miRNAs as therapeutic targets in the liver

Recently, miravirsen, a LNA-modified DNA phosphorothioate antisense oligonucleotide against miR-122, became the first miRNA-targeting drug for clinical use [48]. It was developed to target HCV, as the stability and propagation of this virus is dependent on a functional interaction between the HCV genome and miR-122 [49, 50]. No harmful events were observed in Phase I studies in healthy volunteers, and Phase II studies proceeded to evaluate the safety and efficacy of miravirsen in 36 patients with chronic HCV genotype 1 infection. The patients were randomly assigned to receive 5 weeks of subcutaneous miravirsen injections at 3, 5 or 7 mg per kg body weight or a placebo over a 29-day period. Miravirsen resulted in a dose-dependent reduction in HCV levels, without major adverse events and with no escape mutations in the miR-122 binding sites of the HCV genome [48]. The success of miravirsen is promising, not only as a novel anti-HCV drug, but also as the first trial of miRNA-targeting therapy.

In addition to miravirsen, a clinical trial of MRX34 as a mimic of miR-34 is underway. MRX34 is a liposome-formulated mimic of the tumor suppressor miR-34 (Mirna Therapeutics, Austin, TX, USA). Further study of MRX34 is being conducted by Mirna Therapeutics, which initiated a Phase I study in May 2013 to examine the effects of MRX34 on unresectable primary liver cancer or advanced or metastatic cancer with liver involvement (ClinicalTrials.gov Identifier: NCT01829971). If these oligonucleotide therapies are successful, therapeutic options based on the numerous miRNAs deregulated during hepatocarcinogenesis appear promising [51].

Issues to be resolved in miRNA involvement in hepatocarcinogenesis

As described above, along with recent discoveries of the diverse effects of miRNAs in hepatocarcinogenesis, miRNA-mediated intervention is promising for the development of new diagnostic, preventive and therapeutic tools. However, the data obtained to date are far from complete. The following are some of the critical issues that we believe need to be resolved.

1. The reason for the non-reproducible results among studies should be determined to utilize the available data more reasonably and efficiently.
2. Identification of crucial driver miRNAs among the diverse deregulated miRNAs is critical to develop useful therapeutics in clinics, although even passive miRNAs may be utilized as markers for diagnosis or prediction of prognosis.

3. Comprehensive target gene analyses using in silico systems biology models should be applied.
4. For effective interventions using miRNA, the delivery method, improved oligonucleotide modification and safety must be further considered. Since miRNAs generally have diverse effects due to targeting multiple mRNAs, undesired outcomes, so called off-target effects, may be encountered, even when a specific miRNA is targeted.

Finding solutions to these issues should be considered as critically important for the near future in order to understand more fully the physiological function of miRNAs in hepatocarcinogenesis and utilize this knowledge in translational research.

Conclusions

The discovery of miRNA has, without doubt, opened up new possibilities for understanding the molecular mechanisms of gene regulation. As numerous findings regarding miRNA, from diverse perspectives, have been reported, the speed of discovery in this field is astonishing. In fact, novel therapeutics targeting miRNAs have already been successfully applied in clinical trials. Some miRNAs may be useful as novel biomarkers. Additionally, the discovery of novel concepts in the pathogenesis of hepatocarcinogenesis frequently involves miRNA. On the other hand, several important issues remain to be resolved in this field. Thus, continuous research in this field is still necessary to develop truly innovative concepts in our understanding of pathogenesis related to miRNA and to transform the obtained knowledge into real clinical applications.

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Specific delivery of microRNA93 into HBV-replicating hepatocytes downregulates protein expression of liver cancer susceptible gene MICA

Motoko Ohno[1,*], Motoyuki Otsuka[1,2,*], Takahiro Kishikawa[1], Chikako Shibata[1], Takeshi Yoshikawa[1], Akemi Takata[1], Ryosuke Muroyama[3], Norie Kowatari[3], Masaya Sato[1], Naoya Kato[3], Shun'ichi Kuroda[4] and Kazuhiko Koike[1]

[1] Department of Gastroenterology, Graduate School of Medicine, The University of Tokyo, Tokyo, Japan

[2] Japan Science and Technology Agency, PRESTO, Kawaguchi, Saitama, Japan

[3] Unit of Disease Control Genome Medicine, Institute of Medical Science, The University of Tokyo, Tokyo, Japan

[4] Graduate School of Bioagricultural Sciences, Nagoya University, Nagoya, Japan

[*] These authors contributed equally to this work

Correspondence to: Motoyuki Otsuka , email: otsukamo-tky@umin.ac.jp

ABSTRACT

Chronic hepatitis B virus (HBV) infection is a major cause of hepatocellular carcinoma (HCC). To date, the lack of efficient in vitro systems supporting HBV infection and replication has been a major limitation of HBV research. Although primary human hepatocytes support the complete HBV life cycle, their limited availability and difficulties with gene transduction remain problematic. Here, we used human primary hepatocytes isolated from humanized chimeric uPA/SCID mice as efficient sources. These hepatocytes supported HBV replication in vitro. Based on analyses of mRNA and microRNA (miRNA) expression levels in HBV-infected hepatocytes, miRNA93 was significantly downregulated during HBV infection. MiRNA93 is critical for regulating the expression levels of MICA protein, which is a determinant for HBV-induced HCC susceptibility. Exogenous addition of miRNA93 in HBV-infected hepatocytes using bionanocapsules consisted of HBV envelope L proteins restored MICA protein expression levels in the supernatant. These results suggest that the rescued suppression of soluble MICA protein levels by miRNA93 targeted to HBV-infected hepatocytes using bionanocapsules may be useful for the prevention of HBV-induced HCC by altering deregulated miRNA93 expression.

INTRODUCTION

Hepatitis B virus (HBV) infection is a major global health problem, and more than 350 million people globally are chronic carriers of the virus [1]. A significant number of these carriers suffer from either liver failure or hepatocellular carcinoma (HCC) during the late stages of the disease [2]. In fact, chronic infection with HBV is responsible for 60% of HCC cases in Asia and Africa and at least 20% those in Europe, Japan, and the United States [3].

While nucleoside and nucleotide analogs have been applied in the attempts to suppress HBV replication [4,

5], complete elimination of HBV (including cccDNA) remains difficult [6, 7], and an increased understanding of HBV replication and pathogenesis at the molecular level is essential for clinical management of chronic HBV infection. However, the lack of appropriate cell culture systems supporting stable and efficient HBV infection has been a major limitation. Although transient transfection or viral transfer of HBV genes or genomes are used in the study of specific steps of the HBV cell cycle [8-12], they do not accurately reflect the biology of HBV infection and replication. Thus, humanized mice are used for hepatitis virus research [13-18]. Although these mice are useful, immune deficient, chimeric mice are difficult to handle

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and maintain. Therefore, a more convenient *in vitro* system is required for HBV research.

Primary human hepatocytes can support the complete HBV life cycle *in vitro* [7, 19], but a major drawback is their limited availability. To overcome difficulties regarding availability, we used chimeric mice as sources of primary human hepatocytes, which grow robustly during the establishment of chimeric mice, due to continual liver damage induced by urokinase-type plasminogen activator (uPA) [14, 15].

Another shortcoming of utilizing primary human hepatocytes is their difficulty with gene transduction due to the low transfection efficiency of their primary cell-like nature. Efficient gene delivery methods will significantly improve studies on primary hepatocytes for HBV replication. In addition, cell-specific targeting is required for efficient drug delivery *in vivo*. As a specific gene delivery method to liver-derived cells, bionanocapsules (BNCs) consisted of HBV envelope L particles have been tested for the selective delivery of genes, drugs, or siRNAs into liver-derived cells [20, 21]. Because these BNCs are consisted of HBV L protein, they may be applicable for drug delivery to HBV-infected primary human hepatocytes.

MicroRNAs (miRNAs) are endogenous ~22-nucleotide RNAs that mediate important gene-regulatory events by base-pairing with mRNAs and activating their repression [22]. We previously reported that modifying the expression of miRNAs in liver cells can efficiently regulate the expression levels of the MHC class I polypeptide-related sequence A (MICA) protein [23], which we previously identified as a crucial factor for the susceptibility of hepatitis virus-induced HCC and possibly hepatitis virus clearance [24, 25]. While emerging evidence suggests that miRNAs play crucial roles in chronic HBV infection [26], the comprehensive changes in miRNA expression levels induced by HBV infection in human hepatocytes or in alternative systems reflecting HBV-infected hepatocytes have not been explored.

In this study, we infected primary human hepatocytes isolated from chimeric mice with HBV and identified the transcripts and miRNAs whose expression levels changed. We explored whether BNCs carrying synthesized miRNAs could successfully deliver miRNAs into primary hepatocytes and rescue the modulated miRNA expression due to HBV replication. We found that BNCs carrying synthesized miRNA93 could efficiently restore deregulated soluble MICA protein levels in the supernatant of HBV-replicating primary hepatocytes. These results suggest that miRNA93 delivery into HBV-replicating hepatocytes using BNC methods may enhance HBV immune clearance or suppress HCC by altering miRNA93 levels in HBV-infected cells.

RESULTS

Changes in expression levels of transcripts and miRNAs during HBV replication in human primary hepatocytes

We examined changes in transcript and miRNA expression levels during HBV infection and replication in hepatocytes. Primary human hepatocytes were used for maintainng HBV replication *in vitro*. We first isolated primary hepatocytes from humanized chimeric mice. To examine the infectivity of HBV into the primary hepatocytes *in vitro*, HBsAg and HBV-DNA levels in the cell culture supernatant were measured after the cells were infected with approximately 1.5×10^7 copies of HBV/well in a 24-well plate at day 0. Although both HBsAg and HBV-DNA levels transiently decreased at approximately day 3, levels of both started to increase and were maintained until after day 23 post-infection (Figure 1a and b). These results suggested that human primary hepatocytes isolated from chimeric mice can efficiently support HBV replication *in vitro*, which can be used as an efficient *in vitro* HBV replication system.

To examine comprehensive changes in mRNA and miRNA expression levels in HBV-infected hepatocytes, cells at day 7 post-infection were collected and subjected to cDNA as well as miRNA microarrays. Among 24,460 genes examined, 65 were significantly upregulated by more than 4-fold, and 29 were downregulated to less than 25% (Supplementary Table 1 and 2); however, more than 800 total genes were upregulated or downregulated if the thresholds of the changes were set at 2-fold and 1.5-fold, respectively (Figure 1c; complete datasets have been deposited as GEO accession number: GSE55928). Among the upregulated genes, those associated with the cytochrome family, such as CYP2A7, CYP2C8, CYP2A6, CYP3A4, changed significantly, which was consistent with previous reports [27, 28]. However, few inflammatory cytokines or genes associated with cell growth changed significantly. Based on these results, host factors related to innate immunity may not sense HBV (at least under these replicating conditions), suggesting that this system may mimic the status of hepatitis B patients before seroconversion, in whom inflammation seldom occurs regardless of the high viral load.

Regarding changes in miRNA expression levels during HBV replication, among 2,019 mature miRNAs, 35 were upregulated and 14 downregulated by an increase or decrease of more than two-fold (Figure 1d and Supplementary Tables 3 and 4; complete datasets have been deposited as GEO accession number: GSE55929). Among these miRNAs, miR93-5p was significantly downregulated during HBV replication by more than 50%. Since miRNA93 regulates the expression levels

of the MICA protein [23, 29], which is involved in the susceptibility to hepatocellular carcinoma in chronic hepatitis patients [24, 25], we focused on this miRNA in further analyses.

Efficient delivery of miRNAs into liver cell lines using bionanocapsules

Efficient delivery methods of genes or compounds into targeted tissues or cells are essential to translate the *in vitro* results into clinical settings. Here, we utilized BNCs [21, 30, 31], which were originally developed to deliver genes and drugs with high efficiency and specificity to human liver-derived cells, as an efficient delivery method for miRNAs into human liver cells, including primary hepatocytes. Since BNCs are composed of HBV L proteins, the distribution of these BNCs and infected HBV should be similar. To confirm the efficiency of delivery of miRNAs into liver-derived cells by BNCs, we delivered BNCs carrying let-7g or miRNA93 to the human hepatocellular carcinoma cell lines, Huh7 and HepG2 cells, and to human normal hepatocytes immortalized

with SV40 large T antigen, Fa2N4 cells [28]. The day after delivery of the BNCs, cells were collected and subjected to Northern blotting against let-7g, miRNA93, and U6, the loading control, and the results showed successful delivery of miRNAs into all cell lines tested (Figure 2a). The biological function of the delivered miRNAs was confirmed using luciferase-based reporters, which measured let-7g and miRNA93 functions [23]. Huh7 and Hep3B cells transfected with reporter constructs were delivered with let-7g or miRNA93 using BNCs, followed by a luciferase assay at the next day. Delivered miRNAs significantly decreased the corresponding luciferase activity, suggesting that the delivered miRNAs were functioning within the cells (Figure 2b).

We next examined the delivery of miRNAs into 293T cells (human embryonic kidney cell lines) to explore cell-specificity. Only a small increase in miRNA93 expression levels was observed 24 hours after transfer into 293T cells, based on Northern blots (Figure 2c), indicating that the BNCs had high specificity for hepatocyte-derived cells. The expression of transferred miRNA into Huh7 cells could be observed even 3 days after delivery (Figure 2d), suggesting that the delivered miRNAs are expressed
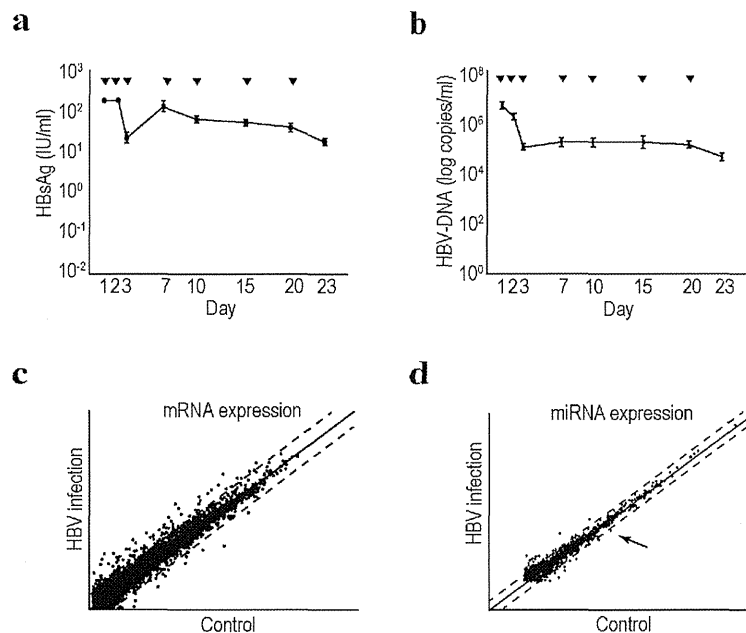

Figure 1: Comprehensive transcriptome and miRNA analyses in HBV-replicating human primary hepatocytes. a, b, Efficient HBV replication in human primary hepatocytes isolated from chimeric mice. Primary human hepatocytes isolated from chimeric mice were seeded into the wells of a 24-well plate. Serum from HBV-infected patients was added to infect the cells with HBV. Media was changed at the indicated days (▼). The supernatant was collected when the media was changed for the analyses of HBsAg levels (a) and HBV-DNA levels (b). Data represent the means ± s.d. of three independent experiments. c, Scatter plot reflecting the transcriptomic results comparing the control and HBV-replicating primary human hepatocytes. Cells at day 7 after HBV infection were used for the analyses. Intensity normalization was performed using global normalization based on the expression levels of all genes analyzed. Dashed lines indicate the thresholds: two-fold increase or 50% decrease in expression levels. The full data are deposited in NCBI GEO database accession: GSE55928. d, A scatter plot of the miRNA microarray results was used to determine the expression levels of comprehensive mature miRNAs. Total RNA from control and HBV-replicating primary hepatocytes at day 7 after infection was used. Dashed lines indicate the thresholds: two-fold increase or 50% decrease in expression levels. Intensity normalization was performed using global normalization based on the expression levels of all miRNAs. The arrow indicates the result for miRNA93. The full data are deposited in NCBI GEO database accession: GSE55929.

for several days.

miRNA delivery into human primary hepatocytes using bionanocapsules

Based on the efficient delivery of miRNA via BNCs into human liver-derived cell lines, we examined the BNC-mediated delivery of miRNAs into non-dividing human primary hepatocytes isolated from chimeric mice, as described above. BNCs could deliver miRNAs efficiently, even into non-dividing human primary hepatocytes, based

on Northern blots (Figure 3a), irrespectively of the use of Polybren (Figure 3a).

Since the expression levels of miRNA93 were downregulated by HBV replication (Figure 1d and Supplementary Table 4), we delivered miRNA93 via BNCs into HBV replicating human hepatocytes to rescue the downregulation of miRNA93 levels and examine the effects of decreased miRNA93 on transcript levels (Figure 3b). The rescue of miRNA93 expression, recovered the baseline-level expression of some genes, such as 17-beta-hydroxysteroid dehydrogenase 14 (HSD17B14) and tripartite motif-containing protein 31 (TRIM 31), which
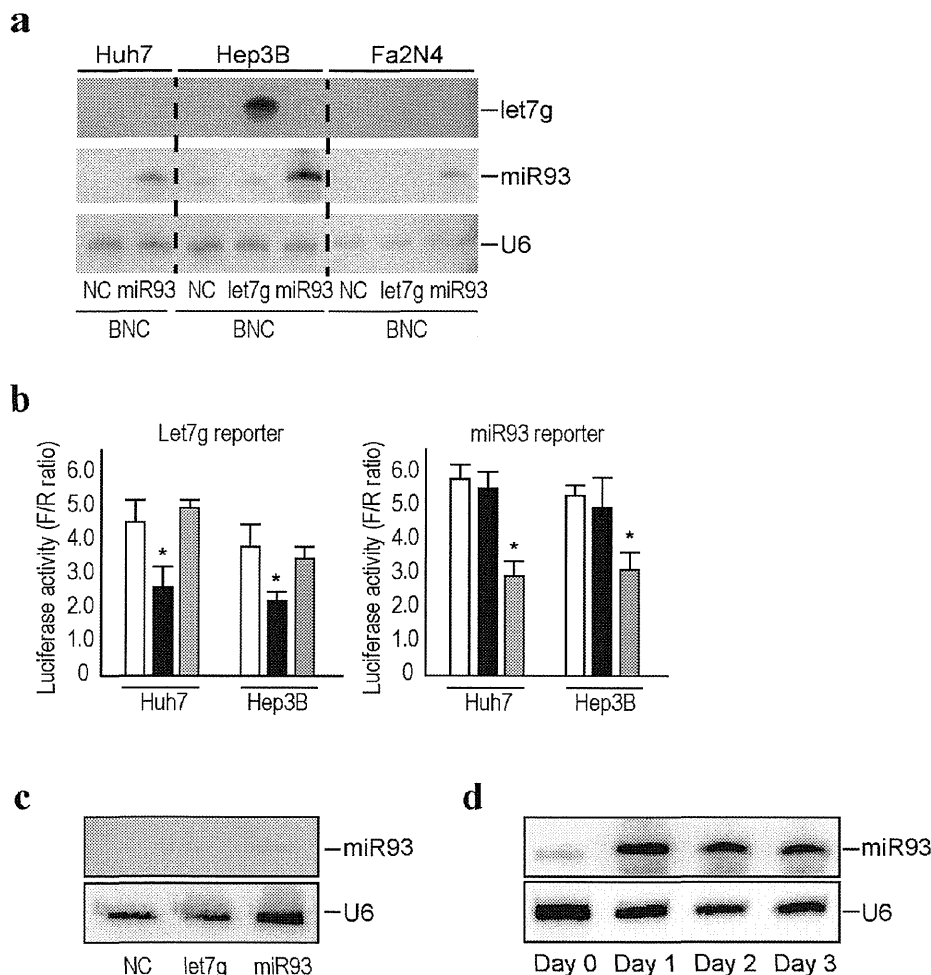

Figure 2: Efficient delivery of miRNAs into liver cell lines using BNCs. a, Northern blotting of miRNAs delivered into liver cells by BNCs. Liver cancer cell lines, Huh7 and Hep3B, and primary hepatocytes immortalized by SV40, Fa2N4, were incubated with BNCs harboring the indicated miRNAs (miRNA93 or let7g) or BNCs without miRNAs (NC). After 24 hours, cells were harvested and subjected to analysis. Membranes were re-probed for let7g, miRNA93, and U6 as a loading control. The results shown are representative of three independent experiments. b, miRNAs delivered using BNCs were biologically functional. Huh7 and Hep3B cells were transfected with the indicated reporter constructs, which indicate the activity of each miRNA function. Twenty-four hours after transfection, cells were mixed with BNCs containing let7g (black bar), miRNA93 (gray bar), or negative control (white bar). Forty-eight hours after transfection, cells were subjected to a dual luciferase assay. Data shown represent the means ± s.d. of the raw ratios (FL/RL), obtained by dividing the firefly luciferase values by the renilla luciferase values, of three independent experiments. *$p < 0.05$. c, Delivery of miRNAs via BNCs were liver cell-specific. The 293T cells (human embryonic kidney cells) were incubated with BNCs containing let7g, miRNA93, or negative control (NC). After 24 hours, cells were subjected to Northern blotting for miRNA93. U6 was used as a loading control. The results shown are representative of two independent experiments. d, miRNA93 expression in Huh7 cells after the delivery of miRNA93 via BNCs. Cells were sequentially collected after incubation with BNCs containing miRNA93 and subjected to Northern blotting. U6 was used as a loading control. The results shown are representative of three independent experiments.

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were increased by HBV replication (Supplementary Table 1), suggesting that the mRNA levels of these genes may be directly or indirectly regulated by miRNA93. Although the enhanced decay of target transcripts by miRNAs has been reported [22, 32], miRNAs generally function as translational repressors [33]. However, these miRNA93 delivery results may not be accurate due to direct or indirect effects of miRNA93. In addition, changes in protein levels may differ from our transcript expression results.

Modulation of MICA protein expression levels by delivery of miRNA93 using BNCs

We previously identified miRNA93 as a critical regulator of MICA protein expression [23], which plays a role in the susceptibility to HBV-induced HCC [25]. MiRNA93 regulates MICA protein levels, but not transcript levels [23, 29]. Although it was found that miRNA93 expression levels decreased during HBV replication in primary hepatocytes (Figure 1d and Supplementary Table 4), MICA transcript levels were not affected (GEO accession number: GSE55928), suggesting that the effects of miRNA93 on MICA may be mediated by translational repression and not by mRNA decay, as we reported previously [23]. To confirm changes in the expression level of the MICA protein on the cell surface of primary hepatocytes induced by HBV infection, cells were subjected to FACS analyses. However, the protein expression levels on the cell surface did not change significantly (Figure 4a). MICA is a soluble protein released into the supernatant after shedding by ADAM10 and ADAM17[34]. Our results suggested that the
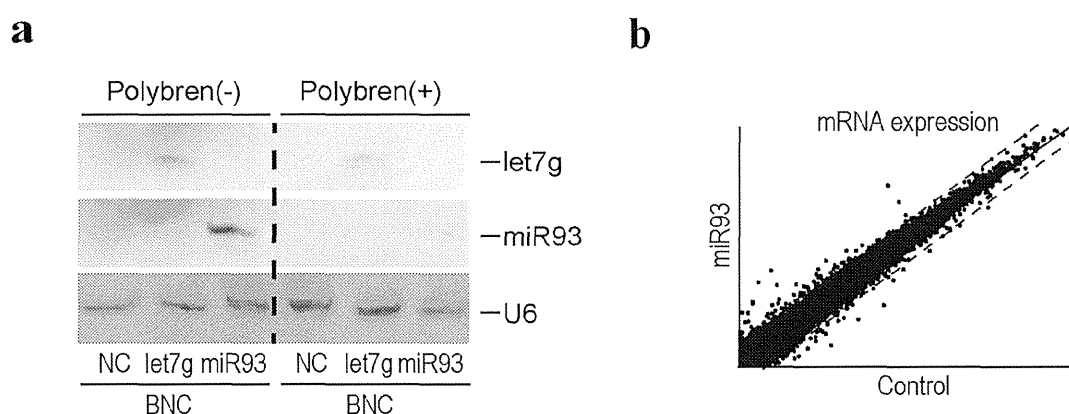

Figure 3: Efficient delivery of functional miRNAs into human primary hepatocytes using BNCs. a, Northern blotting for miRNAs delivered into cells using BNCs. Human primary hepatocytes isolated from chimeric mice were incubated with BNCs containing the indicated miRNAs (miRNA93 or let7g) or BNCs without miRNAs (NC), with or without Polybren. After 24 hours, cells were harvested and subjected to analysis. Membranes were re-probed for let7g, miRNA93, and U6 as a loading control. The results shown are representative of three independent experiments. b, A scatter plot reflecting the transcriptome results between the control and primary human hepatocytes treated with BNCs containing miRNA93. Cells were harvested 24 hours after BNC treatment. Intensity normalization was performed using global normalization based on the expression levels of all genes analyzed. Dashed lines indicate the thresholds: a two-fold increase or 50% decrease in expression levels. The full data are deposited in GEO database accession: GSE55928.
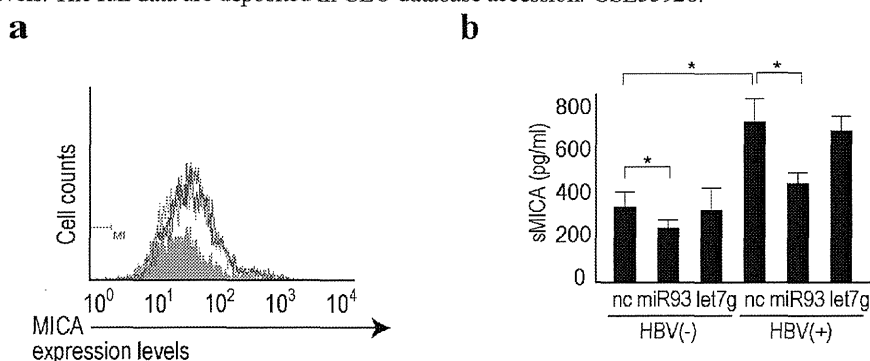

Figure 4: Soluble MICA protein levels were regulated by miRNA93 in human primary hepatocytes. a, Membrane-bound MICA protein expression was not affected by miRNA delivery into human primary hepatocytes. Flow cytometric analysis of membrane-bound MICA protein expression in cells delivered BNC-mediated control (green line), let7g (blue line), or miRNA93 (red line). Gray-shaded histograms represent background staining, assessed using isotype IgG. Representative results from three independent experiments are shown. b, Soluble MICA protein levels in the supernatants of primary hepatocytes after delivery of the indicated miRNAs (let7g or miRNA93) or negative control (NC) with or without HBV replication. Data represent the means ± s.d. of three independent experiments. *p<0.05.

modulated expression of MICA in primary hepatocytes during HBV replication affects this shedding process. To explore this possibility, we examined MICA protein levels in the supernatant using ELISA. As predicted, HBV infection significantly increased the protein concentration of MICA in the supernatant (Figure 4b).

Because an increase in soluble MICA levels in the serum of chronic hepatitis B patients is significantly associated with increased susceptibility to HCC [25], this increase during HBV replication needs to be prevented. Thus, we examined the effects of delivery of BNCs carrying miRNA93 into HBV-infected hepatocytes. Even though the MICA mRNA levels were not significantly affected by miRNA93 delivery based on microarray results (GEO accession: GSE55928), soluble MICA protein in the supernatant significantly decreased according to ELISA (Figure 4b). These results suggested that miRNA93 delivery into the liver decreases soluble MICA levels in the serum, which may be used to prevent HCC in chronic hepatitis B patients.

DISCUSSION

We report that HBV replication in human hepatocytes decreases miRNA93 expression and increases soluble MICA levels. Increased soluble MICA levels in the serum are strongly associated with HBV-related HCC [25], and the increased soluble MICA levels could be antagonized by the delivery of miRNA93 into hepatocytes using BNCs. Thus, BNCs carrying miRNA93 may be used to prevent HCC in patients with chronic HBV infection.

Methods of efficient long-term HBV replication *in vitro* are not commonly available. Although transient transfection assays using fragments or tandem-units of the HBV genome or the full-length HBV genome without vector backbone have been applied [8-12], these models can be analyzed only for short-term replication after transfection. Although stable cell lines carrying HBV genomes are also used, HBV particles are derived from the HBV genome and integrate into the host genome, which differs from natural infection, in which HBV replication mainly relies on HBV cccDNA [6, 7]. Although the most ideal system for HBV infection and replication studies *in vitro* are primary human hepatocytes, they are difficult to obtain. Freshly isolated human hepatocytes from chimeric mice used in this report are relatively easily to obtain, since they proliferate under immune-deficient and liver-damaging conditions. These cells could support HBV replication for a substantial period and are valuable resources for studies on HBV infection and replication.

Another essential tool used in this study is that of BNCs. Primary hepatocytes are generally difficult to transduce with exogenous genes via transfection. Although viral-mediated gene transfer is useful even for primary cells, we chose BNCs as the miRNA delivery method for several reasons. First, since BNCs are composed

of HBV L particles, these BNCs preferentially target primary hepatocytes and theoretically target similar cells as does HBV. Second, since we want to develop future therapeutics based on our experimental results, we avoided using viral materials such as lentiviruses or retroviruses to improve biosafety. Third, although BNCs have been established to transfer genes or drugs [21, 31, 35], transfer of miRNAs has not yet been examined, which prompted us to investigate delivery of miRNAs. We found that BNCs could efficiently deliver miRNAs into primary hepatocytes. Although further studies are required, delivery of miRNAs into hepatocytes via BNCs may be a promising approach to target hepatocytes *in vivo*, as BNCs are efficient delivery vehicles in xenograft models using human liver-derived cells [21].

The present results regarding comprehensive transcriptome analyses using HBV replicating hepatocytes may be applicable for future HBV research. While similar experiments are typically performed using transfection in HBV protein-expressing cells, or other relatively artificial experimental settings, the results here may better reflect the *in vivo* situation for HBV-infected hepatocytes. The expression of approximately 0.3% of genes changed during HBV replication when the threshold was set to a greater than 4-fold increase or to less than a 25% decrease. Although some of these genes were consistent with previous transcriptomic studies [36-38], we observed several novel characteristics. First, few inflammation-related genes were included among genes whose expression levels were significantly changed. The reason for this discrepancy remains unclear, but the results were considered accurate, since inflammation is rare when HBV replicates prior to seroconversion in chronic HBV-infected patients. Thus, HBV may be able to evade the sensing system related to innate immunity [39-41]. It should be explored whether changes in HBV sequences or the presence of host cells other than hepatocytes affect gene expression in hepatocytes *in vivo*. Second, based on comprehensive analysis of transcript changes, many CYP-related genes were upregulated during HBV replication, which is consistent with previous reports [27, 28]. Since the biological significance of these changes remain unclear, further studies are required to explore the biological significance during HBV replication.

Microarray analyses of changes in miRNA expression levels in HBV-replicating cells revealed that miRNA expression levels were not affected by HBV replication (2.4% among 2,000 miRNAs when the threshold was set to more than a two-fold increase or less than a 50% decrease). However, the miRNAs whose expression levels changed may play crucial roles in the regulation of target gene expression without affecting transcript expression levels, for example, targeting of the MICA protein by miRNA93, whose expression levels were downregulated by HBV replication. The results of comprehensive miRNA expression level analysis in

HBV-replicating cells may increase our understanding of deregulated gene expression induced by HBV replication in hepatocytes.

MiRNA93 is a critical regulator of MICA protein expression [23, 29]. Thus, the decreased expression of miRNA93 by HBV suggested that the regulation of MICA expression by miRNA93 has biological significance. Polymorphisms in the MICA gene are associated with HBV and HCV-induced HCC [25, 42], and the increase in soluble MICA in the serum can be used as a susceptibility marker for HBV-induced HCC [25]. The increased levels of MICA protein expression agreed with the decreased miRNA93 expression. However, this increase was observed for soluble MICA protein levels and not membrane-bound MICA. While MICA is post-translationally dependent on the cell context or the status of viral infection [34], MICA may be readily processed from the cell surface in HBV-replicating primary hepatocytes and mainly released as soluble protein. Soluble MICA protein may function as a decoy for the NKG2D receptor in immune cells and as an evasion or immune surveillance system during chronic HBV infection. It may also be associated with HBV-induced HCC since HBV-infected hepatocytes may evade from the immune surveillance. Based on these results, BNCs carrying miRNA93 can be used to eliminate HBV-infected hepatocytes, which may be a novel approach for the prevention of subsequent virus-induced HCC.

MATERIALS AND METHODS

Cells

Primary human hepatocytes isolated fresh using the collagenase perfusion method from chimeric uPA/SCID mice with humanized livers [14, 17] were obtained from Phoenix Bio (Hiroshima, Japan). The purity of human hepatocytes was greater than 95%. A total of 3.0×10^5 cells/well were seeded on a type I collagen coated-24-well plate and maintained in DMEM with 10% FBS, 5 ng/ml EGF, 0.25 μg/ml insulin, 0.1 mM ascorbic acid, and 2% DMSO [43]. These cells were able be maintained at a high density for more than 3 weeks, supporting the long-term replication of HBV infection *in vitro*.

HBV infection *in vitro*

Serum from chronically HBV-infected patients with no HBe antibody before seroconversion was used for *in vitro* infection. Serum containing 9.0 log IU/ml of HBV genotype C in a volume of 3 μl, which is approximately 1.5×10^7 copies of HBV, was added to the 3.0×10^5 cells/well, followed by the addition of 4% PEG 8000 at day 0. Cells were washed, and the media was changed at days 1

and 2 and every 5 days thereafter. The media was collected to measure HBsAg and HBV-DNA at days 1, 2, 3, 7, 10, 15, 20 and 23 to confirm HBV replication. Measurements were performed at the clinical laboratory testing company SRL. Inc. (Tokyo, Japan).

cDNA array and miRNA microarray

Human 25K cDNA microarray and human 2K miRNA microarray analyses were performed using miRNA oligo chips according to the standard protocols (Toray Industries, Tokyo, Japan). The data and the experimental conditions were deposited in a public database (GEO: accession numbers: GSE55928 and GSE55929).

Bionanocapsules for miRNA delivery

Hollow particles consisting of HBV L proteins (pre-S1, pre-S2, and S regions) were used as the BNCs, as described previously [20, 21, 30]. The incorporation of miRNAs (miRNA93 or let-7g) into the hollow space and the delivery of miRNAs into human liver cells were performed as described previously [31]. Briefly, 32 μl BNC was added to 1 ml culture media at a final concentration of 50 nM miRNA 24 h before the indicated assays (unless otherwise specified).

Northern blotting of miRNAs

Northern blotting of miRNAs was performed as described previously. Total RNA was extracted using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. RNA (10 μg) was resolved on denaturing 15% polyacrylamide gels containing 7 M urea in 1× TBE and then transferred to a Hybond N+ membrane (GE Healthcare) in 0.25× TBE. Membranes were UV-crosslinked and prehybridized in hybridization buffer. Hybridization was performed overnight at 42°C in ULTRAhyb-Oligo Buffer (Ambion) containing a biotinylated probe specific for miRNA93 (CTA CCT GCA CGA ACA GCA CTT TG) and let-7g (AAC TGT ACA AAC TACT ACC TCA), which was heated to 95°C for 2 min prior to hybridization. Membranes were washed at 42°C in 2× SSC containing 0.1% SDS, and the bound probe was visualized using the BrightStar BioDetect Kit (Ambion). Blots were stripped by boiling in a 0.1% SDS, 5 mM EDTA solution for 10 min prior to rehybridization using a U6 probe (CAC GAA TTT GCG TGT CAT CCT T).

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Reporter plasmids, transient transfection, and dual luciferase assays

The firefly luciferase reporter plasmid was used to examine let7g and miRNA93 function. pGL4-TK, a renilla luciferase reporter, was used as an internal control [44]. Transfection and dual luciferase assays were performed as described previously [45].

Flow cytometry

The expression levels of MICA on the cell surface were determined using flow cytometry, as described previously [23]. Briefly, cells were hybridized with anti-MICA (1:500; R&D Systems, Minneapolis, MN, USA) and isotype control IgG (1:500; R&D Systems) in 5% BSA/1% sodium azide/PBS for 1 h at 4°C. After washing, cells were incubated with goat anti-mouse Alexa 488 (1:1,000; Molecular Probes, Eugene, OR, USA) for 30 min. Flow cytometry was performed and the data analyzed using Guava Easy Cyte Plus (GE Healthcare, Little Chalfont, UK).

ELISA for MICA

The concentration of MICA in the cell culture supernatant was measured using a sandwich ELISA, according to the manufacturer's instructions (R&D Systems, Minneapolis, MN, USA).

Statistical analysis

Significant differences between groups were determined using the Student's t-test when variances were equal and using Welch's t-test when variances were unequal. P-values less than 0.05 were considered statistically significant.

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Editorial note

This paper has been accepted based in part on peer-review conducted by another journal and the authors' response and revisions as well as expedited peer-review in Oncotarget

AUTHOR CONTRIBUTIONS

M.Ohno and M.Otsuka planned the research and wrote the manuscript. M.Ohno, T.K., C.S., T.Y., and A.T. performed the majority of the experiments. R.M., N.K., M.S. and N.K. measured performed ELISA. S.K. provided materials and wrote the manuscript. K.K. supervised the entire project.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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Research

DNA methylation at hepatitis B viral integrants is associated with methylation at flanking human genomic sequences

Yoshiyuki Watanabe,[1,2,9] Hiroyuki Yamamoto,[1,9] Ritsuko Oikawa,[1] Minoru Toyota,[3] Masakazu Yamamoto,[4] Norihiro Kokudo,[5] Shinji Tanaka,[6] Shigeki Arii,[6] Hiroshi Yotsuyanagi,[7] Kazuhiko Koike,[8] and Fumio Itoh[1]

[1]Division of Gastroenterology and Hepatology, Department of Internal Medicine, St. Marianna University School of Medicine, Kawasaki 216-8511, Japan; [2]Internal Medicine, Kawasaki Rinko General Hospital, Kawasaki 210-0806, Japan; [3]Department of Molecular Biology, Sapporo Medical University School of Medicine, Sapporo 060-8556, Japan; [4]Department of Surgery, Institute of Gastroenterology, Tokyo Women's Medical University, Tokyo 162-8666, Japan; [5]Hepato-Biliary-Pancreatic Surgery Division, Artificial Organ and Transplantation Division, Department of Surgery, Graduate School of Medicine, University of Tokyo 113-8655, Japan; [6]Department of Hepatobiliary Pancreatic Surgery, Graduate School, Tokyo Medical and Dental University, Tokyo 113-0034, Japan; [7]Department of Infectious Diseases, Graduate School of Medicine, University of Tokyo, Tokyo 113-8655, Japan; [8]Department of Gastroenterology, Graduate School of Medicine, University of Tokyo, Tokyo 113-8655, Japan

Integration of DNA viruses into the human genome plays an important role in various types of tumors, including hepatitis B virus (HBV)–related hepatocellular carcinoma. However, the molecular details and clinical impact of HBV integration on either human or HBV epigenomes are unknown. Here, we show that methylation of the integrated HBV DNA is related to the methylation status of the flanking human genome. We developed a next-generation sequencing-based method for structural methylation analysis of integrated viral genomes (denoted G-NaVI). This method is a novel approach that enables enrichment of viral fragments for sequencing using unique baits based on the sequence of the HBV genome. We detected integrated HBV sequences in the genome of the PLC/PRF/5 cell line and found variable levels of methylation within the integrated HBV genomes. Allele-specific methylation analysis revealed that the HBV genome often became significantly methylated when integrated into highly methylated host sites. After integration into unmethylated human genome regions such as promoters, however, the HBV DNA remains unmethylated and may eventually play an important role in tumorigenesis. The observed dynamic changes in DNA methylation of the host and viral genomes may functionally affect the biological behavior of HBV. These findings may impact public health given that millions of people worldwide are carriers of HBV. We also believe our assay will be a powerful tool to increase our understanding of the various types of DNA virus-associated tumorigenesis.

[Supplemental material is available for this article.]

Hepatitis B virus (HBV) infects more than two billion people worldwide, and 400 million chronically infected individuals are at high risk of developing active hepatitis, cirrhosis, and hepatocellular carcinoma (HCC) (Gatza et al. 2005; Lupberger and Hildt 2007). HBV carriers with chronic liver disease are at a 100-fold greater risk of developing HCC, which is the third leading cause of cancer-related death worldwide. The HBV genome is integrated into the host genome in 90% of patients with HCC (HBV-HCC) (Gatza et al. 2005; Lupberger and Hildt 2007). HBV-HCCs have been analyzed by comprehensive genome sequencing and high-resolution genome mapping (Kan et al. 2013; Li and Mao 2013; Nakagawa and Shibata 2013). Moreover, the recent deep sequencing of HBV DNA in patients with HCC revealed increased integration events, structural alterations, and sequence variations (Ding et al. 2012; Fujimoto et al. 2012; Jiang et al. 2012; Sung et al. 2012; Toh et al. 2013). A recent study identified a viral-human chimeric fusion transcript, HBx-LINE1, that functions like a long noncoding RNA to promote HCC (Lau et al. 2014). However, the molecular details and clinical impact of HBV integration on the epigenomes of human cells and HBV remain to be defined.

Methylation of exogenous DNA (including viral DNA) that is integrated into the human genome has been studied over the past decade (Doerfler et al. 2001). Within the human genome, cytosine methylation in CpG dinucleotides (CpG sites), which cluster into islands associated with transcriptional promoters, is an important mechanism for regulating gene expression. Additionally, host cells use methylation as a defense mechanism against foreign agents (e.g., viral DNA) (Doerfler 2008; Doerfler et al. 2001). DNA methylation suppresses the expression of viral genes and other deleterious elements incorporated into the host genome over time. Establishment of de novo patterns of DNA methylation is char-

acterized by the gradual spread of methylation (Orend et al. 1991). Another attractive possibility is that DNA methylation camouflages the virus from the immune system (Tao and Robertson 2003; Hilleman 2004), resulting in a DNA methylation–related blockade of viral antigen presentation that allows the virus to escape immune control (Fernandez et al. 2009).

The DNA methylome of HBV in human cells may undergo dynamic changes at different stages of disease (Fernandez et al. 2009). For example, DNA methylation at the *HBVgp2* locus, which codes for the S viral proteins, reportedly increases during the progression from asymptomatic lesions to benign lesions, to premalignant disease and malignant tumors. However, because of the significant deletions of the integrated HBV genome detected in this previous study (Fernandez et al. 2009), the DNA methylome of HBV needs to be further characterized. Moreover, the molecular mechanisms involved and the clinical impact of the integration of HBV on the human and HBV epigenomes are unknown. To address these issues, we developed a next-generation sequencing (NGS)–based method for methylation analysis of integrated viral genomes (denoted G-NaVI) and applied this method to the integrative genomic and epigenomic analysis of human hepatoma cell lines and tissues with integrated HBV genomes.

Results

DNA methylation levels in PLC/PRF/5 cells and cancerous tissues obtained from HBV-HCC patients

Methylated CpG island (CGI) amplification (MCA) coupled with microarray (MCAM) analysis (Toyota et al. 1999; Oishi et al. 2012) was performed to detect methylated genes in the human PLC/PRF/5 cell line and in six paired specimens of primary HBV-HCC and adjacent tissues. Compared with the DNA methylation of CGIs in the healthy peripheral blood leukocytes of volunteers or the noncancerous tissues, levels of DNA methylation were not remarkable in the PLC/PRF/5 cells and the cancerous tissues obtained from HBV-HCC patients (Supplemental Fig. 1). These results were confirmed by bisulfite pyrosequencing of candidate tumor-related genes.

DNA methylation of CGIs of *HBx*

We then focused on epigenetic changes in the viral genome. Based on the hidden Markov models for sequence analysis performed on the CpG plugin of bioinformatics software Geneious 5.5.8 (see Methods section), a CpG island was found in only the promoter region of the *HBx* gene in the HBV genome (Fig. 1A; Supplemental Fig. 2; Durbin et al. 1998; Kearse et al. 2012). Host signal transduction pathways and gene expression are disrupted by the expression of *trans*-activating factors derived from the HBV genome, such as the HBx protein and PreS2 activators (Gatza et al. 2005; Lupberger and Hildt 2007). Moreover, transgenic mice expressing high levels of HBx in the liver develop HCC (Kim et al.1991; Koike et al. 1994). The DNA methylation levels of the CGIs of *HBx* were analyzed in 10 HBV-HCC samples and 10 adjacent samples, as well as samples of PLC/PRF/5 cells by bisulfite pyrosequencing (Fig. 1A; Supplemental Fig. 2). We performed advanced methylation quantification in long sequence runs by pyrosequencing on PyroMark Q24 Advanced and PyroMark Q24 instruments. Methylation levels of *HBx* varied across samples (Fig. 1B,C) and were generally lower in HCC tissues than in the adjacent

tissues (Fig. 1B). This finding is consistent with a previous report that most HBV genomes, although globally methylated to a greater extent in malignant samples than in premalignant lesions, retain *HBx* in an unmethylated state (Fernandez et al. 2009). Because the pyrosequencing results represent the genome-wide average of DNA methylation levels at the particular CpG site, the results could be affected by the HBV integration site. Therefore, genome-wide methylation analysis of the integrated HBV sequence is necessary in relation to the methylation state of the adjacent human genome. We did not detect an association between *HBx* methylation levels and those of the LINE1 and *Alu*Yb8 repeats (Fig. 1B).

Fluorescence in situ hybridization (FISH) and *Alu* PCR analyses of HBV integration

We developed a FISH technique for detecting HBV DNA in the genome of PLC/PRF/5 cells (Supplemental Figs. 3, 4). Twelve specific primer pairs (FISH probes 1–12) were designed based on the HBV sequences integrated into the genome of PLC/PRF/5 cells; amplification from all primer pairs was confirmed (Supplemental Fig. 4A). These results suggest full-length or partial HBV sequences that are covered by the 12 primer pairs were integrated into the genome of the PLC/PRF/5 cells. The FISH probes were labeled with digoxigenin, and FISH was performed using Carnoy-fixed chromosomal and nuclear specimens. Multiple HBV fluorescent signals (green) were detected in the nuclei (Supplemental Fig. 4B) using probes for *HBx* and its CGI sequences (probes 5 and 6), but not with probes 1–4 or 7–12 (Supplemental Fig. 4C–E). *Alu*-PCR identified one *HBx* integration site in PLC/PRF/5 (Supplemental Fig. 5). The integrated *HBx* sequence was 213 bp and included a promoter region. The *HBx* gene body was located only 13 bases (ATG GCT GCT AGG T) from the transcription start site and was integrated into a noncoding region of the host genome. There were 200 bases of viral DNA sequence upstream of the *HBx* transcription start site. According to the human genome reference sequence (GRCh38) published by the Genome Reference Consortium, this integration site was identified as a noncoding region of host Chromosome 5 1,350,106–1,350,478 that is near the telomerase reverse transcriptase (*TERT*) gene (Supplemental Fig. 5).

NGS analysis of HBV DNA integration site sequences

We developed an NGS analysis technique for sequencing the HBV DNA integration sites (Supplemental Fig. 6A). For efficient genome analysis, we synthesized 12,391 custom baits based on the sequences of the HBV genotypes A to J and on those sequences present in the HBV-transformed PLC/PRF/5 cells that were not related to the human genome sequence (Supplemental Fig. 6B). The average read length was 333.14 bp with a modal length of ~500 bp (Supplemental Fig. 6C). The average read quality was 31.91, corresponding to >99.9% accuracy. We did not detect a common HBV integration site (Fig. 2). The integration sites in the PLC/PRF/5 genome included intergenic (39%), intronic (39%), promoter (8%), and divergent promoter (15%) regions but not exonic (0%) sequences (Fig. 2). HepG2.2.15 cells, which stably express and replicate HBV in a culture system, are derived from the human hepatoblastoma cell line HepG2 (Sells et al. 1987). In the HepG2.2.15 genome, the integration sites included intergenic (29%), intronic (57%), and other (14%) regions but not promoter (0%), divergent promoter (0%), or exonic (0%) sequences (Fig. 2).
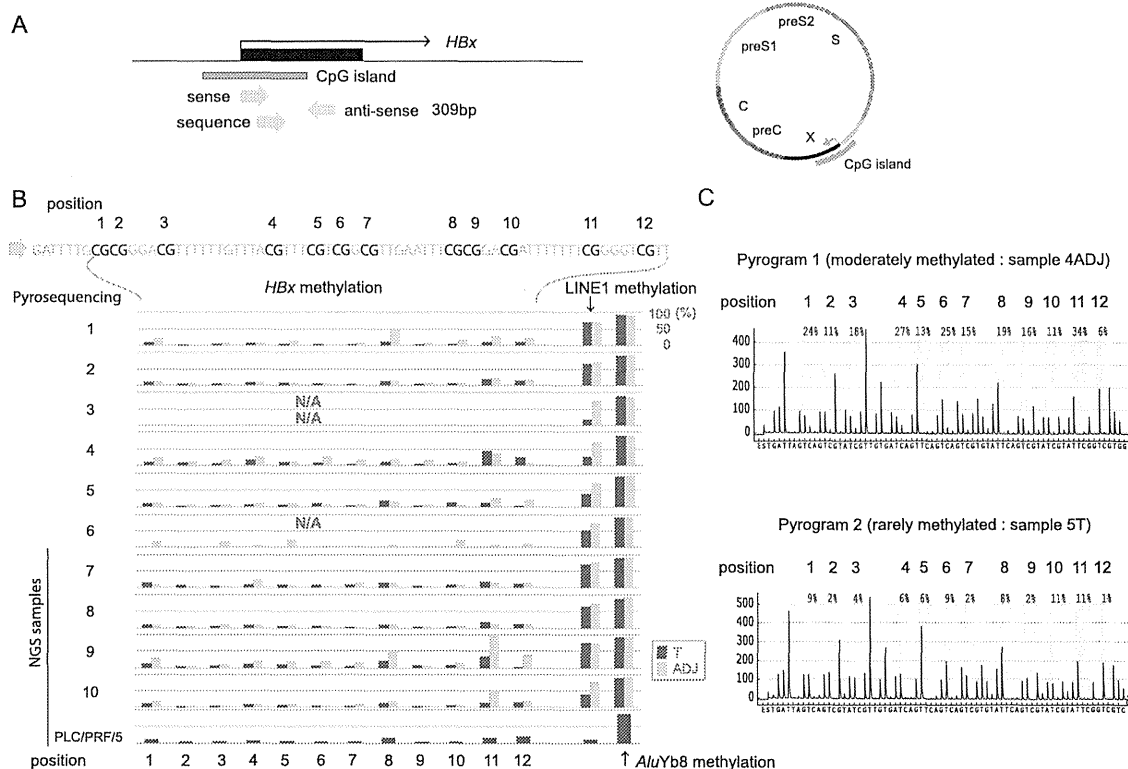
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Figure 1. Methylation analysis of the CGI of the *HBx* gene. (*A*) Schema of the CGI of the *HBx* gene. Three arrows show the pyrosequencing primers used for the methylation analysis. (*B*) DNA methylation levels of the CpGs of the *HBx* gene, LINE1, and *Alu*Yb8 in 10 paired HBV-HCC and adjacent nontumor tissue samples and PLC/PRF/5 DNA were analyzed using bisulfite pyrosequencing. Methylation levels of *HBx* varied across samples and were generally lower in HCC tissues than in the adjacent nontumor tissues. An association between *HBx* methylation levels and those of the LINE1 and *Alu*Yb8 repeats was not observed. N/A, could not be analyzed. DNAs from four paired HBV-HCC and adjacent nontumor tissue samples (sample nos. 7–10), PLC/PRF5, and HepG2.2.15 were further analyzed using the NGS (G-NaVI method). (*C*) Representative pyrograms showing DNA methylation levels of the CpGs of the *HBx* gene. Methylation levels at 12 CpG sites of the *HBx* gene in adjacent nontumor tissue (sample no. 4ADJ) and tumor tissue (sample no. 5T) are shown.

DNA methylation of the integrated HBV genome as well as the adjacent human genome in cell lines

DNA methylation of the integrated HBV genome, as well as the adjacent human genome, was analyzed by bisulfite pyrosequencing. We detected varying levels of methylation of the HBV sequences integrated into the genome of PLC/PRF/5 cells (Fig. 3; Supplemental Fig. 7). Our data suggest DNA methylation in the integrated HBV genome is related to the methylation status of the integration sites within the human genome. We further characterized the methylation status of the HBV genome and human genome by allele-specific DNA methylation analysis (Fig. 3A), which revealed that the HBV genome often showed significant methylation when integrated into highly methylated sites in the human genome; however, the HBV genome remained largely unmethylated when integrated into unmethylated regions such as promoters (Fig. 3B). Integration of the HBV genome did not affect the methylation status of the human genome, including the promoter regions of the *TERT* and *SNX15* genes. Methylation of HBV DNA integrated into HepG2.2.15 cells transformed with HBV DNA (using a head-to-tail dimer) was further analyzed by bisulfite pyrosequencing, which revealed that the HBV genome generally showed significant methylation when integrated into highly methylated regions of the human genome; however, the HBV genome remains largely unmethylated when integrated into unmethylated regions (Fig. 3A).

DNA methylation levels in orthologous loci

We examined methylation levels of orthologous loci in HepG2.2.15 cells and in peripheral blood lymphocytes (PBLs) of a healthy volunteer and compared them to the methylation levels at the same (empty) target sites of PLC/PRF/5 cells. Methylation levels of orthologous loci in HepG2.2.15 cells and PBLs were generally similar to those of PLC/PRF/5 cells (Fig. 3B). Similarly, we examined methylation levels of orthologous loci in PLC/PRF/5 cells and in PBLs of a healthy volunteer and compared them to the methylation levels at the same (empty) target sites of HepG2.2.15 cells. Methylation levels of orthologous loci in PLC/PRF/5 cells and PBLs were also generally similar to those of HepG2.2.15 cells (Fig. 3B).

DNA methylation of the integrated HBV genome and the adjacent human genome in HCC tissues

To determine whether our results are relevant to human tumors, we used bisulfite pyrosequencing to investigate the methylation status of the HBV and human genomes in surgical specimen pairs of HCC and adjacent nontumor tissues. We detected no common HBV integration site (Fig. 4; Supplemental Fig. 8). Recurrent HBV integration into the *SLC6A13* gene was observed in cancerous tissues. Integration sites were rarely detected in exonic regions of the DNA from HBV-HCC samples (Fig. 4; Supplemental Fig. 8). Similar to the results obtained from the PLC/PRF/5 and HepG2.2.15 cells, our analysis revealed that the HBV genome became significantly
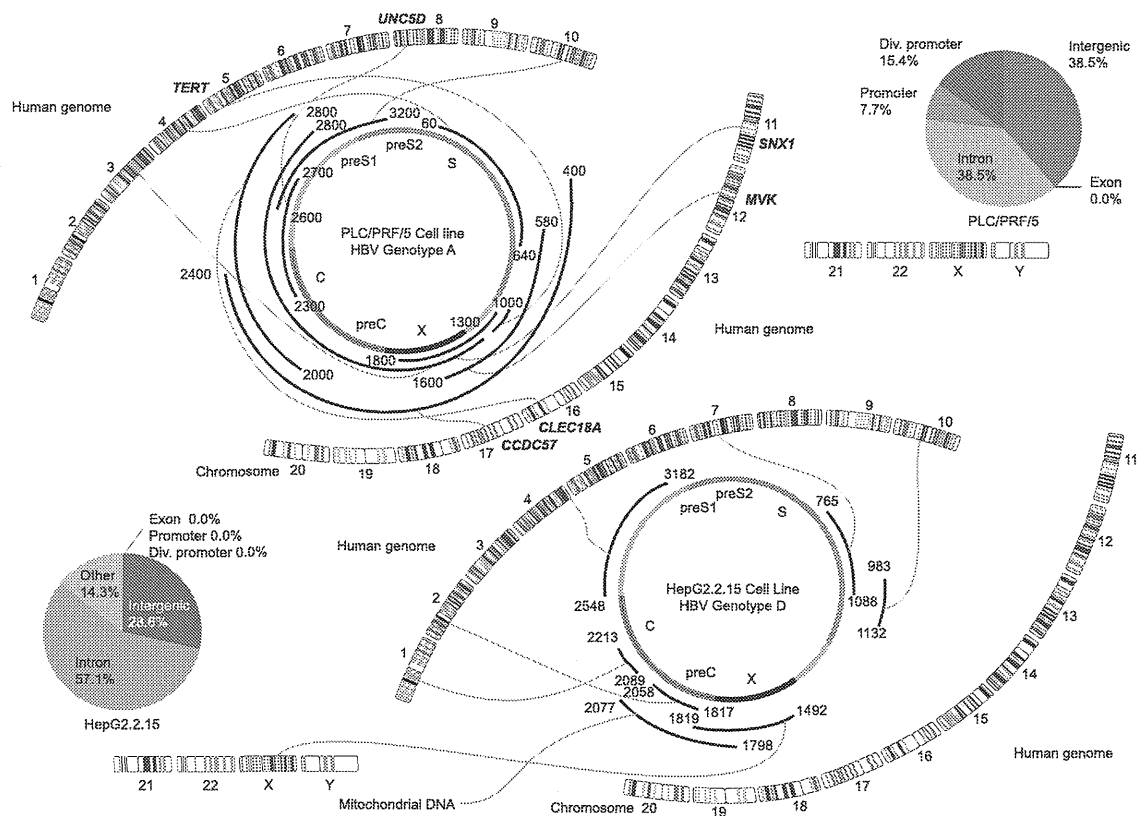
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Figure 2. Distribution of the integration sites in the HBV genome and human chromosomes represented by Circos plots of the PLC/PRF/5 genome and the HepG2.2.15 genome. HBV DNA integration was analyzed using the G-NaVI method in the genome of PLC/PRF/5 cells and HepG2.2.15 cells. A common HBV integration site was not detected. Integration sites were not detected in exonic regions of the DNA from cell lines (Venn diagrams). The HBV genes (*PreC, Precore; C, Core; PreS, Presurface; S, Surface; X, X*) and the 24 human chromosomes are shown.

methylated when integrated into highly methylated human genome regions but not when integrated into unmethylated human genome regions (Fig. 4).

Correlation between the methylation pattern of the integrated HBV DNA and the human genome

DNA fragments, including 200 bp of the HBV DNA and 200 bp of the human genome around the boundary, were analyzed for average methylation, GC content, and repetitive sequences. A statistically significant correlation was observed between the average methylation of the HBV DNA and that of the human genome in cell lines and clinical samples (Fig. 5A–C; Supplemental Table 2). In contrast, average methylation did not correlate with GC content or repetitive sequences in the human and viral genome (Fig. 5D,E; Supplemental Table 2).

Using Bander software, we analyzed the chromatin structure at the integrated HBV site in PLC/PRF/5 and HepG2.2.15. Open chromatin and heterochromatin were observed more frequently at the integrated HBV in PLC/PRF/5 and HepG2.2.15, respectively (Supplemental Table 3). The difference may reflect the fact that PLC/PRF/5 is a naturally derived HBV-positive cell line and HepG2.2.15 is an HBV DNA-transfected cell line.

Discussion

We developed an NGS-based method for structural methylation analysis of integrated viral genomes. This method is a novel approach that enables the enrichment of viral fragments for sequencing using unique baits based only on the sequence of the HBV genome. We detected all regions of the human genome that harbored integrated HBV genomes without conducting unnecessary sequencing of regions where the HBV genome was not integrated. Because this technique only requires sequencing a small region of DNA around the integrated HBV sequences, a sufficient number of sequence reads can be acquired.

Methylation of viral DNA in infected cells may alter the expression patterns of viral genes related to infection and transformation (Burgers et al. 2007; Fernandez et al. 2009) and may clarify why certain infections are either cleared or persist with or without progression to precancer (Mirabello et al. 2012). To the best of our knowledge, we have, for the first time, established that the de novo patterns of DNA methylation in the integrated HBV genome are related to the methylation status of the integration sites within the human genome. A statistically significant correlation between the average methylation of the HBV DNA and that of the human genome in cell lines and clinical samples has greatly substantiated our findings. It is possible that the HBV genome becomes inactivated by methylation, when it is integrated into highly methylated host sites; therefore, HBV methylation may not contribute to tumor development. However, after integration into unmethylated human genome regions such as promoters, the HBV DNA remains unmethylated and may eventually play an important role in tumorigenesis (Fig. 6). Because multiple HBV integration sites were present in each of the analyzed samples, there remains the possibility of an asso-
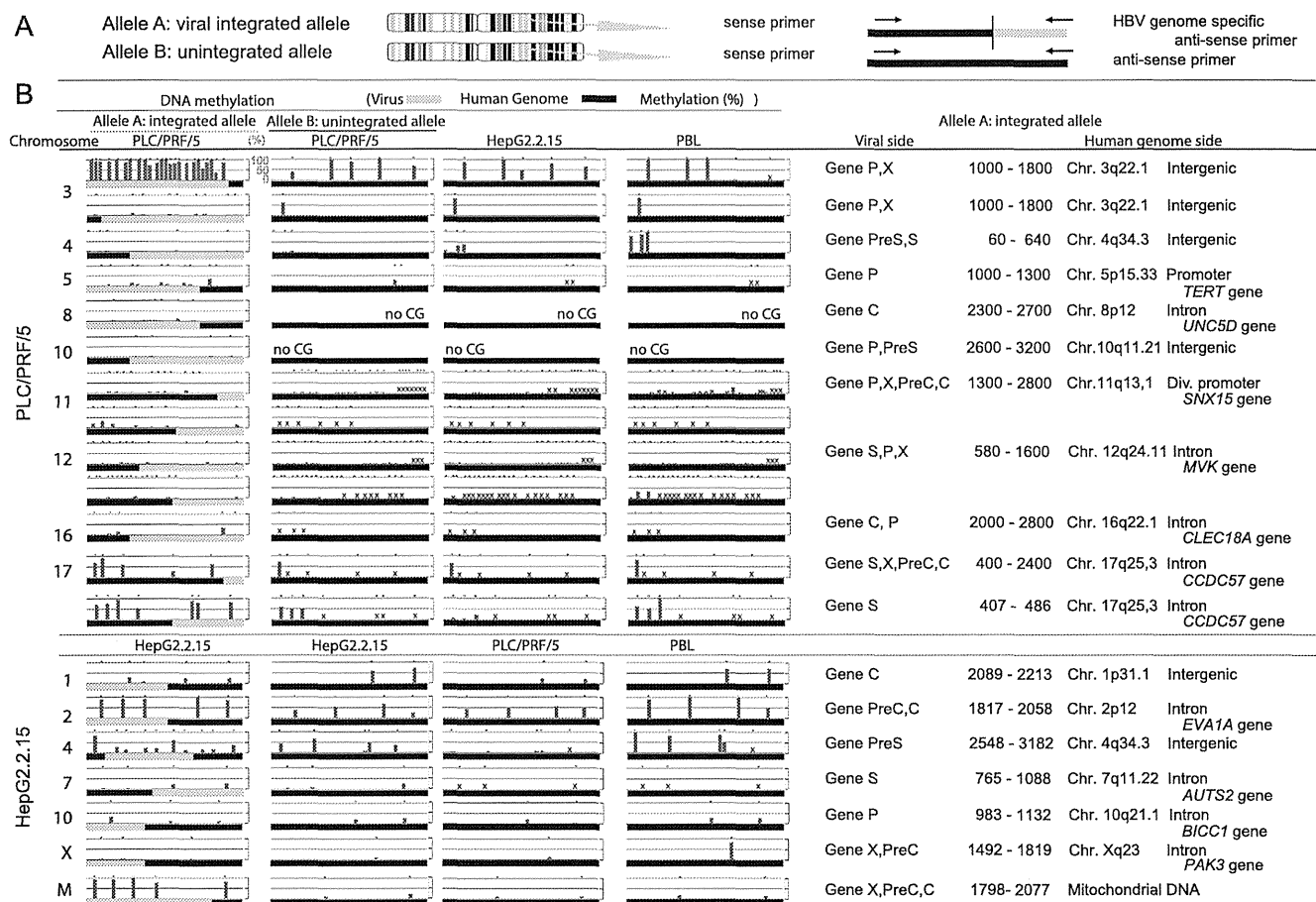
Figure 3. Allele-specific methylation analysis of the PLC/PRF/5 genome and the HepG2.2.15 genome. (*A*) A schema of allele-specific methylation analysis. (*B*) The methylation levels of the HBV and human genomes for the integrated and unintegrated alleles. Detailed results of the HBV integrants (*PreC, Precore; C, Core; PreS, Presurface; S, Surface; X, X*) and flanking host genomes (position, chromosome, location of the genome, and gene names) are shown. DNA methylation of the integrated HBV genome as well as the flanking human genome was examined by allele-specific DNA methylation analysis using bisulfite pyrosequencing. The HBV genome often showed significant methylation when integrated into highly methylated sites in the human genome; however, the HBV genome remained largely unmethylated when integrated into unmethylated regions. Methylation levels of orthologous loci in HepG2.2.15 cells and in PBLs of a healthy volunteer were examined and compared to the methylation levels at the same (empty) target sites of PLC/PRF/5 cells. Methylation levels of orthologous loci in HepG2.2.15 cells and PBLs were generally similar to those of PLC/PRF/5 cells. Similarly, methylation levels of orthologous loci in PLC/PRF/5 cells and in PBLs of a healthy volunteer were examined and compared to the methylation levels at the same (empty) target sites of HepG2.2.15 cells. Methylation levels of orthologous loci in PLC/PRF/5 cells and PBLs were generally similar to those of HepG2.2.15 cells. (×) The desired quantitative methylation levels were not obtained because of technical difficulties with the sequences that were being analyzed.

ciation between methylation and viral transcript levels. The biological impact of methylation on viral transcript levels or viral function, induced by viral insertions, also needs to be further addressed.

Methylation levels of orthologous loci in other samples at the same (empty) target sites of PLC/PRF/5 were generally similar to those of PLC/PRF/5. Similar results were observed in HepG2.2.15. These data suggest that a "before and after" relationship exists between methylation levels at preexisting target sites and those within viral insertions. At the same time, we cannot rule out the possibility that the integration of the virus subsequently affects the methylation established at the flanking target site, perhaps by acting in trans on the empty target site-containing allele. Therefore, this issue needs to be further addressed.

Differences in the integrated viral sequences could have a direct impact on the amount of cytosine methylation observed. In cases where the integration site is a highly active promoter, comparisons of methylation statuses may not be informative. Addi-

tional studies, using a large number of samples, are needed to address this issue.

Our results are notable because other studies have detected a statistically significant enrichment of HBV integration into regulatory regions, particularly promoters, in tumors (Sung et al. 2012; Toh et al. 2013); this observation may be explained by the relatively open chromatin structure of promoter regions. Average methylation did not correlate with GC content or repetitive sequences in the human and viral genomes. The relationship between methylation of HBV sequences and chromatin structure remains to be clarified because of the limitation of the Bander software used in this study. Although the mechanism needs clarification, the significant enrichment of HBV integration into regulatory regions would favor integrated HBV nonmethylation and lead to tumorigenesis. Alternatively, while the integration of HBV into the host genome may be random, HBV integration into regulatory regions is positively selected during tumorigenesis (Toh et al. 2013).