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Short hairpin RNA induces methylation of hepatitis B virus covalently closed circular DNA in human hepatoma cells

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ABSTRACT

Small interfering RNAs not only modulate gene expression at a post-transcriptional level, but also induce transcriptional gene silencing by RNA interference-mediated heterochromatin formation and RNA-directed DNA methylation (RdDM). However, although established in plants, there have been controversies whether RdDM operates in mammals. Hepatitis B virus (HBV) covalently closed circular DNA (cccDNA) serves as a template for viral RNA transcription, and transcriptional activity of HBV cccDNA is regulated by methylation in patients with chronic HBV infection. In this study, we stably expressed short hairpin RNA (shRNA) against HBV in human hepatoma cells to determine whether shRNA induces methylation of HBV cccDNA. HepAD38 cells which permit replication of HBV under control of tetracycline-responsive promoter were transduced with lentiviral vectors which encode sh-1580, a shRNA against the hepatitis B viral protein HBx. Bisulfite sequencing PCR analysis revealed that sh-1580 induced CpG methylations at a higher rate compared to control (31.3% vs. 12.8%, $p < 0.05$). The sh-1580-induced CpG methylation was localized near the target sequence of sh-1580 in more than a half of the clones. Methylation-induced transcriptional suppression was confirmed by *in vitro* transcription assay. These results confirm the feasibility of RdDM of HBV cccDNA in human cells. Lentiviral vector-mediated transfer of shRNA may be used as a tool for novel transcriptional modulation by epigenetic modification of HBV cccDNA.

1. Introduction

The RNA interference (RNAi) is a double-stranded RNA-dependent mechanism which regulates eukaryotic gene expression in a wide variety of organisms [1]. Small interfering RNAs (siRNAs), a representative effector of RNAi, control gene expression at a post-transcriptional level by forming RNA-induced silencing complexes, which in turn recognize target RNA sequence and either induce mRNA degradation or translational repression [2,3]. In addition to post-transcriptional gene silencing (PTGS), it has become apparent over the last decade that siRNA also induces transcriptional gene silencing (TGS) by RNAi-mediated heterochromatin formation and RNA-directed DNA methylation (RdDM) [4]. Whereas siRNA-induced heterochromatin formation has been characterized in animal cells [5], there have been conflicting results regarding whether RdDM operates in mammals [4].

Human DNA viruses are cytosine-methylated in host cells, especially during latency [6], and methylation of viral genome may be an innate antiviral defense. However, the mechanisms of viral DNA methylation during natural human infection have not been fully elucidated. Interestingly, several recent reports suggest that small RNAs (siRNAs and microRNAs) may induce methylation of integrated or episomal viral DNA [7,8]. Hepatitis B virus (HBV) is a partially double-stranded circular DNA virus which is one of the most important causes of liver cirrhosis and hepatocellular carcinoma worldwide. After entering hepatocytes, the viral genome becomes fully double-stranded covalently closed circular DNA (HBV cccDNA) and functions as a episomal template for viral transcription [9]. HBV cccDNA is methylated in the human liver [10,11], and we found that methylation of cccDNA regulates the transcriptional activity of cccDNA in chronic HBV infection [12]. DNMT3 may be responsible for the methylation of HBV cccDNA [13], but the exact mechanisms are still elusive.

Abbreviations: cccDNA, covalently closed circular DNA; HBV, hepatitis B virus; siRNA, small interfering RNA; shRNA, short hairpin RNA; PTGS, post-transcriptional gene silencing; RdDM, RNA dependent DNA methylation; RNAi, RNA interference; TGS, transcriptional gene silencing.

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In this work, we showed that lentiviral vector-mediated transfer of short hairpin RNA (shRNA) against HBV is capable of inducing methylation of HBV cccDNA and transcriptional suppression in human hepatoma cells.

2. Materials and methods

2.1. Cells and reagents

HepAD38 cells which produce HBV under the control of tetracycline-responsive CMV-IE promoter were a generous gift from professor C. Seeger (Fox Chase Cancer Center, PA) [14]. 293FT cells were purchased from Invitrogen (Carlsbad, CA). Polybrene (hexadimethrine bromide) and tetracycline were purchased from Sigma–Aldrich Chemical Co. (St. Louis, MI).

2.2. Lentivirus-mediated transfer of shRNA against HBV to HBV-replicating cells

A shRNA sequence against HBV X gene (sh-1580) was cloned into HIV-based plasmid to generate a 3rd generation lentiviral vector as described previously [15,16]. Briefly, a target sequence in the HBX region of HBV genome (nt 1580–1604, TGCACTTCGCTTCACCTCTGCACGT) [17] was assembled in the downstream of the human Pol III U6 promoter by PCR using pTZ U6 + 1 plasmid as a template and the following 3′ primer [15,16,18]: 5′-GGAAGATCTAGAAAAAATGCACTTCGCTTCACCTCTGCACGTT*CTCTTGA*AAACGTGCAGAGGTGAAGCGAAGTGCACCGGTGTTTCGTCCTTTCCACAAG-3′. For a negative control, a scrambled shRNA was prepared with the following 3′ primer [19]: 5′-GGAAGATCTAGAAAAAAGCACCTATAACAACGGTAG*CTACACAAA*CTACCGTTGTTATAGGTGCCGGTGTTTCGTCCTTTCCACAAG-3′. Underlined nucleotides indicate HBV target (sense) and scrambled RNA, respectively, and italicized nucleotides indicate loop sequences of shRNA. Amplified shRNA expression cassettes were cloned into a lentiviral backbone plasmid (pHIV7-GFP PL) as described [15]. The recombinant plasmid was cotransfected with core packaging plasmids (pMDLg/pRRE and pRSV-Rev) and envelope plasmid (pMD2.VSVG) to a packaging 293FT cell line. The medium was replaced with a fresh medium 16 h after transfection, and the supernatant was collected after 24 h of further incubation. Real-time quantitative RT-PCR was performed to determine the lentiviral titers as described [20].

2.3. Transduction of lentiviral vectors in HepAD38 cells

HepAD38 cells were maintained as described [14]. On the day of transduction, cells were plated at a density of 5×10^5 cells in 60-mm cell culture dishes and 1×10^9 particles of lentivirus were added to the medium along with polybrene at final concentration of 4 μg/ml. From the 4th day following transduction, the cells were maintained without tetracycline for five days to induce replication of HBV, and then tetracycline was replenished again in the culture medium until harvest. Cells were harvested on 14th days after transduction for the evaluation of HBV replication and HBV cccDNA methylation.

2.4. Bisulfite sequencing PCR analysis of HBV cccDNA

HBV cccDNA was extracted by using a modified Hirt extraction procedure, followed by digestion with Plasmid-safe DNase (Epicentre, Madison, WI) as described [12]. One-hundred nanograms of the enzyme-treated DNA samples were subject to bisulfite modification by using Imprint DNA Modification Kit as recommended by the manufacturer (two-step modification procedure, Sigma Cat. No MOD50). The modified DNA was PCR amplified using the following primer pairs which were designed by MethPrimers software in order to amplify the bisulfite-modified HBX gene region (nt 1327 – 1670): F, 5′-GGGATTGATAATTTTGTTGTTTTTTT-3′; R, 5′-TCCAAAAATCCTCTTATATAAAACCTTAA-3′ [12,21]. The amplicons were cloned into pDrive TA cloning vector (Qiagen) and sequenced. Modified DNA sequences were analyzed by the software BiQ Analyser as recommended [22].

2.5. Southern and Northern blotting

Cytoplasmic HBV DNA and RNA were isolated from the harvested HepAD38 cells as described [12]. Southern blotting was performed using the digoxigenin-labeled single-strand DNA probe as described [12]. HBV RNA was subject to Northern blotting as described [23].

2.6. In vitro transcription assay

Transcriptional activity of HBV cccDNA was assessed by *in vitro* transcription assay as described [12]. Briefly, 5×10^5 copies of HBV cccDNA from lentivirus-treated HepAD38 cells were incubated with the following reaction mixture: 12 mM HEPES (pH 7.6), 12% (v/v) glycerol, 0.12 mM EDTA, 60 mM KCl, 7.5 mM $MgCl_2$, 500 μM each of ATP, CTP, GTP, and UTP, 7 mM creatine phosphate, 0.3 mM DTT, 0.3 mM PMSF, 10U RNAsin (Promega, Madison, WI) and 7 mg/ml of Huh7 cell nuclear extract. Alpha-amanitin was used to exclude the possibility of contamination with pre-existing HBV RNA. The reaction mixture with HBV cccDNA was incubated at 30 °C for 45 min, and then treated with DNase I for 30 min at 37 °C to remove template cccDNAs. The RNA was reverse transcribed using random hexamers, and PCR amplified by using the following primer pairs: forward, 5′-CACCTCTGCCTAATCATC-3′; reverse, 5′-GGAAAGAAGTCAGAAGGCAA-3′.

3. Results and discussion

3.1. shRNA against HBV induces methylation of HBV cccDNA in HepAD38 cells

Whereas the effect of the exogenous siRNA lasts for <1 week in rapidly dividing cell lines [24], our previous study showed that suppressive effect of HBV-specific shRNA can be maintained up to 4 weeks after lentiviral transduction [16]. Repeated experiments confirmed that suppressive effect of shRNA against HBX region (sh-1580) last at least 2 weeks after lentiviral transduction (Fig. 1).

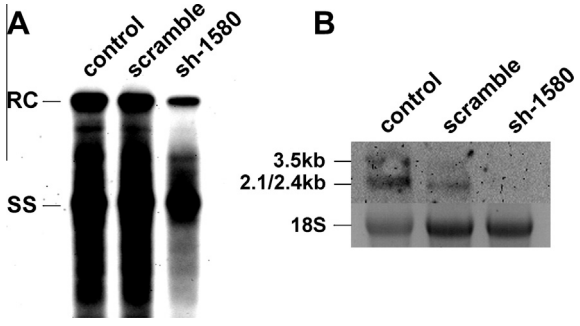

Fig. 1. Suppressive effect of lentivirus-delivered shRNA on HBV replication. HepAD38 cells were transduced with lentiviral vectors which encode scrambled RNA (scramble) or shRNA against HBV (sh-1580). HBV replicative intermediates and mRNA were extracted from the cells and analyzed by Southern (A) and Northern (B) blotting, respectively. The positions of HBV pregenomic RNA (pgRNA) (3.5 kb) and preS/S RNA (2.4/2.1 kb) are indicated, and 18S ribosomal RNA was shown as the loading control. RC, relaxed circular DNA; SS, single-strand DNA; control, HepAD 38 cells without lentiviral transduction.

Next, HBV cccDNA was isolated from shRNA-treated HepAD38 cells and DNA methylation was analyzed by bisulfite sequencing. CpG methylation was found in the HBX region which contains the target sequence of sh-1580 in 3.8% and 12.8% of clones in control HepAD38 cells and HepAD38 cells transduced with scrambled shRNA, respectively. In comparison, cells transduced with sh-1580 showed increased levels of CpG methylation (31.3%, Fig. 2A and B). Interestingly, CpG methylation was found in the immediate upstream and downstream of sh-1580 target site in a half of the methylated clones from sh-1580-treated cells (Fig. 2A).

3.2. Transcriptional activity of HBV cccDNA is suppressed by shRNA-induced methylation

In vitro transcription assay was performed to evaluate the effect of CpG methylation on the transcriptional activity of HBV cccDNA. PCR amplification of HBV cDNA from *in vitro* transcription showed that wild cccDNA and cccDNA treated with scrambled shRNA showed similar transcriptional activity, whereas the transcription of HBV RNA was not observed from cccDNA isolated from sh-1580-transduced cells (Fig. 3). This result indicates that methylation directly inhibits transcriptional activity of HBV cccDNA.

In plants, RdDM is a well-established phenomenon by which double-stranded RNA associated with Argonaute4 induces *de novo* methylation of both CpG and non-CpG cytosine residues by DRM2 DNA methyltransferase [25–27]. Since there are mammalian homologues of plant-specific machinery for RdDM, small RNAs may guide *de novo* methylation in mammalian cells [4]. However, there has been controversy regarding the presence of RdDM in mammals [28]. The discordant reports used different target genes,

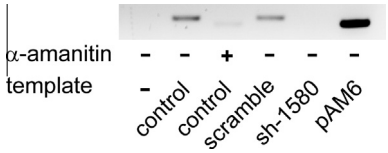

Fig. 3. *In vitro* transcription assay of HBV cccDNA HBV cccDNA from HepAD38 cells transduced with lentiviral vectors encoding scrambled shRNA (scramble) or sh-1580 was used as a template for nuclear run-off reaction, and HBV RNA was amplified by RT-PCR from the run-off transcripts. "Control" indicates HBV cccDNA from HepAD38 cells without lentiviral transduction. Alpha-amanitin was used to exclude the possibility of carry-over amplification of HBV cccDNA. pAM6 is a positive control template for HBV PCR.

and it is conceivable that those target sites might have as yet undefined differences that affect RdDM [28]. Alternatively, cellular differentiation [29] or duration of expression [30] may affect the efficacy of RdDM by siRNA. We speculate that duration of induction may be an important determinant of RdDM in mammals, because previous studies which observed mammalian RdDM employed shRNAs with prolonged expression profiles compared to siRNAs [15,30–32]. RdDM in mammals may be a low-efficiency event and need prolonged induction for over 10 days compared to heterochromatin remodeling [33]. We also observed that lentiviral vector-delivered shRNAs induce methylation of HBV cccDNA at the frequency of <40% (Fig. 2B), and that the frequencies do not increase significantly up to 7 days of induction (data not shown). Since lentiviral vectors ensure efficient delivery and prolonged expression of shRNA [18,34], lentiviral vector-mediated transfer of shRNA may be an appropriate approach for future methylation

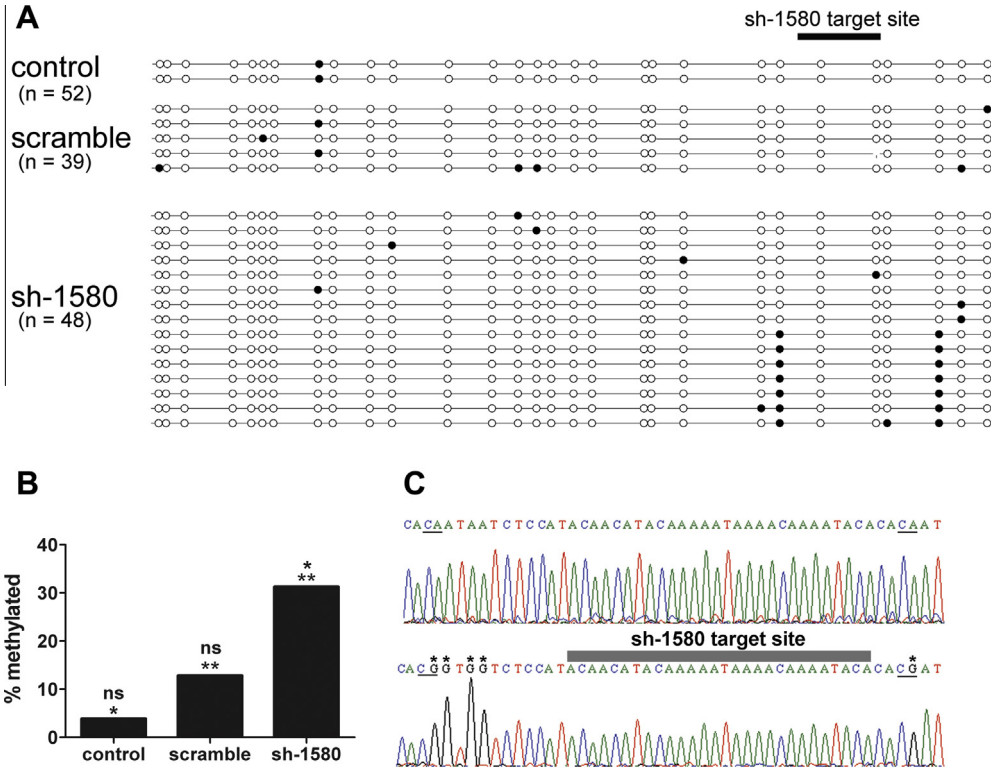

Fig. 2. Lentivirus-delivered shRNA induces methylation of HBV cccDNA in HepAD38 cells. Methylation status of HBV cccDNA from lentivirus-transduced HepAD38 cells was assessed by bisulfite sequencing PCR analysis (A). Total number of sequenced clones in each group was indicated in the parentheses, and clones without CpG methylation were omitted in the alignment. Open and filled circles denote unmethylated and methylated CpG dinucleotides, respectively. The horizontal black bar indicates the target sequence homologous to sh-1580. Control, HepAD 38 cells without lentiviral transduction. The percentages of methylated HBV cccDNA clones were plotted for comparison (B). $^{*}p < 0.01$; $^{**}p < 0.05$; ns, not significant. Representative results of bisulfite sequencing analysis (C) showed conversion of CG to TG (CA in a complementary sequence, underlines in upper panel) in HBV cccDNA without CpG methylation. In contrast, CpG and non-CpG methylation were present ('G' marked with asterisks in a complementary sequence, in lower panel) in the immediate upstream and downstream area of sh-1580 target sites of HBV cccDNA from cells with sh-1580 transduction.

studies and possible therapeutic applications for HBV infection (see below).

Our results also reveal interesting methylation patterns: in about half of methylated clones from sh-1580-treated cells, CpG methylations are confined to the immediate flanking CpG dinucleotides of the target sequence with little spreading of methylation (Fig. 2A and C), a typical pattern of RdDM in plants [35]. Methylations of non-CpG cytosine, another characteristic of RdDM in plants [35], are also present adjacent to the target sequence (Fig. 2C). These findings may suggest that shRNA-loaded effector complex directs DNA methyltransferase to the specific target region, analogous to RdDM in plants. Further studies are warranted to explore the exact targeting mechanisms of RdDM in mammalian cells.

Although there has been much progress in antiviral therapy against HBV in the last decade, viral relapse is the rule rather than the exception after stopping the treatment because HBV cccDNA is insensitive to antiviral drugs [36]. Transcriptional activity of HBV cccDNA determines the viral replication levels during the natural history of chronic HBV infection [37], and we have demonstrated that methylation is one of the determinants of transcriptional activity of HBV cccDNA [12]. Therefore, sustained transcriptional suppression by cccDNA methylation would be a novel therapeutic alternative to prolonged viral suppression by antiviral drugs.

The main limitation of our study is that it is still unknown whether shRNA-induced methylations of HBV cccDNA actually occur in the non-neoplastic human liver. Although DNMT3a and DNMT3b, enzymes responsible for *de novo* methylation, are overexpressed in HBV-infected HepG2 cells [13], *de novo* methylation is generally limited to the early developmental stages and malignancies in animals [38]. Long-term culture model using primary liver cells would be necessary, considering the requirement for prolonged incubation time for RdDM in human cells.

In conclusion, lentivirus–mediated shRNA against HBV can induce the methylation of HBV cccDNA in a human hepatoma cell line. This result confirms the possibility of RNA-directed DNA methylation of episomal viral genome in human cells, and may provide a tool for novel transcriptional modulation of HBV replication by epigenetic modification.

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