Methylation profile of hepatitis B virus is not influenced by interferon α in human liver cancer cells

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Abstract. Interferon (IFN) α is used for the treatment of chronic hepatitis B virus (HBV) infection, but the molecular mechanisms underlying its antiviral effect have not been fully elucidated. Epigenetic modifications regulate the transcriptional activity of covalently closed circular DNA (cccDNA) in cells with chronic HBV infection. IFN-α has been shown to modify cccDNA-bound histones, but it is not known whether the anti-HBV effect of IFN-α involves methylation of cccDNA. The present study aimed to determine whether IFN-α induced methylation of HBV cccDNA in a cell-based model in which HepG2 cells were directly infected with wild-type HBV virions. Methylation status of HBV cccDNA was assessed using global DNA methylation ELISA assay, methylation-specific PCR and bisulfite sequencing. IFN-α suppressed HBV DNA and RNA transcripts, but methylation profiles were similar between the control and IFN-α treated groups. Chromatin immunoprecipitation results revealed binding of DNA methyltransferases (DNMT) 3A and DNMT3B to HBV cccDNA and treatment with IFN-α suppressed the recruitment of DNMT3B to cccDNA. Taken together, these results suggest that IFN-α does not induce methylation of HBV cccDNA. Therefore, it was concluded that methylation is unlikely to contribute to the anti-HBV effect of IFN-α in HepG2 cells, and that alternative mechanisms need to be sought to enhance cccDNA methylation as a novel therapy against HBV.

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Introduction

Hepatitis B virus (HBV) is one of the commonest causes of chronic hepatitis worldwide (1). The chronicity of HBV infection is related to the fact that HBV produces very stable viral genome in the host cells (1). The HBV genome is partially double-stranded relaxed circular DNA (rcDNA), which is converted to complete double-stranded covalently closed circular DNA (cccDNA) in the nucleus of infected hepatocytes (2). HBV cccDNA remains the main hurdle in the eradication of infected HBV as current antiviral agents cannot eradicate this episomal viral minichromosome (3). HBV cccDNA persists in the liver throughout the natural course of chronic HBV infection.

Serum HBV DNA, a standard surrogate marker of HBV replication in hepatocytes, decreases over time during the natural course of infection in numerous patients with chronic hepatitis B (CHB) (4). In addition, some of them lose serum HBsAg which is a sensitive marker of HBV infection and controlled by a separate promoter (2). These findings suggest that the transcriptional activity of HBV cccDNA may decrease in the late stage of infection. Indeed, recent studies confirm transcriptional suppression of cccDNA in HBeAg-negative chronic hepatitis B (5,6). The transcriptional activity of HBV cccDNA is controlled by four promoters (precore/pregenomic, S1, S2 and X) and two enhancer sequences (enhancer I and enhancer II) (7). These cis-regulatory elements recruit several ubiquitous and liver-specific transcription factors and contribute to the regulation of cccDNA transcription (8,9).

In addition to the trans-acting DNA-binding proteins, structural changes in cccDNA, i.e., epigenetic modifications, have been implicated as regulatory mechanisms of cccDNA transcription (10,11). Electron microscopy examination of cccDNA reveals typical 'beads-on-a-string' nucleosomal organization (12). HBV nucleoprotein is composed of HBV core protein and histones (13). Similar to the case of mammalian cellular DNA, posttranslational modifications (PTMs) of histone proteins modulate the transcription of HBV cccDNA (8). Acetylation of cccDNA-bound H3/H4 histones was first reported to promote HBV replication (14). Thereafter, a genome-wise map of PTMs revealed that stimulatory PTMs, such as H3K4me3, H3K27ac and H3K122ac are enriched, whereas suppressive PTMs, including H3K9me3 and H3K27me3, are underrepresented in cccDNA chromatin (15).

Recently, succinylation of H3K79 was reported to upregulate cccDNA transcription (16). Thus, PTMs of histone proteins are now considered to be major regulators of cccDNA transcription.

Methylation of HBV cccDNA has also been pursued as a regulatory mechanism for cccDNA transcription (17-20). There are two CpG island sequences (II and III) which are conserved across the eight major genotypes (21) and are hotspots for methylation of cccDNA isolated from human liver tissues (22). In particular, CpG II is located near the core promoter and methylation densities on CpG II are correlated with HBeAg status (17), transcriptional activity of cccDNA (20) and serum HBV DNA levels (20,22). *De novo* methylation of mammalian DNA is mediated by DNA methyltransferases (DNMT) 3A and DNMT3B, but the mechanisms underlying the methylation process of cccDNA are poorly understood (23).

Interferon (IFN) α has long been used as an antiviral therapy for CHB. The antiviral effect of IFN-α is modest and observed in only a portion of patients compared to that of nucleos(t)ide analogues (1). However, patients responding to interferon treatment typically show durable viral suppression after cessation of therapy (24), suggesting induction of persistent antiviral milieu in hepatocytes. Diverse interferon-stimulated genes (ISGs) work at the transcriptional and post-transcriptional levels to keep HBV replication in check (25-28), but the downstream intracellular mechanisms responsible for the durable interferon response remain to be elucidated. Previous studies have shown that IFN-α induces modification of cccDNA-bound histones (15,16,29,30). These epigenetic modifications may explain the transcriptional suppression of cccDNA and off-treatment sustained HBV suppression by IFN-α (31). However, while IFN-α treatment reduces the stimulatory PTMs (H3K4me3, H3K9ac H3K27ac and H3K122ac), H3K9me3 and H3K27me3, the canonical repressive PTMs, are not enriched by IFN-α, suggesting that additional active epigenetic mechanisms may contribute to selective silencing of the precore/pregenomic HBV promoter by IFN-α (15,28). Considering the suppressive role of CpG II methylation during the natural course of HBV infection, it is hypothesized that IFN-α treatment may induce changes in the methylation status of cccDNA, a hypothesis not yet tested. Thus, the aim of the present study was to elucidate the effect of IFN-α on the methylation status of HBV cccDNA in a cell-based HBV replication system.

Materials and methods

In vitro HBV replication model. Infectious HBV particles were collected from HepAD38 cells (a gift from Professor C. Seeger, Fox Chase Cancer Center, PA), which allow HBV replication under the control of an inducible tetracycline promoter (32). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM)/F12 medium (Welgene, Inc.) containing 10% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin, 0.3 μg/ml tetracycline and 400 μg/ml G418 at 37˚C in a 5% carbon dioxide chamber. HBV replication was induced by omitting tetracycline from the culture medium for 5-7 days. The culture supernatant was centrifuged at 12,000 x g for 5 min at room temperature to remove cellular debris and then ultracentrifuged at 25,000 x g for 4 h at 4˚C.

The pellets were resuspended in PBS and the viral titer was measured using reverse transcription-quantitative (RT-q) PCR. HepG2 cells, a human liver cancer cell line (cat. no. 88065, passage number 5-10, Korean Cell Line Bank, Seoul, South Korea), were infected with HBV particles at a titer of 50 multiplicity of infection (MOI) in the presence of DMSO (cat. no. H 9268; final concentration of 0.2%; Sigma-Aldrich; Merck KGaA) because DMSO facilitates intracellular entry of HBV and enhances intracellular replication (33,34). The cells were then maintained in DMEM containing 10% fetal bovine serum, penicillin and streptomycin. On the following day, IFN-α (Intron A; MSD International GmbH; 15 MU/ml) was replenished at a final concentration of 1,500 U/ml for four days before harvest.

Isolation of HBV rcDNA and cccDNA. HBV-infected HepG2 cells that were grown in a 60-mm tissue culture dish at a density of $5x10^5$ cells/ml were lysed with 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 nuclear pellet was used for cccDNA extraction (as follows) and the supernatant was used to isolate core particle-associated HBV rcDNA (35). Briefly, the cytoplasmic fraction was treated with 1/4 volume of 35% PEG8000 in 1.75 M NaCl, incubated on ice for 30 min and centrifuged at 16,000 x g for 10 min. The precipitated viral particles were dissolved in DNA extraction buffer (10 mM Tris-HCl, pH 8.0; 100 mM NaCl; 1 mM EDTA; 0.5% SDS; 200 μg/ml proteinase K) at 45˚C for 1 h and viral DNA was recovered by phenol-extraction and ethanol-precipitation (36). HBV cccDNA was extracted by dissolving the nuclear pellet in the DNA extraction buffer, followed by phenol-extraction and ethanol-precipitation. Contaminating genomic DNA was removed using treatment with Plasmid-Safe DNase (Epicentre; Illumina, Inc.).

Quantification of HBV using RT-qPCR, Southern blotting and northern blotting. SYBR Green RT-qPCR was performed to quantify HBV rcDNA and cccDNA. Primers for HBV rcDNA were GAGTGTGGATTCGCACTCC (forward) and GAGGCGAGGGAGTTCTTCT (reverse) (37) and for cccDNA were GCGGCTCCCCGTCTGTGCC, GTCTGTGCCTTCTCATCTGC (forward) and GTCCATGCCCCAAAGCAACC (reverse) (38) at a final concentration of 200 nM. The Thermal Cycler Dice Real Time System (Takara Bio, Inc.) was used according to the manufacturer's instructions. The PCR cycling program for HBV rcDNA consisted of 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 and 72˚C for 30 sec. The cycles for HBV cccDNA were initial denaturation at 95˚C for 15 min, followed by 45 amplification cycles at 95˚C for 15 sec, 63˚C for 10 sec and 72˚C for 25 sec. The mitochondrial DNA gene was used for normalization: GCCTGCCTGATCCTCCAAAT (forward) and AAGGTAGCGGATGATTCAGCC (reverse). The Thermal Cycler Dice Real Time System (ver. 6.0.1A, Takara Bio, Inc.) was used for analysis according to the manufacturer's instructions.

Southern and northern blots for HBV rcDNA and RNA were performed using a digoxigenin-labeled RNA probe as previously reported (39,40). Briefly, 15 μg of HBV rcDNA

was electrophoresed on a 1% agarose/Tris-Borate EDTA gel, which was depurinated with HCl (0.25 M) for 15 min and denatured in NaOH (0.5 M) for 15 min. DNA was transferred to a Hybond-N+ membrane (Roche Diagnostics GmbH) by capillary transfer. The membrane was UV cross-linked and hybridized with digoxygenin-tagged HBV RNA probes in Dig Easy Hybridization Buffer (Roche Diagnostics GmbH; 20 ng/ml stock diluted to 1:5,000) overnight at 60˚C. The membrane was washed twice for 5 min at room temperature in 2X SSC, 0.1% SDS and for 15 min at 60˚C in 0.1X SSC, 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-digoxygenin-alkaline phosphate antibody (Roche Diagnostics GmbH) followed by Immun-Star AP Substrate (Bio-Rad Laboratories, Inc.) treatment, and exposed to X-ray film.

For northern blotting, the cytoplasmic fraction was isolated as described above and treated with TRIzol® (Thermo Fisher Scientific, Inc.) and 5 μg of RNA was separated on 1% agarose gel. Equal loading was examined by staining the gels with ethidium bromide and the RNA was transferred onto nylon membranes. Transferred RNA was detected in the same way as Southern blotting analysis.

Analysis of methylation profiles of HBV. The 5-mC dot blot assay was performed to assess the global methylation of HBV cccDNA using mouse anti-5-methylcytosine antibody (cat. no. 33D3; Active Motif, Inc.) as previously reported (41). Briefly, 50 ng of HBV cccDNA was blotted onto a Hybond-N+ membrane, UV-cross linked and subjected to immunodetection with 1:5,000 diluted anti-5mC antibody.

Global methylation of cccDNA was quantified by MethylFlash Global DNA Methylation (5-mC) ELISA Easy kit (cat. no. P-1030; EpiGentek Group Inc.) according to the manufacturer's protocols. A total of 100 ng of cccDNA was used for the assay.

Bisulfite modification of HBV cccDNA was performed as previously reported (42). Methyl Primer Express (v1.0; Applied Biosystems; Thermo Fisher Scientific, Inc.) was used to design primers for methylation-specific PCR for CpG island II, which is relevant for transcriptional control of HBV replication as described previously (21,43): For unmethylated DNA, GTG GGATGTTTTTTGTTTAT (forward) and AACAAAAAA TCCACATAAAA (reverse; for methylated DNA, GCGGGA CGTTTTTTGTTTAC (forward) and AACGAAAAATCC GCGTAAAA (reverse) were used. The PCR mixture contained TOPreal qPCR 2X PreMIX (Enzynomics Co., Ltd.), primers (200 nM) and 50 ng of bisulfite-modified DNA in a final volume of 50 μl. PCR cycles were as follows: Initial activation at 95˚C for 5 min, followed by 35 cycles of 94˚C for 30 sec, 55˚C for 30 sec, 72˚C for 30 sec and a final 5 min extension at 72˚C. The PCR amplicons were quantified via densitometric analysis of 1% ethidium bromide-stained agarose gels. PCR was performed to measure the relative amount of methylation using the same primer pairs for methylation and normalized using the unmethylated primer pair. Bisulfite sequencing was performed to detect the methylation of CpG island II (39). The cloned sequences were analyzed using a BiQ Analyzer (Max Planck Institut Informatik; https://biq-analyzer.bioinf.mpi-inf.mpg.de/) (44).

Chromatin immunoprecipitation (ChIP) assay, western blotting and luciferase assay for DNA methyltransferases. ChIP assay was performed as previously reported (45), with minor modifications. Briefly, formaldehyde (cat. no. F8775; Sigma-Aldrich; Merck KGaA) was added directly to trypsinized HepG2 cells in PBS at a final concentration of 1% at room temperature for 10 min for fixation. The process was stopped with the addition of 0.125 M final concentration of glycine. HepG2 cells were collected using centrifugation at 300 x g for 3 min at 4˚C and rinsed with cold phosphate-buffered saline. The cell pellets were resuspended in MC lysis buffer [10 mM Tris-Cl, pH 7.5; 10 mM NaCl; 3 mM MgCl, 0.5% (v/v) Nonidet P-40], incubated on ice for 15 min and centrifuged at 300 x g for 3 min at 4˚C. The nuclei were resuspended in FA lysis buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 1% Triton X-100, 0.1% sodium deoxycholate, 0.1% SDS, 1X protease inhibitor) and incubated at room temperature for 10 min. Prior to sonication, 50 mg of glass beads (G1277, Sigma-Aldrich; Merck KGaA) was added to each sample. The samples were sonicated on ice with the Sonic Dismembrator Model 100 (Thermo Fisher Scientific, Inc.) with a microtip probe set to a power output of 4-6 W for 10 cycles of 10 sec pulse/50 sec rest and then microcentrifuged at 300 x g for 3 min at 4˚C. Dynabeads Protein G (Thermo Fisher Scientific, Inc.) were blocked with bovine serum albumin/salmon sperm DNA and incubated for 10 min at room temperature with 5 μg of appropriate antibodies or negative control normal mouse IgG (cat. no. sc-2025; Santa Cruz Biotechnology, Inc.), DNMT1 (cat. no. ab13537; Abcam), DNMT3a (sc-20703; Santa Cruz Biotechnology, Inc.) and DNMT3b (cat. no. sc-20704, Santa Cruz Biotechnology). Sonicated chromatin was added to the Dynabead-antibody complexes and rotated at 4˚C for ~16 h. Whole cell lysate were prepared as positive control. The beads were washed and cross-linked by addition of NaCl to a final concentration of 250 mM, followed by boiling for 15 min and treatment with proteinase K. HBV was detected using PCR with specific primers as described previously (14). Western blotting was performed as follows: Total protein was extracted from HepG2cells using RIPA buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.5% Sodium deoxycholate, 0.1% SDS). The protein was quantified by bicinchoninic acid assay. Protein (100 μg) was separated per lane via 8% SDS-PAGE and transferred onto a polyvinylidene difluoride membrane (Roche Diagnostics GmbH). The membranes were blocked with 2.5% skimmed milk in PBS with 0.1% Tween20 for 1 h at room temperature. The membranes were incubated with the following primary antibodies overnight at 4˚C: Mouse monoclonal anti-β-actin (cat. no. sc69879; dilution 1:2,000; Santa Cruz Biotechnology, Inc.), mouse monoclonal anti-DNMT 3a (cat. no. ab13888; dilution 1:1,000; Abcam), mouse monoclonal anti-DNMT 3b (cat. no. ab13604; dilution 1:1,000; Abcam). The membranes were subsequently incubated with horseradish peroxidase-conjugated goat anti-mouse IgG secondary antibody (cat. no. sc2005; dilution 1:2,000; Santa Cruz Biotechnology, Inc.) for 1 h at room temperature. Signals were detected by exposure of the blot to x-ray film using SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific, Inc.). ImageJ software (version 1.46; the National Institutes of Health) was used for measuring the intensity of bands in film image. The promoter
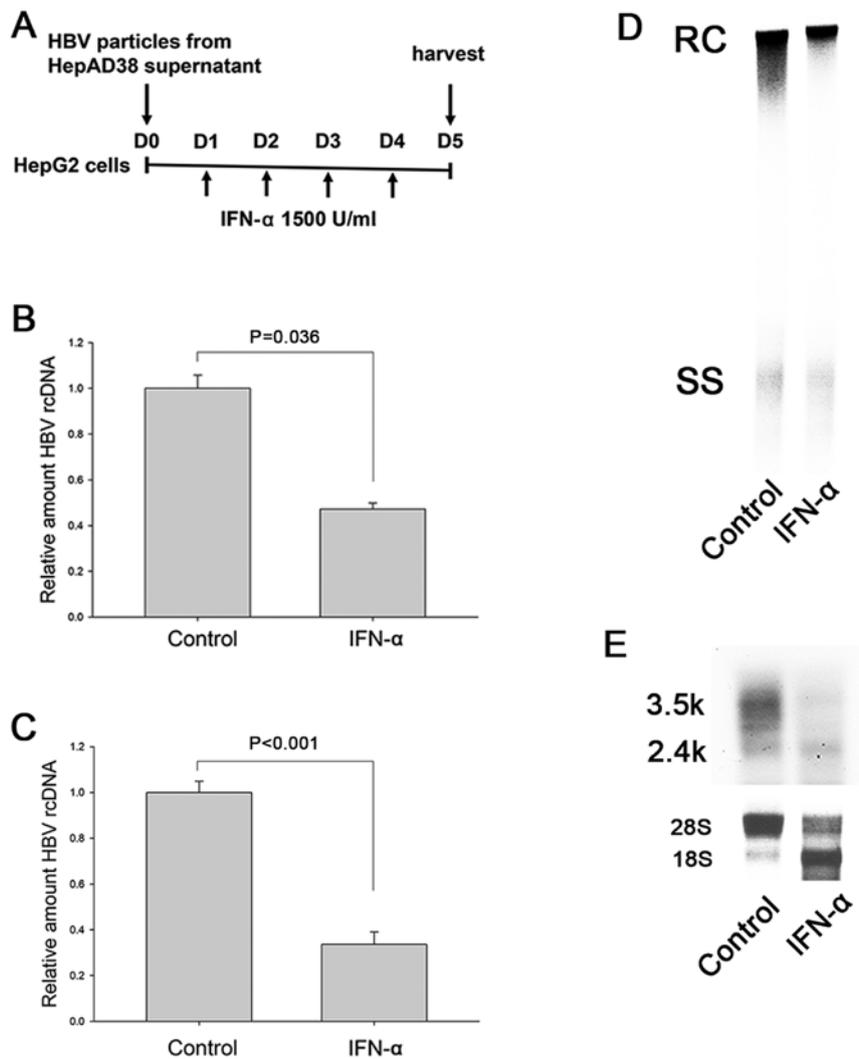
Figure 1. IFN-α suppresses HBV transcripts in HepG2 cells. (A) HBV virion particles were collected from the supernatant of HepAD38 cells and added to HepG2 cells at a titer of 50 MOI. On the following day, IFN-α was added at a final concentration of 1,500 U/ml for four days before harvest. (B) and (C) PCR showed suppression of HBV DNA by IFN-α treatment in cytoplasm and culture supernatant, respectively. (D) Southern blot assay revealed suppression of HBV rcDNA following IFN-α treatment. (E) Northern blot assay revealed suppression of HBV pregenomic/precore RNA (3.5 kb) by IFN-α treatment. Error bars represent standard error of the mean (n=3). HBV, hepatitis B virus; MOI, multiplicity of infection; RC/rcDNA, relaxed circular DNA; SS, single-strand HBV DNA replicative intermediates.

of DNMT3b was cloned into the pGL3-basic vector and used for the luciferase assay with normalization to *Renilla* luciferase in the pRL-TK plasmid according to the instructions of the manufacturer. Briefly, 24 h after transfection of pGL3-DNMT3b promoter (1 μg) and pRL-TK (100 ng) into HepG2 cells (5x10^5 cells) using Lipofectamine 2000® (Thermo Fisher Scientific), luciferase activity was quantified by using a Dual-Luciferase Reporter Assay kit (Promega Corporation).

Statistical analysis. Continuous data were analyzed by using unpaired Student's t test except for comparison of real-time PCR data which were made by pairwise fixed realloca-tion randomization test using REST software (REST 2009 version 2.0.13; Qiagen GmbH; https://www.gene-quantifica-tion.de/rest-2009.html) (46). At least three experiments were repeated and the results were presented as the mean ± stan-dard deviation. Comparison of bisulfite sequencing data was made by Fisher's exact test. P<0.05 was considered to indicate a statistically significant difference.

Results

IFN-α suppresses HBV replication in HepG2 cells. In the cell-based HBV replication model of the present study HepG2 cells were infected with concentrated HBV virion particles, rather than HBV-expressing plasmids, in order to exclude the possibility of carrier plasmid DNA serving as a source of methylation and of engineered promoters being responsive to IFN (47). IFN-α was replenished daily for four days to ensure sufficient time for methylation as that in our previous study (Fig. 1A) (42). The model showed that IFN-α treat-ment suppressed the intracellular HBV DNA (Fig. 1B and D) and HBV DNA secretion to culture supernatant (Fig. 1C). IFN-α also reduced HBV RNA, suggesting the possibility of transcriptional suppression (Fig. 1E).

IFN-α does not affect methylation profiles of HBV cccDNA in HepG2 cells. Since methylation of HBV cccDNA has been identified as a mechanism of regulating HBV replication both
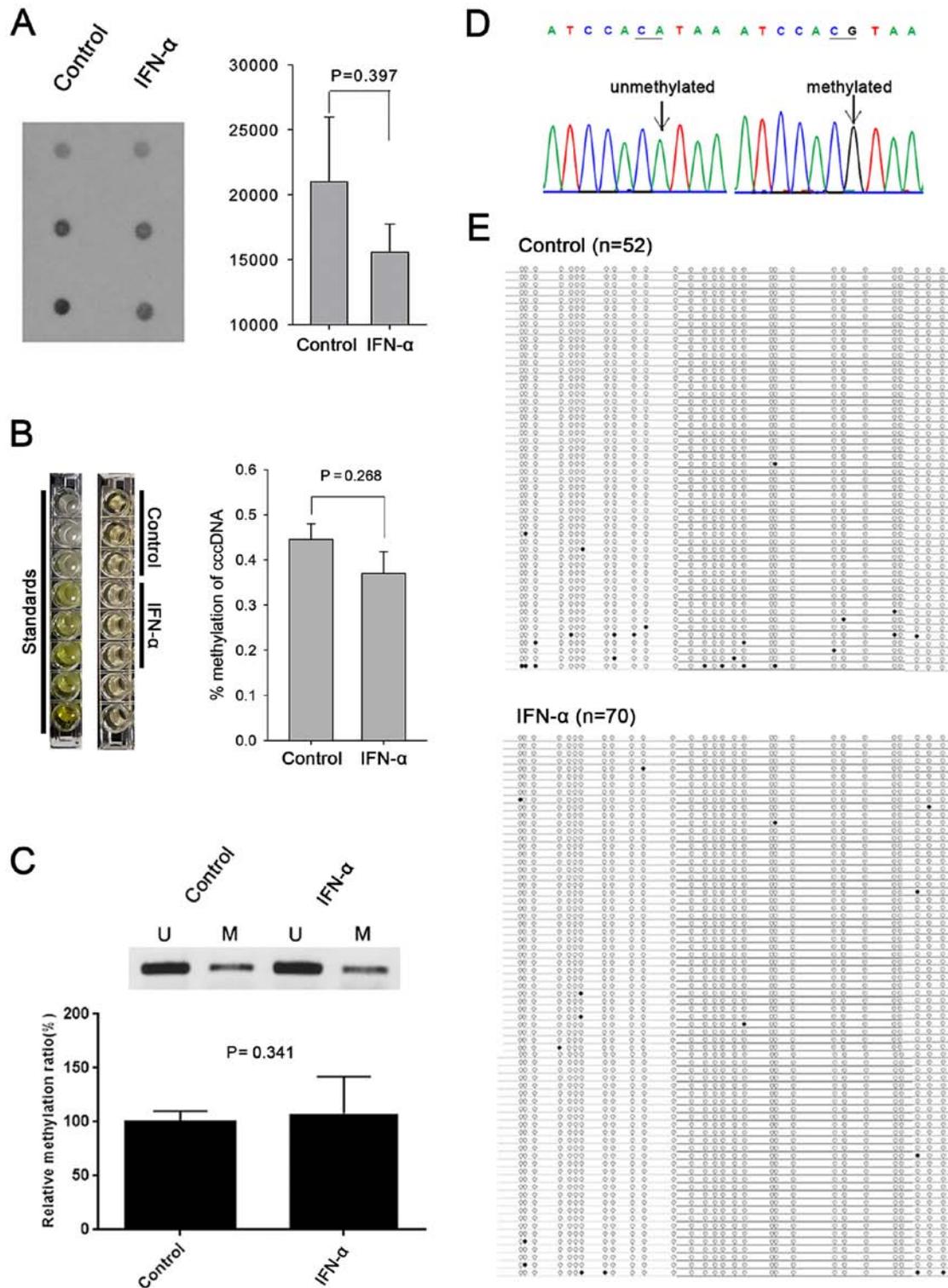
Figure 2. Methylation profile of HBV cccDNA is not influenced by IFN-α in HepG2 cells. (A) 5-mC dot blot analysis showed similar degrees of global methylation of HBV cccDNA between control and IFN-α treated groups (triplicates, left panel; densitometric measurement, right panel). (B) Global DNA methylation ELISA assay also found no difference between the two groups (triplicates, left panel, ELISA reading values, right panel). (C) Methylation-specific PCR revealed no difference in CpG island II methylation between the control and IFN-α treated groups. Bars in lower panel indicate ratio of densitometric measurements of methylated-to-unmethylated bands. (D) Bisulfite sequencing converted CG to TG in HBV cccDNA without CpG methylation (CA in a complementary sequence, left panel), whereas methylated CpG sequences were not modified by bisulfite treatment (right panel). (E) Bisulfite sequencing analysis showed that the frequencies of CpG island II methylation were similar between the control and IFN-α treated groups (11/52 vs. 13/70; P=0.723). Error bars represent standard error of the mean (n=3). HBV, hepatitis B virus; cccDNA, covalently closed circular DNA.

in vitro and *in vivo* (17-20,48), the present study sought to determine whether the anti-HBV effect of IFN-α was also mediated through cccDNA methylation. First, global methylation level was assessed using 5-mC dot blot assay, which showed that IFN-α did not affect the overall level of HBV cccDNA methylation (Fig. 2A). Global DNA methylation ELISA assay
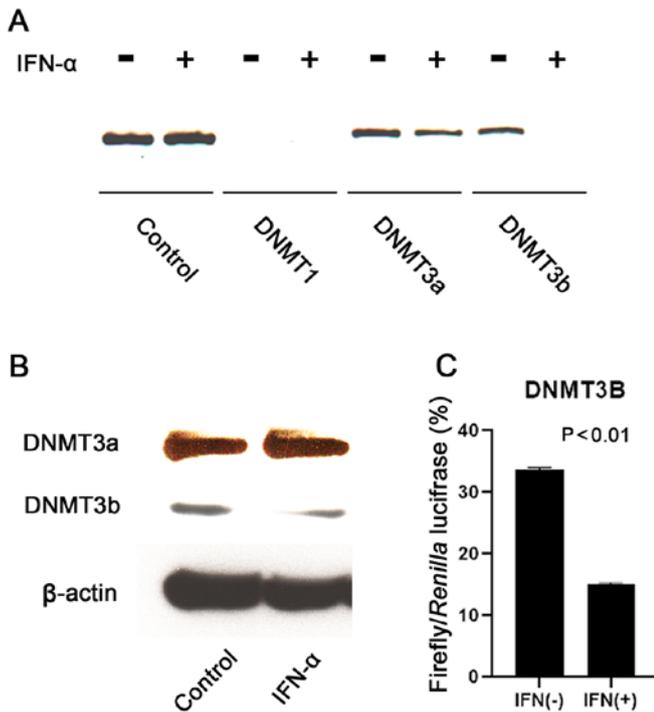
Figure 3. IFN-α suppresses the association between DNMT3b and HBV cccDNA. (A) Chromatin immunoprecipitation assay showed that DNMT3a and DNMT3b bound to HBV cccDNA in HepG2 cells. Treatment with IFN-α did not affect DNMT3a binding, but suppressed DNMT3b binding to cccDNA. (B) Western blot analysis revealed that IFN-α did not affect the expression of DNMT3a, but downregulated DNMT3b in HepG2 cells. (C) Luciferase assay showed decreased promoter activity of DNMT3b in response to IFN-α treatment in HepG2 cells (P<0.01). The y-axis indicates the relative activity (%) of firefly luciferase driven by the DNMT3b promoter, normalized to *Renilla* luciferase driven by the HSV TK promoter. Error bars represent standard error of the mean (n=5). DNMT, DNA methyltransferase; HBV, hepatitis B virus; cccDNA, covalently closed circular DNA.

also confirmed no difference of the degree of methylation between control and IFN-α treated group (Fig. 2B). Next, methylation-specific PCR showed that IFN-α did not alter the level of methylation in CpG island II, which regulates the X promoter of HBV cccDNA (Fig. 2C) (49). Bisulfite sequencing also confirmed a similar degree of methylation in CpG island II of HBV cccDNA regardless of interferon treatment (11/52 for control and 13/70 for IFN-α; P=0.723; Fig. 2D and E).

IFN-α suppressess DNMT3b binding to HBV cccDNA. Finally, the present study determined the effect of IFN-α on the association of DNMTs with HBV cccDNA. ChIP assay confirmed that DNMT1 did not bind to HBV cccDNA, but DNMT3a and DNMT3b were associated with cccDNA (Fig. 3A). Notably, IFN-α suppressed the binding of DNMT3b to cccDNA. Western blotting demonstrated decreased DNMT3b protein expression induced by IFN-α (Fig. 3B). The luciferase assay indicated that IFN-α suppressed DNMT3b promoter activity in HepG2 cells (Fig. 3C).

Discussion

Methylation of cccDNA has recently received considerable attention as a mechanism of transcriptional regulation in human HBV infection (10). The present authors and other researchers

have shown that methylation of cccDNA suppresses the transcriptional activity of cccDNA in cell-based models (17,18,20). In addition, cccDNA methylation is frequently observed in the immunologically controlled stages of chronic HBV infection with low viremia (20,22). However, the underlying mechanisms of *de novo* methylation of cccDNA are largely unknown.

The anti-HBV effect of IFN-α simulates the host immune response against chronic HBV infection; i.e., sustained inhibition of viral gene transcription (5), loss of HBeAg and decrease in HBsAg titers (50). Epigenetic regulation may serve as a shared mechanism between sustained anti-HBV response to IFN-α and the low viremia phase of chronic HBV infection (20,30). While IFN-induced PTMs of histone proteins have been well established (14,15,29), only one previous report that assessed the effect of IFN-α on cccDNA methylation was found, which found a total lack of methylation in both control and IFN-treated cccDNA (30). In that study, however, CpG island I was sequenced, which is barely methylated in the advanced stage of chronic HBV infection and is not related to transcriptional control of cccDNA in previous studies (22,51).

The present study hypothesized that cccDNA methylation may also contribute to the IFN-mediated transcriptional suppression of HBV. To test this hypothesis, our previous cell-based model was used to generate a condition for cccDNA methylation, with some modifications (39). First, direct infection with wild-type HBV virions (52,53) was used instead of plasmid-mediated *in vitro* HBV models (30), because CpG-containing plasmids may trigger inflammatory cytokine responses and become inadvertently methylated (54). The model of the present study, although it has low efficacy for HBV infection due to the lack of sodium taurocholate cotransporting polypeptide (37), has an advantage over transfection of greater-than-genome HBV plasmid or HBV-producing cell lines in which newly generated HBV cccDNA cannot be differentiated from the transfected plasmids or pre-formed cccDNA. Second, conditionally HBV-producing cell lines are avoided because the tetracycline-responsive promoter contains functional interferon-inducible response elements (47). The baseline methylation of HBV cccDNA was confirmed without interferon treatment (Fig. 2), indicating the presence of methylation machinery against natural HBV infection, as previously suggested (20,42).

IFN-α suppressed HBV replication in the cell model of the present study. Contrary to the authors' prediction, however, extensive bisulfite sequencing experiments did not detect significant differences in the level of cccDNA methylation between control and IFN-α-treated groups. Therefore, the anti-HBV effect of IFN-α is unlikely to be mediated by DNA methylation. Recently, DNA methylation has been proposed as a target of epigenetic therapy for CHB (10). Despite the negative result of the present study it is hypothesized that modulation of cccDNA methylation may still be pursued to enhance the therapeutic efficacy of IFN.

Mammalian *de novo* DNA methylation is mediated by DNMT3A and DNMT3B (55). DNMT3a is suggested to induce *de novo* HBV cccDNA methylation (19). The ChIP data confirmed the association of both DNMT3a and DNMT3b with HBV cccDNA (Fig. 3A). The data also suggested that innate interferon response is not the main mechanism of HBV methylation and that ISGs are not sufficient for methylation

of cccDNA. which is in contrast to the fact that downstream signaling of interferon modifies cccDNA-bound histones (56). Thus, the underlying mechanisms of *de novo* cccDNA methylation by DNMTs still remain largely unknown. The data of the present study does not necessarily exclude the potential participation of ISGs as a mechanism underlying DNA methylation in mammalian cells. Whether additional inflammatory cytokines such as TNFα and IFNγ or small RNAs may cooperate with IFN signaling to induce cccDNA methylation needs to be clarified by further studies.

There have been reports demonstrating that HBV induces *de novo* methylation of ISGs, leading to suppression of anti-HBV innate signaling of IFN (57,58). It is not known whether innate IFN response counter-attacks HBV epigenetically, but it is plausible considering the fact that IFN-α may induce reversible DNA demethylation of ISGs (59). The finding of the present study that IFN-α suppressed DNMT3b expression and DNA binding may also be in line with the hypothesis, but further studies are warranted.

Since HepG2 cells were maintained in the presence of fetal bovine serum (FBS) throughout the experiments, the possibility that various cytokines in FBS may affect the results cannot be excluded. Serum-free culture condition is difficult to maintain in our cell model because our previous data showed that ≥5 days were needed before methylation of HBV cccDNA was induced (39). Other related studies also used similar study design with serum in culture media (16,30) and it is hypothesized that possible effect of cytokines may have been adjusted by comparison with the control with same culture condition (except for IFN). However, potential interactions between IFN and other cytokines need to be explored in future studies.

The present study has some limitations. First, it did not sort HepG2 cells according to intracellular HBV replication after treatment with HBV particles. However, it was presumed that the efficacy of HBV infection should be similar between control and IFN-treated cells. In addition, the degree of methylation was assessed by normalization to total cccDNA, so that the results are independent of the efficacy of HBV infection. Second, methylation of cccDNA was evaluated in liver cancer cells and the possibility that normal hepatocytes may show different pattern of methylation cannot be excluded.

In conclusion, methylation of HBV cccDNA was not induced by IFN-α, which suggested that it is not responsible for the antiviral effect of IFN-α against HBV in HepG2 cells and that alternative mechanisms need to be sought to enhance cccDNA methylation as a novel therapy against HBV.

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Availability of data and materials

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

Authors' contributions

IYM and JWK designed the study; IYM performed the experiments; IYM and JWK analyzed the data and contributed to the final manuscript. Both authors read and approved the final manuscript and confirm the authenticity of all the raw data.

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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