-181b, which is upregulated in HepG2 2.2.15 cells, was also detected as a positive control according to the report from Liu et al. (2009).

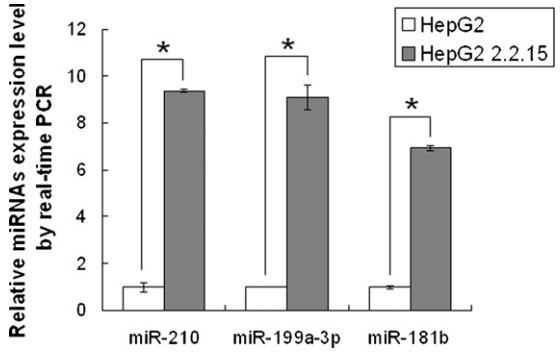

Fig. 4. MiR-199a-3p and miR-210 were overexpressed in HepG2 2.2.15 compared to HepG2 cells. The mRNA level of miR-199a-3p and miR-210 in HepG2 2.2.15 and HepG2 cells were detected by stem-loop RT followed by real-time PCR. Expression levels were normalized to that of the small nuclear RNA U6. The miRNA level in HepG2 was set to 1. $^{*}P < 0.05$.

4. Discussion

Recent reports have highlighted the role of miRNAs as critical effectors in intricate networks of host–pathogen interactions (Voinnet, 2005; Berkhout and Jeang, 2007; de Vries and Berkhout, 2008). To address whether miRNAs influence HBV replication and gene expression, we first employed a loss of function approach, transfecting HepG2 2.2.15 cells with chemically synthesized antisense oligonucleotides of 328 identified human miRNAs. The results showed that miR-199a-3p and miR-210 inhibitors can increase HBsAg expression and HBV replication without significant effecting host cell proliferation, and that ectopic expression of miR-199a-3p and miR-210 results in reduction of HBsAg expression and suppression of HBV replication. In contrast to the effects of the miRNAs on HBsAg antigen levels, HBeAg levels did not change whenever miR-199a-3p and miR-210 were blocked or overexpressed.

Since the identification of miRNA target genes may help to identify their functions, we employed bioinformatics to predict miRNA targets. Algorithm analysis indicated that miR-199a-3p and miR-210 binding sites are present in transcripts of the HBV gene. MiR-199a-3p and miR-210 target the HBV S protein coding region and pre-S1 region, respectively. Since the HBV genome is highly compact and its four open reading frames (ORFs) overlap extensively, miR-199a-3p and miR-210 also target the ORF of HBV polymerase (Table 1). In addition, the replication of the HBV genome occurs via an RNA intermediate (Miller et al., 1984) which contains putative target sites of miR-199a-3p and miR-210. MiR-199a-3p and miR-210 may suppress the production of HBV HBsAg and HBV viron by post-transcriptional gene silencing. Our results showed that both miR-199a-3p and miR-210 could decrease HBsAg expression and the production of HBV particle in the supernatant of HepG2 2.2.15 cells. However, HBeAg, being a soluble antigen that is not the target of miR-199a-3p and miR-210, can be secreted unaffectedly into the culture medium.

In HBV chronic infections, HBV infects hepatocytes and maintains a low level of virus production over a long period. The factors that determine the development of chronic infection with HBV have not been fully identified. Immune system tolerance to the surface protein of HBV appears to be one of the factors involved in the development of the carrier state. Here we identified two host-encoded anti-HBV miRNAs, miR-199a-3p and miR-210. We speculated that up-regulation of miR-199a-3p and miR-210 in HepG2 2.2.15 cells compared to HepG2 cells may be involved in the maintenance of a suitable lower level of virus antigen and virion production, which may contribute to a persistent chronic HBV infection or HBV latency state.

To date, miRNAs have been shown to play a role in the antiviral defense of plants and invertebrates. Here, we provide evidence that human miRNAs can inhibit HBV replication by targeting important HBV genes. Our work reveals the existence of an intricate physiological interplay between the cell's miRNAs and HBV replication that suggests the identification of anti-HBV miRNAs and their target genes may have future diagnostic and therapeutic implications.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.antiviral.2010.08.008.

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