MicroRNA-20 induces methylation of hepatitis B virus covalently closed circular DNA in human hepatoma cells

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Abstract. Methylation was suggested to suppress the transcriptional activity of hepatitis B virus (HBV) covalently closed circular DNA (cccDNA) in hepatocytes. This may be associated with its low replicative activity during the inactive stage of chronic HBV infection; however, the exact mechanisms of methylation in HBV infection remain unknown. We have previously shown that short hairpin RNAs induced the methylation of the HBV genome in hepatoma cell lines. We also reported that the microRNA (miR) 17-92 cluster negatively regulates HBV replication in human hepatoma cells. In addition, miR-20a, a member of the miR 17-92 cluster, has sequence homology with the short hairpin RNA that induces HBV methylation. In the present study, we investigated whether miR-20a can function as an endogenous effector of HBV DNA methylation. The results indicated that overexpression of miR-20a could suppress the replicative activity of HBV and increased the degree of methylation of HBV cccDNA in the HepAD38 hepatoma cell line. Argonaute (AGO)1 and AGO2, effectors of the RNA-induced silencing complex, were detected in the nucleus of HepAD38 cells; however, only AGO2 was bound to HBV cccDNA. In addition, intranuclear AGO2 was determined to be bound with miR-20a. In conclusion, miR-20a may be loaded onto AGO2, prior to its translocation into the nucleus, inducing the methylation of HBV DNA in human hepatoma cells, leading to the suppression of HBV replication.

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Abbreviations: HBV, hepatitis B virus; cccDNA, covalently closed circular DNA; miR, microRNA; AGO, Argonaute; RISC, RNA-induced silencing complex; DNMT, DNA methyltransferase; RdDM, RNA-directed DNA methylation; ChIP, chromatin immunoprecipitation; RNP-IP, ribonucleoprotein immunoprecipitation

Key words: HBV, methylation, miR, AGO, cccDNA

Introduction

Hepatitis B virus (HBV) poses global health burden by infecting >250 million people worldwide (1). Although the global prevalence of acute HBV infection is decreasing due to effective immunization, the prevalence chronic hepatitis B (CHB) and other HBV-associated chronic liver diseases have not reduced during the past decade (2). One of the main challenges in HBV eradication is the persistence of the viral minichromosome, also known as covalently closed circular DNA (cccDNA) which can stably remain in the nucleus of infected hepatocytes even after prolonged effective antiviral therapy (3,4). Thus, HBV cccDNA has emerged as the main target molecule for the complete cure of chronic HBV infection (5).

The natural course of CHB has characteristic stages with respect to viral loads and hepatic inflammation: The initial immune tolerant phase with high viral loads and normal hepatic inflammation markers is followed by an immune clearance phase with a flare of hepatic inflammation and decrease in viral DNA levels; finally, the immune control phase with low or undetectable circulating viral loads ensues (6,7). A progressive decrease in the serum HBV viral loads may be attributed to reductions in the amount of nuclear HBV cccDNA; however, HBV cccDNA persists throughout the natural course of CHB, even in patients with complete virologic and serologic clearance, suggesting that the viral productivity of HBV cccDNA may decrease over time (4,7). In addition, analysis of intrahepatic HBV RNA levels has revealed the transcriptional activity of cccDNA to decrease in the immune control phase of CHB (8,9). The transcriptional activity of HBV cccDNA is modulated by a variety of non-specific and liver-specific transcription factors (8). Additionally, epigenetic modifications of HBV cccDNA, such as histone modifications and methylation of HBV cccDNA may also affect cccDNA transcription (10-12). Previously, studies by our group and other researchers have reported that the methylation of HBV cccDNA contributes to the transcriptional control of HBV cccDNA in CHB (13-15); however, the underlying mechanism by which the HBV cccDNA is methylated is yet to be elucidated.

MicroRNAs (miRNAs/miRs) regulate mammalian gene expression at the post-transcriptional level by directing

RNA-induced silencing complex (RISC) to complementary mRNA targets, and subsequently inducing translational repression and/or mRNA destabilization (16,17). miRNAs may also regulate gene expression at the transcriptional level indirectly by modifying the expression of DNA methyltransferases (DNMTs) (18,19). In plants, miRNAs directly induce transcriptional silencing via DNA methylation by recruiting Argonaute (AGO)-containing effector complexes to the target DNA (20-24). Whether miRNAs can directly induce transcriptional silencing in mammalian cells remains unknown; however, similar miRNA machineries may also function, analogous to RNA-directed DNA methylation (RdDM) by small interfering RNA (25) or Piwi-interacting small RNA (26,27) in mammalian cells.

Our previous study demonstrated that short hairpin RNA induced the methylation of HBV cccDNA in hepatoma cells (28). Interestingly, the target sequence of the short hairpin RNA overlaps with the potential target site of *Homo sapiens*-miR-20a, a member of the oncogenic miR-17-92 cluster (29-31). In addition, our previous report has shown that the miR-17-92 cluster negatively regulates HBV replication in human hepatoma cells (32), and the HBV X sequence exhibited conserved homology to miR-20a across HBV genotypes (Fig. 1).

Considering the structural homology between short hairpin RNAs and miRNA precursors, we hypothesized that miR-17-92 may also induce the methylation of HBV cccDNA. To investigate this hypothesis, miR-17-92 precursors were transfected into human hepatoma cells and the methylation profiles of the HBV genome were determined.

Materials and methods

In vitro HBV replication model and isolation of HBV cccDNA. HepAD38 cells that support HBV replication under the control of a tetracycline-inducible promoter (a generous gift from Professor C. Seeger (Fox Chase Cancer Center, Philadelphia, PA, USA) were used (33). HBV replication was induced by omitting tetracycline from the DMEM/F12 culture medium (Welgene, Inc., Gyeongsan, South Korea) for 5-7 days. All cells were maintained in 5% CO_2 at 37˚C. HBV cccDNA was purified as previously reported (28), precipitation of protein-bound DNA was omitted to allow the utilization of both the protein-free and protein-bound form of cccDNA. Briefly, HepAD38 cells were lysed by the addition of 0.4 ml of cell lysis buffer [50 mM Tris-HCl (pH 8.0), 1 mM EDTA, 0.2% (v/v) Nonidet P-40 and 0.15 M NaCl]. The lysate was centrifuged at 16,000 x g at 4˚C for 2 min, and the supernatant (cytoplasmic fraction) was collected for the extraction of HBV relaxed circular DNA (rcDNA, described below). The pellet of the nuclear fraction was treated with the same volume of nuclear lysis buffer (6% SDS, 0.1 N NaOH) at 37˚C for 15 min, followed by phenol extraction using an volume of phenol-chloroform-isoamyl alcohol mixture (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) and ethanol-precipitation with double the volume of absolute alcohol (Sigma-Aldrich; Merck KGaA). Contaminating genomic DNA was removed using Plasmid-Safe DNase (Epicentre Biotechnologies, Madison, WN, USA), according to the manufacturer's instructions, and the absence of contaminating genomic DNA was confirmed by the negative PCR results obtained using a primer pair for β-globin: Forward, 5'-GTGCACCTGACTCCT GAGGAGA-3' and reverse, 5'-CCTTGATACCAACCTGCC CAG-3'.

Overexpression of the miR-17-92 cluster. Plasmids expressing human miRNA precursor were purchased from OriGene Technologies, Inc. (Rockville, MD, USA): pCMV-MIR control (6.2 kb, cat. no. PCMVMIR), pCMV-MIR17-5p (6.3 kb, cat. no. SC400201), pCMV-MIR18a (6.3 kb, cat. no. SC400218), pCMV-MIR19a (6.3 kb, cat. no. SC400253), pCMV-MIR20a (6.3 kb, cat. no. SC400269) and pCMV-MIR92a (6.3 kb, cat. no. SC400682). Plasmids were transfected into HepAD38 cells using Lipofectamine® 2000 (Thermo Fisher Scientific, Inc., Waltham, MA, USA) at a concentration of 10 μg per 60 mm dish, according to the manufacturer's instruction (Thermo Fisher Scientific, Inc.). Following transfection, the cells were incubated at 37˚C in 5% CO_2 for 5 days prior to analysis for HBV replication and methylation as described below.

Quantification of cytoplasmic HBV DNA by quantitative (q) PCR and Southern blotting. HepAD38 cells grown on a 60-mm culture dish at a density of $5x10^5$ cells/ml in 4 ml medium were lysed and nuclei were pelleted as previously reported (34). The cytoplasmic fraction was treated with 1/4 volume of 35% PEG 8000 in 1.75 M NaCl, incubated on ice for 30 min and centrifuged at 16,000 x g for 10 min at 4˚C. The pelleted HBV core particles were dissolved in proteinase K buffer [10 mM Tris-HCl (pH 8.0), 100 mM NaCl, 1 mM EDTA, 0.5% SDS, 200 μg/ml proteinase K] and incubated at 45˚C for 1 h. The core-associated rcDNA was recovered by phenol extraction and ethanol precipitation as aforementioned.

qPCR was performed as previously reported to quantify HBV rcDNA (35). PCR amplification was performed using SYBR® Green (Enzynomics, Inc., Daejeon, South Korea) and the following primer pair, at final concentration of 200 nM, was sued to amplify nucleotides 2,268-2,286 (Genbank accession number AF286594): Forward, 5'-GAGTGTGGATTC GCACTCC-3' and reverse, 5'-GAGGCGAGGGAGTTCTTC T-3'. The Thermal Cycler Dice Real Time System (Takara Bio, Inc., Otsu, Japan) was used according to the manufacturer's instructions. The thermocycling conditions of qPCR comprised an initial denaturing step at 95˚C for 15 min, followed by 45 amplification cycles at 95˚C for 10 sec, 60˚C for 20 sec, 72˚C for 30 sec. Mitochondrial DNA was amplified with the following primers and used for normalization: Forward, 5'-GCCTGCCTGATCCTCCAAAT-3' and reverse, 5'-AAGGTAGCGGATGATTCAGCC-3'.

Southern blotting for the analysis of HBV rcDNA was performed as previously reported (13). Briefly, 15 μg of HBV rcDNA was electrophoresed on a 1% agarose/Tris-Borate EDTA gel, followed by partial depurination with HCl (0.25 M) for 15 min and denaturation in NaOH (0.5 M) for 15 min. DNA was transferred in 20X standard saline citrate (SSC) buffer to a Hybond-N+ membrane (Roche Diagnostics GmbH, Mannheim, Germany) by capillary blotting. The membrane was UV-cross-linked and hybridized with digoxygenin-tagged HBV RNA probes in Dig Easy Hybridization Buffer (20 ng/ml

stock diluted 1:5,000; Roche Diagnostics GmbH, Mannheim, Germany;) overnight at 60˚C. Following hybridization, the membrane was washed twice for 5 min at room temperature in 2X SSC, 0.1% SDS and twice for 15 min at 60˚C in 0.1X SSC and 0.1% SDS. The membrane was blocked with blocking reagent (Roche Diagnostics GmbH) for 30 min at room temperature and incubated for 30 min in blocking buffer containing 187.5 mU/ml (1:4,000 v/v) anti-digoxigenin-alkaline phosphatase (AP; Roche Diagnostics GmbH). The membrane was treated with Immun-Star AP Substrate (Bio-Rad Laboratories, Inc., Hercules, CA, USA) and was exposed to X-ray film for 1 h. In order to estimate the viral productivity of cccDNA, the loading amounts of HBV DNA were normalized by the copy numbers of HBV cccDNA determined by RT-qPCR as previously reported (36). Densitometry analysis was performed using Quantity One software (version 4.6.2; Bio-Rad Laboratories, Inc.).

Quantification of cytoplasmic HBV pregenomic RNA (pgRNA) using reverse transcription (RT)-qPCR and dot blot analysis. Total RNA was extracted from the cytoplasmic fraction of HepAD38cells using AccuZol reagent (Bioneer Corp., Daejon, South Korea), according to the manufacturer's instructions, and treated with 5 U/μl DNase (Takara Bio, Inc., Otsu, Japan). RT was performed using 10 μg of total RNA and random hexamers with MMLV (iNtRON Biotechnology, Seongnam, South Korea) as recommended. RT was performed using the following thermocycling conditions: 65˚C for 5 min, 4˚C for 5 min, 25˚C for 10 min, 37˚C for 50 min, 85˚C for 5 min and 37˚C for 20 min. qPCR was then performed with the same primers for rcDNA amplification, with GAPDH used for normalization: Forward, 5'-GCACCGTCAAGGCTGAGAAC-3' and reverse, 5'-ATGGTGGTGAAGACGCCAGT-3'.

A dot blot assay was performed in a manner to Southern blotting. Briefly, 2 μg of cytoplasmic RNA was obtained as aforementioned and was denatured using 1M NaOH and fixed on the Hybond-N+ membrane by microwave heating for 2 min. Hybridization and signal detection with digoxygenin-tagged HBV RNA probe was performed as described for Southern blotting.

RNA stability assay. Actinomycin D (5 μg/ml, Sigma-Aldrich; Merck KGaA) was added to the culture medium of HepAD38 cells to block transcription at 24 h following transfection of miRNA mimics (125 nmol). Total RNA was extracted at 1, 3 and 6 h following actinomycin D treatment under the aforementioned culture condition and HBV pgRNA was measured by RT-qPCR as aforementioned.

Assessment of HBV cccDNA methylation by methylation-specific PCR and bisulfite sequencing. HBV cccDNA was isolated as described above and subjected to bisulfite modification using the Imprint DNA Modification Kit according to the manufacturer's instructions (MOD50; Sigma-Aldrich; Merck KGaA Darmstadt, Germany). The primers for methylation-specific PCR were designed based on the universal CpG island II sequence of HBV (37) by using Methyl Biosystems Primer Express (v1.0, Applied; Thermo Fisher Scientific, Inc.): Nonmethylation forward, 5'-GTGGGATGTTTTTTGTTTAT 3', reverse, 5'-ACAAAATACACACAATCCCAA-3'; methylation forward, 5'-GCGGGACGTTTTTTGTTTAC-3' and methylation reverse, 5'-ACGAAATACACACGATCCGA-3' at final concentration of 200 nM. The C1000™ Thermal Cycler (Bio-Rad Laboratories, Inc., Hercules, CA, USA) was used, according to the manufacturer's instructions. The thermocycling conditions for qPCR comprised initial denaturation at 95˚C for 15 min, followed by 45 amplification cycles at 95˚C for 10 sec, 55˚C for 20 sec and 72˚C for 30 sec. Bisulfite sequencing was also performed by PCR-based TA cloning of bisulfite-modified HBV cccDNA CpG island II as previously reported (28). Briefly, the PCR product were purified by AccuPrep® PCR Purification Kit (Bioneer Corporation, Daejeon, South Korea) and 50 ng of purified DNA was ligated with 50 ng of pDrive cloning vector (Qiagen GmbH). Bisulfite sequencing was performed using the T7 promoter primer: 5'-TAATACGACTCACTATAGGG-3'.

Chromatin immunoprecipitation (ChIP) and ribonucleoprotein immunoprecipitation. The association of AGO with HBV cccDNA was assessed using ChIP (38,39). Briefly, plasmids expressing FLAG-AGO1 and FLAG-AGO2 protein (Addgene plasmid cat. no. 21533 and 21538, respectively; Addgene, Inc., Cambridge, MA, USA; gifts from Professor Edward Chan, University of Florida) (40) were transfected into HepAD38 cells as aforementioned. On day 5, cells were cross-linked using 1.42% formaldehyde for 15 min at room temperature. Nuclei were isolated and sonicated as described (38). Chromatin samples, 100 μg in 100 μl of dilution buffer (0.01% SDS, 1.1% TritonX-100, 1.2 mM EDTA, 16.7 mM Tris/HCl, pH 8.0, 167 mM NaCl), were treated with either 8 μg of anti-FLAG M2 antibody (cat. no. F1804, Sigma-Aldrich; Merck KGaA) or normal mouse IgG (cat. no. sc-2025, Santa Cruz Biotechnology, Inc., Dallas, TX, USA) as a control, with rotation for 10 min at room temperature and then pre-incubated with Dynabeads Protein G (Thermo Fisher Scientific, Inc.) as recommended. Following several steps of washing, Chelex100 (cat. no. 142-1253, Bio-Rad Laboratories, Inc.) slurry was added directly to the washed beads (39). DNA was eluted by boiling the Chelex slurry, and HBV cccDNA was detected using the following primers by qualitative PCR: Forward, 5' CTGAATCCCGCGGACGACCC-3' and reverse, 5'-ACCCAAGGCACAGCTTGGAGG-3' at final concentration of 200 nM after treatment with Plasmid-safe DNase. The thermocycling conditions for PCR comprised of an initial denaturation at 95˚C for 5 min, followed by 35 cycles at 94˚C for 1 min, 60˚C for 1 min, 73˚C for 1 min and a final extension of 73˚C for 5 min (41).

The presence of the nuclear AGO-miR complex was assessed by ribonucleoprotein immunoprecipitation (RNP-IP). Briefly, 5 μg of plasmid expressing FLAG-AGO2 protein was co-transfected along with 10 μg of either pCMV-MIR control or pCMV-MIR20a plasmid into HepAD38 cells using Lipofectamine® 2000 as aforementioned. On day 5, cells were harvested and cross-linked as described previously (42). RNase Out (final concentration 100 U/ml, cat. no. 10777019, Thermo Fisher Scientific, Inc.) and dithiothreitol (final concentration 1 mM) were added to the nuclear fraction of samples as described (38). Nuclear RNA was released from cross-linked proteins by digestion with 200 μg/ml proteinase K (Takara Bio, Inc., Seoul,

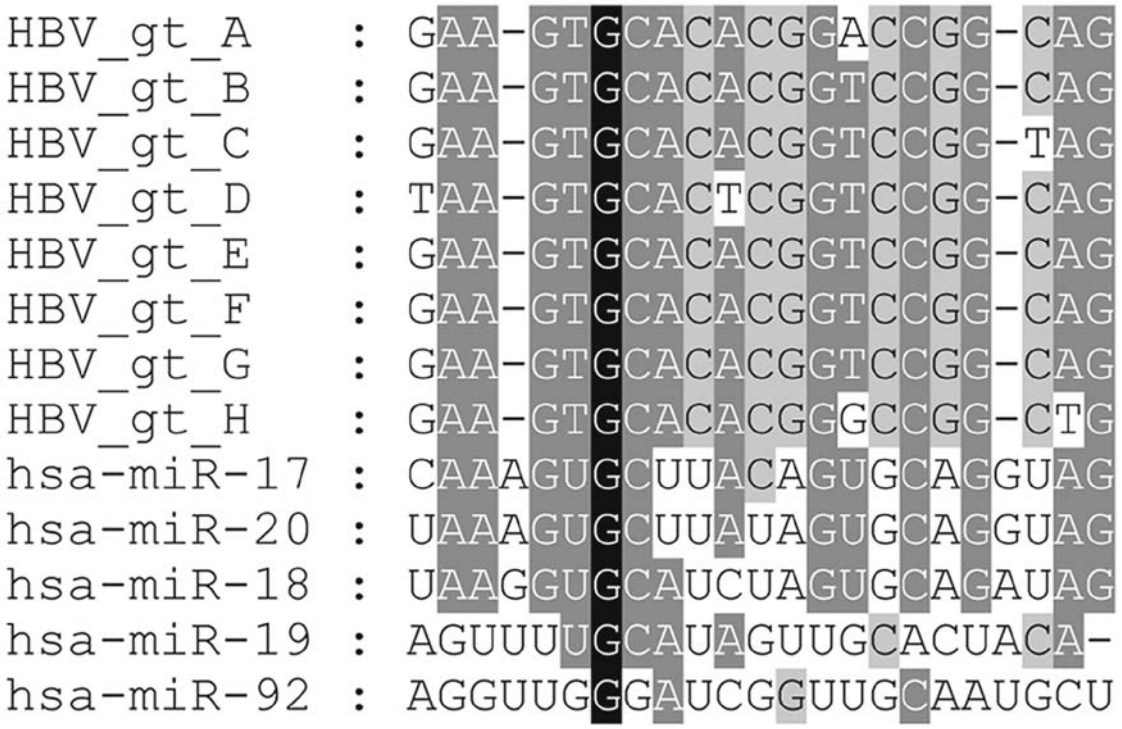

Figure 1. Alignment of HBV and miR-17-92 cluster miRs. Hepatitis B sequences present nucleotides 1,567-1,587 on the X gene of each genotype. Black, dark gray and light gray shades indicate 100, 80 and 60% match with conserved sequence, respectively. HBV, hepatitis B virus; hsa, *Homo sapiens*; miR, microRNA.

South Korea) for 1 h at 55˚C, followed by TRIzol® extraction (Thermo Fisher Scientific, Inc.). Stem-loop RT-qPCR was performed to detect miR-20a with primers designed as reported (43): Stem-loop RT primer, 5'-GTCGTATCCAGT GCAGGGTCCGAGGTATTCGCACTGGATACGACCTAC CT-3'; miR-20 forward, 5'-ACACTCCAGCTGGGTAAA GTGCTTAT-3' and reverse, 5'-CCAGTGCAGGGTCCG AGGTA-3'. RT was performed using 0.2 μg of total RNA and stem loop RT primer (50 nM), dNTPs (250 nM each), MMLV (200 U/ml; Intron Biotechnology, Inc.), RNase inhibitor (40 U/ml; Intron Biotechnology, Inc) in RT buffer [10% glycerol, 50 mM Tris-HCl (pH 8.3), 3 mM MgCl, 20 mM DTT and 75 mM KCl] with the following thermocycling conditions: 16˚C for 30 min, 42˚C for 30 min and 85˚C for 5 min. The qPCR thermocycling conditions were as follows: Initial denaturation at 95˚C for 10 min, followed by 40 cycles at 60˚C for 1 min and 95˚C for 15 sec.

Statistical analysis. qPCR data of HBV rcDNA, HBV RNA and methylation-specific PCR were analyzed via the $2^{-\Delta\Delta Cq}$ method (44). All samples were quantified in triplicate. At least three experiments were repeated and the results were presented as the mean ± standard deviation. P-values were calculated by a pairwise fixed reallocation randomization test using REST software (Qiagen, Inc, Valencia, CA, Inc.) (45); P<0.05 was considered to indicate a statistically significant difference.

Results

miR-20a suppresses the replication of HBV cccDNA at the transcriptional level in HepAD38 cells. Our previous study showed that the miR-17-92 cluster negatively regulated HBV replication in human hepatoma cells (32). qPCR and Southern blotting indicated that miR-17-5p and miR-20a significantly reduced the levels of HBV DNA in HepAD38 cells (Fig. 2A and B). The reduction was associated with significantly suppressed HBV RNA levels (Fig. 2C and D). Analysis of RNA stability revealed that degradation of HBV RNA was not accelerated by miR-17-5p and miR-20a (Fig. 2E). miR-17-5p and miR-20a did not significantly affect the stability of HBV RNA, indicating that the miRs may act on the transcription of HBV RNA in a different manner other than affecting its stability.

miR-20a induces the methylation of HBV cccDNA in HepAD38 cells. Additionally, whether miR-17-5p and miR-20a induced the methylation of HBV cccDNA was determined as these two members of the miR-17-92 cluster were suggested to suppress the replication of HBV at the transcriptional level. Methylation-specific PCR revealed that overexpression of miR-17-5p and miR-20a induced a significant increase in the degree of methylation in HBV cccDNA compared with the control (Fig. 3A). Bisulfite sequencing revealed that the frequency of methylation induced by miR-17-5p did not significantly different from control (P=0.348), whereas miR-20a induced a significantly higher level of methylation in the putative target site of HBV cccDNA (P=0.001; Fig. 3B).

AGO2 translocates to the nucleus of HepAD38 cells and binds to HBV cccDNA. In order to elucidate the mechanism underlying cccDNA methylation by miR-20a, we sought to determine whether AGOs, the main effectors of RISC,

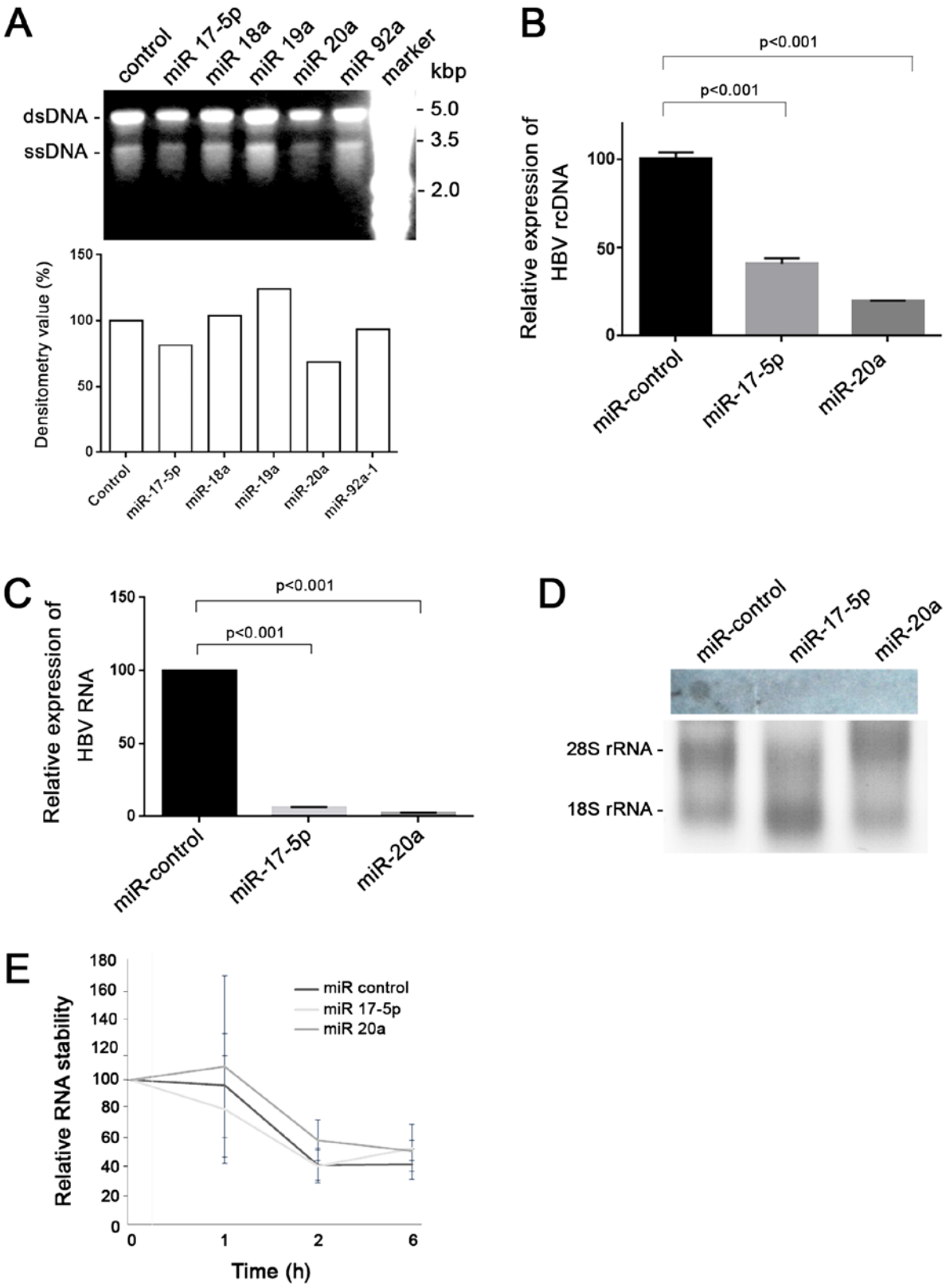

Figure 2. Overexpression of miR-17-5p and miR-20a suppresses HBV replication in HepAD38 cells. (A) Southern blotting analysis of cytoplasmic HBV rcDNA was performed after transfection of miR-17-92 precursor-expressing plasmids. Loading amounts were adjusted according to the copy numbers of HBV cccDNA as determined by qPCR. Therefore, the relative quantity of HBV DNA indicated the viral productivity per unit cccDNA. Compared with the control, miR-17-5p and miR-20a suppressed HBV replication by 18 and 32%, respectively, as evaluated by densitometry analysis. The image used for densitometry analysis is representative of three independent experiments. (B) Cytoplasmic HBV rcDNA levels were determined by qPCR in HepAD38 cells transfected with miR-17-5p and miR-20a precursor-expressing plasmids. Error bars indicate standard deviation. (C) Cytoplasmic HBV RNA was measured by RT-qPCR in HepAD38 cells transfected with miR-17-5p and miR-20a precursor plasmids. The error bars indicate the standard deviation. (D) Dot blot assay of HBV RNA indicated the suppression of HBV RNA by miR-17-5p and miR-20a. (E) Analysis of HBV RNA stability in HepAD38 cells transfected with miR-17-5p and miR-20a precursor plasmids. Actinomycin D was added to culture medium and RNA was harvested at the time points as indicated. Error bars indicate standard deviation. dsDNA, double stranded DNA; HBV, hepatitis B virus; miR, microRNA; rcDNA, relaxed circular DNA; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; ssDNA, single stranded DNA.

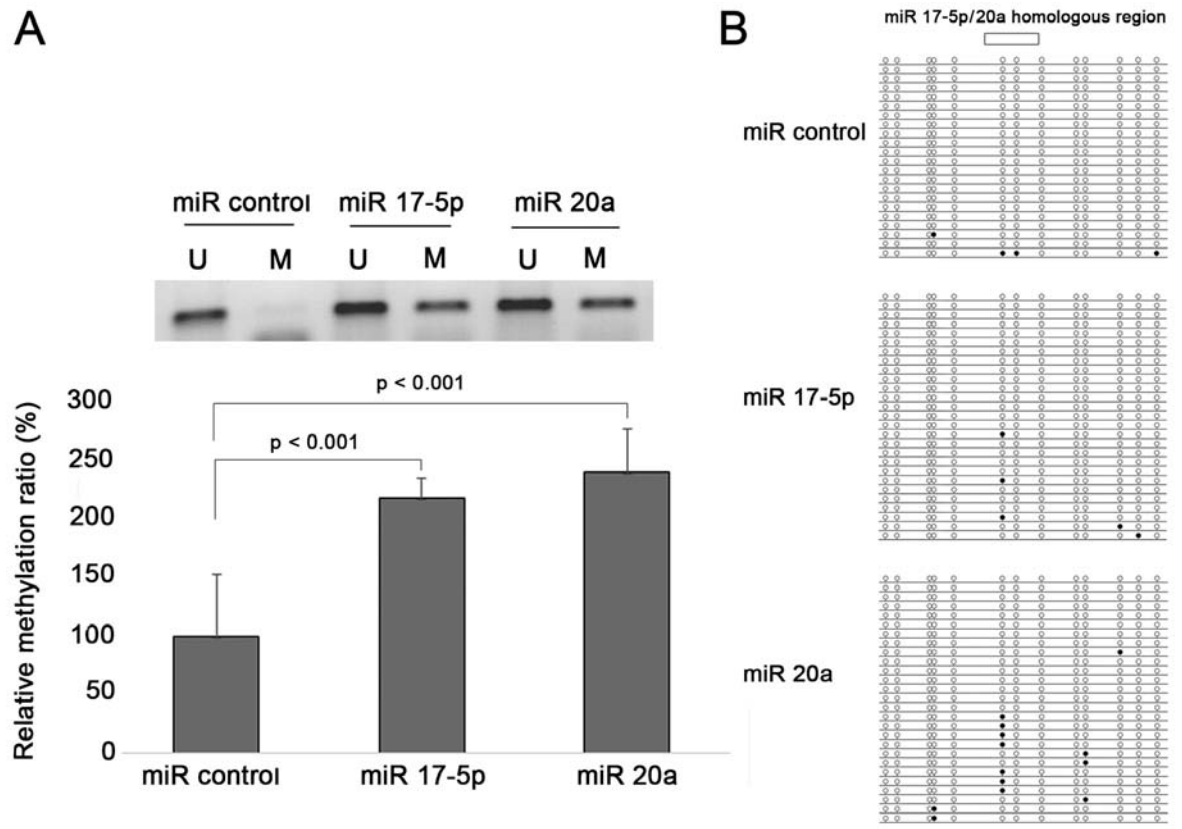

Figure 3. miR-20a induces the methylation of HBV cccDNA. (A) Methylation-specific PCR assay. HepAD38 cells were transfected with miRNA precursor-expressing plasmids and cccDNA was isolated at day 7 for bisulfite modification. Bisulfite-modified HBV cccDNA was amplified with methylation-specific PCR primer sets. (upper panel). The relative amount of methylated to unmethylated cccDNA was assessed (lower panel). The results are representative of three independent experiments; error bars indicate the standard deviation. (B) Bisulfite sequencing analysis of HBV cccDNA. CpG island II was amplified from bisulfite-modified HBV cccDNA, TA-cloned and sequenced. Metheylated and unmethylated CpG dinucleotides were marked by open and filled circles, respectively. Compared with the control, overexpression of miR-17-5p exhibited a similar level of methylation (P=0.348), whereas miR-20a exhibited a significantly higher degree of methylation (P=0.001). cccDNA, covalently closed circular DNA; miR, microRNA; U, unmethylated primer set; M, methylated primer set; HBV, hepatitis B virus; PCR, polymerase chain reaction.

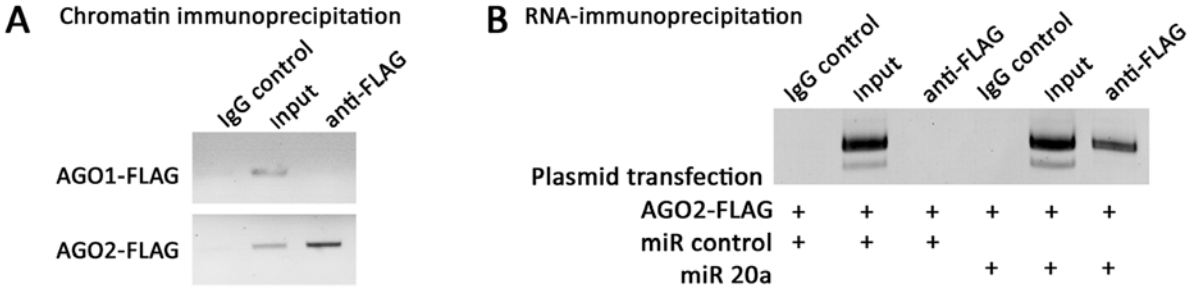

Figure 4. Nuclear AGO2 is associated with HBV cccDNA and miR-20a. (A) Chromatin immunoprecipitation analysis was performed using HepAD38 cells transfected with AGO1-FLAG (upper gel) or AGO2-FLAG plasmids (lower gel). Immunoprecipitated DNA was amplified with HBV cccDNA-specific primers. AGO1 did not bind HBV cccDNA (anti-FLAG lanes, upper gel), whereas AGO2 was associated with HBV cccDNA (anti-FLAG lanes, lower gel). The results were representative of three independent experiments. (B) ribonucleoprotein immunoprecipitation analysis was performed using HepAD38 cells transfected with AGO2-FLAG plasmid, along with an miR-20a-expressing plasmid. RNA was extracted from anti-FLAG immunoprecipitates and amplified for miR-20a. The results were representative of two independent experiments. AGO, Argonaute; miR, microRNA; HBV, hepatitis B virus.

are directed to nuclear HBV cccDNA. The ChIP assay showed that AGO1 and AGO2 were present in the nucleus (Fig. 4A, input lanes). On the contrary, AGO2 but not AGO1 was associated with HBV cccDNA (Fig. 4A).

Nuclear AGO2 associates with miR-20. As AGO2 was associated with nuclear HBV cccDNA, RNP-IP was performed to determine whether miR-20a interacts with intranuclear AGO2. The results demonstrated that overexpression of miR-20a promoted interactions between miR-20a-bound AGO2 in the nuclei of HepAD38 cells (Fig. 4B).

Discussion

Although methylation has been identified *in vitro* (13,28) and *in vivo* (13-15) as a mechanism of transcriptional regulation in

HBV replication, how HBV cccDNA is methylated remains unknown. Our previous study showed that miR-20a interacts directly with the HBV X/polymerase gene sequence (32). The potential target sequence is also shared by a short hairpin RNA which was proposed to induce the methylation of HBV cccDNA (28). These findings prompted us to evaluate the epigenetic effect of miR-20a in the present study. The results indicated that human miR-20a may modulate the *de novo* methylation of nuclear HBV DNA in a hepatoma cell line. To the best of our knowledge, this is the first study to investigate methylation of the HBV genome by overexpressing miRNA.

RNA-directed transcriptional gene silencing (TGS) has been reported in mammalian cells by independent researchers (25,46-48), but the underlying mechanisms have not yet been fully understood. Human miRNA is loaded onto RISC which includes Dicer, AGOs, TAR RNA binding protein and EMSY interactor protein (49), and miR-loaded RISC acts as an effector of slicer-dependent and slicer-independent post-transcriptional silencing (50). In addition, it has been reported that AGO1 and AGO2 are transported to the nuclear compartment in human cells (51). The detection of AGO2 and RNA interference factors in human nuclei suggests their participation in TGS (52-54). Additionally, the nuclear translocation of miR-bound AGO2 has been associated with gene silencing (55); AGO2 and miRNAs are recruited to the promoter region leading to TGS (48,56). In the present study, miR-20a-loaded AGO2 was determined to translocate to the nucleus, which supports the hypothesis that TGS may occur via miR-bound AGO2.

Our data also demonstrated that AGO2 can bind to the nuclear HBV genome (cccDNA). This observation, along with the reported translocation of miR-20a-bound AGO2 into the nucleus, suggests that miR-20a may direct AGO/RISC to the homologous target of HBV DNA. We speculate that miR-guided AGO/RISC may function as an effector of *de novo* methylation in mammalian cells, in a manner analogous to RdDM in plants (57). HBV X protein (HBx) upregulates DNMT3 (58), which is the principal enzyme for mammalian *de novo* DNA methylation (59), and directs DNMT3 to target DNA (60). Based on this, we proposed that HBV cccDNA first binds to miR-guided AGO2-RISC, which in turn recruits HBX-mediated DNMT3 to AGO2-bound cccDNA. However, further investigation is required by studying the interaction between miR-20a-loaded RISC, DNMT3a HBx and HBV cccDNA.

RT-qPCR and hybridization assays in the present study indicated that miR-17-5p and miR-20a suppressed HBV RNA levels but did not affect RNA stability. These findings suggest that miR-induced methylation inhibits the transcription of HBV cccDNA. We previously reported that methylation suppressed the transcriptional activity of HBV cccDNA by an *in vitro* nuclear run-off assay (13). Bisulfite sequencing demonstrated that miR-17-5p markedly induced gene methylation. Since the miR-17-92 cluster was identified in mammalian nuclei (61-64), and the mature sequences of miR-17-5p and miR-20a differ by only two ribonucleotides, miR-17-5p may also induce methylation that could reduce the transcriptional activity of HBV cccDNA. This was indicated by the suppressed viral replication of DNA as observed by Southern blotting and the increased methylation observed by methylation-specific PCR.

The methylation of HBV cccDNA has been reported to contribute to the transcriptional suppression of HBV replication (13-15); however, the exact mechanisms of methylation are yet to be elucidated. The results of the present study may provide insight into the role of miRNAs as an innate epigenetic modulator in chronic HBV infection. Furthermore, our findings may improve understanding of the endogenous mechanisms of TGS in mammalian cells. As the persistence of HBV cccDNA is the major obstacle in curing chronic HBV infection (4,65), these miRNA-induced epigenetic modifications may have therapeutic potential in the development of novel treatments against chronic hepatitis B.

Of note, there are several limitations of the present study to be addressed: i) The recruitment of DNMT by the miR-20a/AGO2 complex to HBV cccDNA needs to be confirmed; ii) the presence of miR-induced methylation machinery should be determined in non-neoplastic hepatocytes infected with HBV; and iii) strategies to circumvent the risk for hepatocellular carcinoma should be developed, considering the oncogenic potential of miR-17-92 (31).

In conclusion, the present study proposed that, in human hepatoma cells, miR-20a is loaded onto AGO2, which is then translocated into the nucleus to induce the methylation of HBV DNA, leading to the suppression of HBV replication.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

IYM, ESJ, SHJ and JWK designed the study; IYM, JHC and JWC performed the experiments; IYM, JHC, JWC, ESJ, SHJ and JWK analyzed data. All authors contributed to the final manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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