reported that PLNE is a relatively common finding in patients with primary biliary cirrhosis among other liver diseases.[15]

On the other hand, the reports regarding PLNE in patients with chronic HBV infection have been scarce. The purpose of the present study was to evaluate the clinical significance of PLNE in chronic HBV infection.

METHODS

Patients and screening for PLNE

WE ENROLLED 502 consecutive patients with chronic HBV infection who underwent ultrasonography (US) between November 2012 and April 2013 at the Department of Clinical Laboratory, the University of Tokyo Hospital. Patients with chronic HBV infection were defined as those positive for hepatitis B surface antigen for at least 6 months. Patients who were positive for HCV RNA and had a history of other hepatobiliary disease were excluded. All patients took laboratory blood tests at the time they underwent US. Aspartate aminotransferase (AST) platelet ratio index (APRI) was used to assess liver fibrosis, and APRI of more than 1.5 was classified as bridging fibrosis or cirrhosis (F stage 3–4).[18] The criteria to identify PLNE were previously described; PLNE was defined as a lymph node at the perihepatic area measuring 1 cm or more in the longest axis.[17] In the analysis to examine the association between PLNE and clinical findings, we excluded the patients receiving IFN or nucleoside analog treatment, or having HCC or a history of HCC from the primary analysis.

The present study was carried out in accordance with the ethical guidelines of the Declaration of Helsinki and was approved by the Institutional Research Ethics Committees of the authors' institutions.

Study end-points

We examined the association between PLNE and clinical findings such as liver fibrosis, hepatocellular injury or the presence of HCC in patients with chronic HBV infection (the primary end-point of this study). We previously reported the prevalence of PLNE in the patients with CHC[17] or the patients underwent general health examinations (general population).[19] Using the results of our previous studies, we then compared the prevalence of PLNE in the patients with chronic HBV infection to patients with CHC or general population (the secondary end-point).

Statistical analysis

Continuous variables were presented as the mean ± standard deviation (SD), while categorical variables were expressed as frequencies (%). Categorical data were analyzed using the χ^2-test or Fisher's exact test, and stepwise logistic regression model analyses were used to adjust the contribution of PLNE by other covariates such as sex or age. For continuous data, the univariate associations were evaluated using Student's *t*-test. Stepwise regression model analyses were used to adjust the contribution of PLNE by other covariates such as sex or age. The Cochran–Armitage trend test was used for assessing increasing or decreasing trends in binomial proportions. All statistical analyses were two-sided, and the threshold of the reported *P*-values for significance was accepted as less than 0.05. All statistical analyses were performed using R statistic software version 2.15.2 (http://www.r-project.org).

RESULTS

Patient characteristics and association between PLNE and clinical findings

TO EXAMINE THE association between PLNE and clinical findings, patients receiving IFN or nucleoside analog treatment, and those with HCC or past history of HCC were excluded from the primary analysis, because antiviral treatment or HCC could directly influence PLNE. As a result, the data of 288 among 502 patients were primarily analyzed. Characteristics of these patients are shown in Table 1. Overall, 51.0% (147) were male, and the mean age was 53.72 years. PLNE were detected in 27 of 288 (9.4%) patients, and the mean length of the longest axis was 1.6 cm (range, 1.0–3.2).

Table 2 shows the relationships between PLNE and various clinical findings. The presence of PLNE was significantly associated with a higher APRI value of more than 1.5 ($P = 0.01$), a higher serum AST level ($P = 0.001$), a higher serum alanine aminotransferase (ALT) level ($P < 0.0001$), and a lower platelet count ($P = 0.048$) after adjustment for sex and age, suggesting that PLNE may be observed in patients with more liver fibrosis and more hepatocellular injury. We also compared the correlation between PLNE and clinically diagnosed liver cirrhosis. Diagnosis of cirrhosis is based on the presence of clinical and laboratory features of portal hypertension (the presence of esophageal varices and/or collateral circulation at endoscopy and ultrasound) and/or liver stiffness measurement value more than

Table 1 Patient characteristics ($n = 288$)

Parameter	Values
Mean age (years)	53.72 ± 14.74
Sex	
Male	147 (51.0%)
Female	141 (49.0%)
PLNE	
Present	27 (9.4%)
Absent	261 (90.6%)
Perihepatic lymph node diameter†	1.6 (1.0–3.2)‡
APRI score	
>1.5	7 (2.4%)
≤1.5	281 (97.6%)
Mean HBV DNA (log^{10} copies/mL)	3.97 ± 2.13
Mean AST (U/L)	27.83 ± 17.37
Mean ALT (U/L)	30.09 ± 30.68
Mean TB (mg/dL)	0.93 ± 0.57
Mean albumin (g/dL)	4.20 ± 0.34
Mean platelet count ($\times 10^4/\mu L$)	20.43 ± 6.01
Mean γ-GT (U/L)	39.26 ± 108.55
Mean PT-INR	0.95 ± 0.14

†In PLNE positive patients.
‡Median (range).
Continuous variables are represented as the mean ± standard deviation and categorical variables were as number and frequencies (%).
γ-GT, γ-glutamyltransferase; ALT, alanine aminotransferase; APRI, aspartate aminotransferase-to-platelet ratio index; AST, aspartate aminotransferase; HBV, hepatitis B virus; PLNE, perihepatic lymph node enlargement; PT-INR, prothrombin time international normalized ratio; TB, total bilirubin.

16.9 kPa which was reported to be the optimal diagnostic accuracy.[20] Of the patients, 18.5% (5/27) with PLNE and 2.7% (7/261) without PLNE were clinically diagnosed with liver cirrhosis. The prevalence of the patients with clinically diagnosed liver cirrhosis was significantly higher in the patients with PLNE ($P = 0.0009$) after adjustment for sex and age (data not shown). In addition, there was a trend that serum HBV DNA level was higher in patients with PLNE than in those without, although not statistically significant (Table 2).

We then compared the prevalence of PLNE among asymptomatic carrier (APRI, ≤1.5; ALT, ≤30), patients with chronic hepatitis (APRI, ≤1.5; ALT, >30) and patients with cirrhosis (APRI, >1.5). PLNE was detected in 8.3% (17/206) of asymptomatic carriers, 9.3% (7/75) of patients with chronic hepatitis and 42.9% (3/7) of patients with cirrhosis (Fig. 1). The progression of liver disease was significantly associated with higher prevalence of PLNE ($P = 0.03$), as demonstrated by the Cochran–Armitage trend test. We also examined the association between hepatitis B e (HBe) status and the prevalence of PLNE. PLNE was detected in 18.8% (6/32) of the patients positive for HBe antigen (HBeAg) and 8.3% (21/251) of the patients negative for HBeAg. Prevalence of PLNE was higher in the patients with positive for HBeAg, however, the difference did not reach statistical significance ($P = 0.11$).

Comparison of the frequency of PLNE in patients with HCC and/or its history and those without HCC

Because our current findings suggest the associations between PLNE and liver fibrosis, hepatocellular injury or serum HBV DNA level in patients with chronic HBV infection, we wondered whether PLNE might be observed more frequently in those with HCC and/or its past history than in those without. On the other hand, PLNE was reportedly a negative risk for hepatocarcinogenesis in CHC patients. Thus, these results prompted us to examine how PLNE could be associated with HCC in patients with chronic HBV infection.

To address this question, we compared the frequency of PLNE in patients with HCC and/or its history and in those without in our original sample ($n = 502$). Table 3 shows the patient characteristics and the associations between prevalence of HCC and clinical findings. PLNE was detected in 1.4% (1/69) of the patients with HCC and/or its history and 9.2% (40/433) of the patients without HCC, where the patients receiving IFN or nucleoside analog treatment were also included in the analysis. As shown in Figure 2 and Table 3, the frequency of PLNE was significantly lower in patients with HCC and/or its history than in those without ($P = 0.03$). Then, we tested PLNE and the following variables on multivariate analysis: age, sex, APRI score, ALT, total bilirubin, albumin and γ-glutamyltransferase. As a result, the association between PLNE and lower probability of HCC was noted, although not statistically significant ($P = 0.057$). In this multivariate analysis, higher prevalence of HCC was significantly associated with older age ($P = 0.01$), male sex ($P = 0.009$), higher APRI score ($P = 0.0002$) and lower ALT level ($P = 0.0006$) (data not shown).

Comparison of the frequency of PLNE with liver diseases of other etiology

Perihepatic lymph node enlargements were detected in 27 of 288 (9.4%) of the patients without treatment for

Table 2 Associations between PLNE and clinical findings ($n = 288$)

Variable	n (proportion)/mean (SD)		P	
	PLNE positive group ($n = 27$)	PLNE negative group ($n = 261$)	P-value	Adjusted P-value‡
APRI >1.5†	3 (11.1%)	4 (1.5%)	0.02	0.01§
HBV DNA (log^{10} copies/mL)	4.70 (2.97)	3.89 (2.01)	0.2	0.13¶
AST (U/L)	38.0 (33.5)	26.8 (14.5)	0.09	0.001¶
ALT (U/L)	50.1 (70.1)	28.0 (22.4)	0.11	<0.0001¶
TB (mg/dL)	0.93 (0.44)	0.93 (0.58)	0.98	-
Albumin (g/dL)	4.14 (0.29)	4.20 (0.34)	0.33	-
Platelet count ($\times 10^4/\mu L$)	18.6 (6.96)	20.6 (5.87)	0.12	0.048¶
γ-GT (U/L)	44.6 (84.1)	38.7 (110.9)	0.74	-
PT-INR	0.99 (0.09)	0.95 (0.14)	0.11	-
AFP (ng/mL)	3.22	4.68	0.15	-

†Odds ratio (95% confidence interval) for PLNE positive group was 7.85 (1.59–38.76).
‡Adjusted for sex and age at enrollment (independent variables). The dependent variables of each P-value are the items in the leftmost fields of corresponding rows (the proportion of having APRI >1.5, AST, ALT, TB and so on).
§P-value by stepwise logistic regression analysis.
¶P-value by stepwise regression analysis.
γ-GT, γ-glutamyltransferase; AFP, α-fetoprotein; ALT, alanine aminotransferase; APRI, aspartate aminotransferase-to-platelet ratio index; AST, aspartate aminotransferase; HBV, hepatitis B virus; PLNE, perihepatic lymph node enlargement; PT-INR, prothrombin time international normalized ratio; SD, standard deviation; TB, total bilirubin.

HBV or history of HCC and in 41 of 502 (8.2%) of the whole patients, and the proportion of PLNE was not significantly different between these two patient groups ($P = 0.65$, χ^2-test). In addition, in patients receiving IFN or nucleoside analog treatment and/or having HCC or its past history, PLNE was found in 7.0% patients. Thus, PLNE may be observed in as much as 10% of patients with chronic HBV infections. Then, we compared the frequency of PLNE in subjects with different backgrounds; in our previous studies, PLNE was detected in 20.0% (169/846) of patients with CHC[17] and in 1.6% (69/4234) of subjects who underwent a general health examination.[19] As shown in Figure 3, the frequency of PLNE in patients with chronic HBV infection was significantly higher than that in subjects undertaking a general health examination ($P < 0.0001$) but lower than that in CHC patients ($P < 0.0001$).

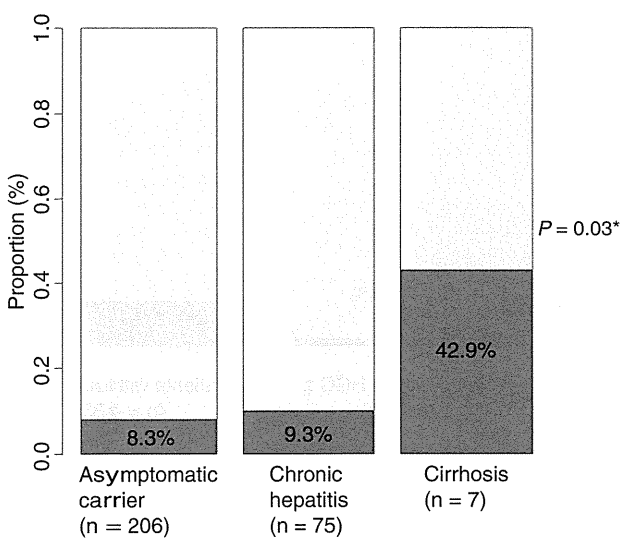

Figure 1 Bar plot of the proportion of perihepatic lymph node enlargement (PLNE) positive patients in asymptomatic carriers, patients with chronic hepatitis and patients with cirrhosis. *P-value by the Cochran–Armitage trend test. □, PLNE negative; ■, PLNE positive.

DISCUSSION

ALTHOUGH PLNE IS one of the common findings in chronic liver disease,[8–12,16,17] it has yet remained unclear how frequent PLNE would be observed or what would be the clinical significance of PLNE in patients with chronic HBV infection. In the current study, in patients without HBV treatment or HCC, PLNE was significantly associated with a higher probability of having an APRI of more than 1.5, a higher serum AST level and a higher ALT serum level. Also, a significantly increasing trend of PLNE prevalence across asymptomatic carriers, patients with chronic hepatitis and patients with

Table 3 Patient characteristics according to prevalence of HCC in the original cohort ($n = 502$)

Variable	n (proportion)/mean (SD)		P
	HCC positive group ($n = 69$)	HCC negative group ($n = 433$)	
PLNE positive	1 (1.4%)	40 (9.2%)	0.03
APRI >1.5	8 (11.6%)	14 (3.2%)	0.005
HBV DNA (log^{10} copies/mL)	1.19 (1.67)	3.00 (2.36)	<0.0001
AST (U/L)	30.4 (17.8)	28.3 (27.3)	0.40
ALT (U/L)	27.2 (19.1)	29.5 (38.1)	0.42
TB (mg/dL)	1.02 (0.45)	0.95 (0.56)	0.29
Albumin (g/dL)	4.08 (0.41)	4.20 (0.39)	0.03
Platelet count ($\times 10^4/\mu L$)	14.0 (5.41)	19.7 (6.10)	<0.0001
γ-GT (U/L)	48.8 (50.2)	40.9 (98.9)	0.31
PT-INR	1.00 (0.12)	0.98 (0.25)	0.60

γ-GT, γ-glutamyltransferase; ALT, alanine aminotransferase; APRI, aspartate aminotransferase-to-platelet ratio index; AST, aspartate aminotransferase; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; PLNE, perihepatic lymph node enlargement; PT-INR, prothrombin time international normalized ratio; SD, standard deviation; TB, total bilirubin.

cirrhosis was observed, suggesting that PLNE may be associated with progress of liver fibrosis and severer hepatocellular damage in patients with chronic HBV infection. Furthermore, the frequency of PLNE in patients with chronic HBV infection was significantly lower than that in CHC patients. To the best of our knowledge, this is the first study showing the clinical significance of PLNE in chronic HBV infection.

It is well known that liver fibrosis and hepatocellular injury are associated with HCC occurrence generally in chronic liver disease.[21] Furthermore, recent evidence has revealed that serum HBV DNA level is a risk for hepatocarcinogenesis in chronic HBV infection.[22] In the current study, PLNE was associated with liver fibrosis, hepatocellular injury and serum HBV DNA level, however, PLNE was less frequently observed in patients with HCC and/or its history than in those without HCC in our original sample ($n = 502$). Although this paradoxical result is interesting, its mechanism remains to be clarified. Of note, we previously reported that PLNE was a negative risk for HCC occurrence in CHC patients.[17] Thus, the result in the present study may imply the similar association between PLNE and the development of HCC in patients with chronic liver disease in general. One probable explanation for this paradoxical result is that the presence of PLNE may reflect a stronger immune response to hepatitis virus, which could exert also an antitumor immune response.

On multivariate analysis regarding HCC prevalence and clinical parameters, lower ALT levels were significantly associated with higher prevalence of HCC. This

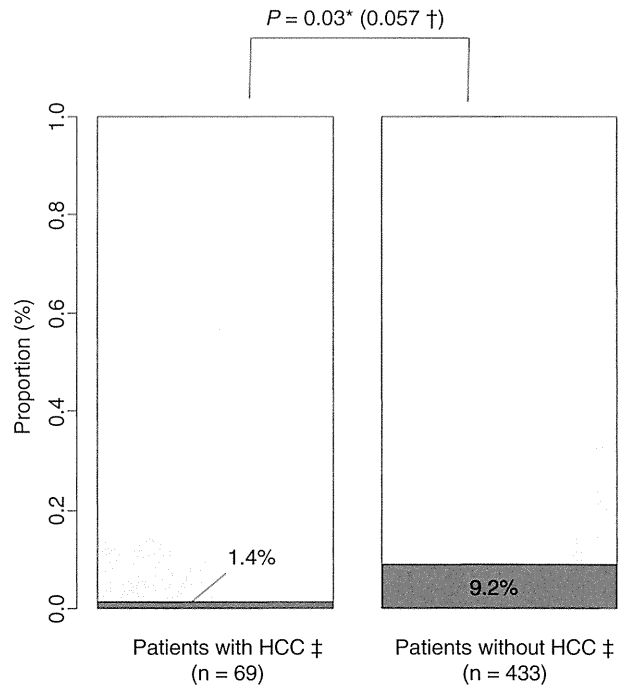

Figure 2 Bar plot of the proportion of perihepatic lymph node enlargement (PLNE) positive patients with and without hepatocellular carcinoma (HCC). *P-value by Fisher's exact test. †P-value by logistic regression test (adjustment for age, sex, aspartate aminotransferase-to-platelet ratio index score, alanine aminotransferase, total bilirubin, albumin and γ-glutamyltransferase). ‡Patients receiving interferon or nucleoside analog treatment were also included. □, PLNE negative; ■, PLNE positive.

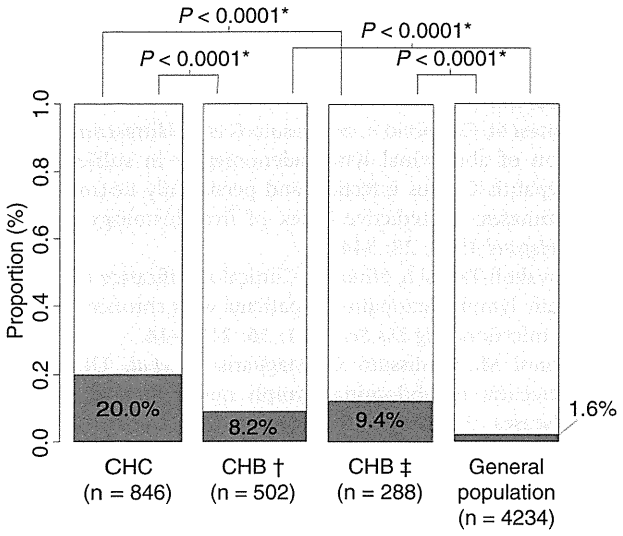

Figure 3 Bar plot of the proportion of perihepatic lymph node enlargement (PLNE) positive patients in chronic hepatitis B (CHB), chronic hepatitis C (CHC) and general health examination. **P*-value by the χ^2-test. †Total patients; ‡Patients without history of hepatocellular carcinoma or treatment for CHB. □, PLNE negative; ■, PLNE positive.

unexpected result may be explained by the treatment effect for HBV. Nucleotide analog treatment has been generally applied in symptomatic patients but not asymptomatic ones in our cohort, in which HCC occurred more frequently in these symptomatic patients. Indeed, the proportion receiving nucleotide analog treatment was higher in HCC positive patients compared with HCC negative patients (71.0% vs 33.4%, $P < 0.0001$, data not shown), which may explain the lower ALT levels in HCC positive patients. Lower HBV DNA levels in HCC positive patients compared to HCC negative patients (1.19 vs 3.00, $P < 0.0001$, Table 3) may be similarly explained by nucleotide analog treatment.

It should be noted that controversy still exists regarding whether PLNE could be associated with hepatocellular damage or liver fibrosis in patients with chronic HCV infection.[6–9,11,13,14] These results raise a possibility that the clinical significance of PLNE may be distinct regarding the association with hepatitis activity between chronic HBV infection and chronic HCV infection. The distinct PLNE frequency between chronic HBV infection and chronic HCV infection observed in the current study may be in line with this concept. Hyperplasia of regional lymph nodes are generally considered to reflect inflammatory responses in the adjacent organs. Especially in chronic HCV infection, PLNE are thought to reflect the immunological response of the host.[11] Indeed, HCV-specific IFN-γ production and proliferative response of T cells were found commonly in perihepatic lymph nodes,[23] suggesting that PLNE indicates an active host immune response in chronic hepatitis C. The humoral immune response plays an essential role in HBV and HCV infection.[24] Almost 40% of patients infected with HCV were reported to develop at least one extrahepatic manifestation during the course of the disease.[25] CHC patients were shown to be associated with mixed cryoglobulinemia, chronic thyroiditis, Sjögren's syndrome or membranoproliferative glomerulonephritis.[26–31] On another front, extrahepatic manifestations of hepatitis B were reported to be present in 1–10% of HBV-infected patients, which is lower than that of HCV-infected patients, including serum sickness-like syndrome, acute necrotizing vasculitis (polyarteritis nodosa), membranous glomerulonephritis or popular acrodermatitis of childhood (Gianotti–Crosti syndrome).[32,33] Thus, there may be an apparent incompatibility between patients with chronic HBV and HCV infection in terms of the component of immune system. The result of the present study may support this concept.

In patients not receiving IFN or nucleoside analog treatment, and those without having HCC or its past history for comparison with other background ($n = 288$) to avoid an influence of antiviral treatment or HCC occurrence, the frequency of PLNE was 9.4%. Although these patients appear to be biased toward a less severely ill patient population, the frequency of PLNE in these patients was not significantly different from that in the original 502 patients (8.2%). Of note, the frequency of PLNE in CHB patients was much lower than that in chronic hepatitis C patients, as previously reported. As suggested earlier, the different components of the immune system in patients with chronic HBV and HCV infections could explain this difference.

This study is limited by the absence of some important clinical details such as information about the histological findings of fibrosis and inflammation. Although the APRI is a useful index for the prediction of fibrosis, the limitation of this score has been reported in previous studies.[34,35] However, we also showed that the prevalence of the patients with clinically diagnosed liver cirrhosis was also significantly higher in the patients with PLNE ($P = 0.0009$), which may minimize this limitation. Another limitation to consider is the cross-sectional design of the present study, which does not allow causal inferences and limits any assumptions about the duration of the existence of any of the criteria,

such as APRI score, ALT level or HCC occurrence. Also, clinical findings at the onset of PLNE were not accessible. Moreover, for comparison of the frequency of PLNE in patients with HCC and/or its history and those without, only one HCC patient with PLNE positivity was included in the present study. Thus, the sample may not be large enough, especially for adjustment of other covariates. Further prospectively designed study with a larger sample is needed to elucidate the association between PLNE and the risk of hepatocarcinogenesis.

In conclusion, in spite of the positive association between the presence of PLNE and progressed fibrosis or higher hepatocellular injury, the presence of PLNE was negatively associated with the prevalence of HCC in patients with chronic HBV infection.

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TOPIC HIGHLIGHT

WJH 6th Anniversary Special Issues (1): Management of hepatocellular carcinoma

Diagnostic and therapeutic application of noncoding RNAs for hepatocellular carcinoma

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Abstract

MicroRNAs (miRNAs) are small, noncoding RNA molecules that regulate gene expression posttranscriptionally, targeting thousands of messenger RNAs. Long noncoding RNAs (lncRNAs), another class of noncoding RNAs, have been determined to be also involved in transcription regulation and translation of target genes. Since deregulated expression levels or functions of miRNAs and lncRNAs in hepatocellular carcinoma (HCC) are frequently observed, clinical use of noncoding RNAs for novel diagnostic and therapeutic applications in the management of HCCs is highly and emergently expected. Here, we summarize recent findings regarding deregulated miRNAs and lncRNAs for their potential clinical use as diagnostic and prognostic biomarkers of HCC. Specifically, we emphasize the deregulated expression levels of such noncoding RNAs in patients' sera as noninvasive biomarkers, a field that requires urgent improvement in the clinical surveillance of HCC. Since nucleotide-based strategies are being applied to clinical therapeutics, we further summarize clinical and preclinical trials using oligonucleotides involving the use of miRNAs and small interfering RNAs against HCC as novel therapeutics. Finally, we discuss current open questions, which must be clarified in the near future for realistic clinical applications of these new strategies.

Key words: MicroRNA; Long noncoding RNA; Hepatocellular carcinoma; Clinical trials; Biomarker

Core tip: In this review, we summarize the latest findings on deregulated microRNAs (miRNAs) and long noncoding RNAs in hepatocellular carcinomas (HCCs) with a focus on their clinical use as novel diagnostic and prognostic

biomarkers. In addition, we summarize the current status of clinical and preclinical oligonucleotide therapies including miRNAs and small interfering RNAs as novel HCC therapeutics. This review will enable the readers to understand the current status of clinical applications and knowledge of noncoding RNAs in HCC management.

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INTRODUCTION

Noncoding RNAs contain multiple classes of RNAs that are not transcribed into proteins. While most noncoding RNAs studied to date are microRNAs (miRNAs), many noncoding RNAs with various lengths have also been reported.

MiRNAs are short, single-stranded RNAs that are expressed in most organisms[1-3]. Through gene expression regulation at a posttranscriptional level, miRNAs are involved in various physiological and pathological processes[4,5]. Since the discovery of miRNA lin-4 in Caenorhabditis elegans[6,7], as of August 2014, 1881 miRNA precursors and 2588 mature miRNA sequences in humans are deposited in miRBase, a miRNA database by the Sanger Institute[8]. MiRNAs are dysregulated in nearly all types of cancer[9,10], and specific signatures of aberrantly expressed miRNAs in specific cancers may have diagnostic and therapeutic implications[11,12].

Long noncoding RNAs (lncRNAs) also play crucial roles in transcription and translation[13,14]. Similar to miRNAs, their dysregulation is also associated with human cancers[15]. One of the most well-studied lncRNAs is the *HOX* transcript antisense intergenic RNA (HOTAIR). Class I homeobox genes (*HOX* in humans) encode 39 transcriptional factors initially described as master regulators of embryonic development[16] and display a unique gene network organization. HOTAIR, a 2.2-kb-long RNA residing within the *HOXC* locus, was initially described in breast cancer tissues, where it is highly expressed[17]. In addition to HOTAIR, many other lncRNAs are dysregulated in cancer tissues. Thus, lncRNAs may also be candidates for biomarker discovery and therapeutic applications in hepatocellular carcinomas (HCCs)[18].

In contrast to miRNAs and lncRNAs, short interfering RNAs (siRNAs) are double-stranded RNAs that degrade mRNAs through perfect matches with their target sequences. Although human telomerase reverse transcriptase was recently found to function as an RNA-dependent RNA polymerase and contribute to RNA silencing[19], its activities are not dominant in mammals. Additionally, endogenously produced siRNAs may play functional roles under limited circumstances in humans[20]. However, the exogenous application of synthesized siRNAs is an attractive method that could be used to intervene in crucial gene expression under pathological conditions, including cancers[21].

HCC is the third leading cause of cancer-related mortality worldwide[22]. Although advances have been made in early detection and interventional therapies, a continuing need exists to develop novel approaches for the management of advanced HCC[23]. While many reports have described deregulated expression levels or functions of miRNAs and lncRNAs in HCCs, we will focus on the potential clinical use of noncoding RNAs in the very near future for novel diagnostic and therapeutic applications in the management of HCCs.

NONCODING RNAS AS BIOMARKERS FOR HCC

Deregulated expression levels of noncoding RNAs in HCC tissues

Although several published reports have described deregulated expression levels of miRNAs and lncRNAs in HCC tissues[18,24,25], the data thus far vary greatly. The differences may be because of several reasons, including the use of different techniques or samples as controls, normal liver tissues *vs* nonneoplastic tissues around tumors, background livers with various fibrosis staging, inflammation activities, or etiologies, such as hepatitis B, hepatitis C, or steatohepatitis, as well as the age or sex of the tissue-derived patients; any of these factors may cause the differential expression status of miRNAs. Regardless of these limitations, the plenty data about dysregulated miRNAs in HCCs suggests that noncoding RNAs play crucial roles in hepatocarcinogenesis[24].

Deregulated expression of noncoding RNAs in HCC as prognostic/diagnostic markers

Deregulated expression levels of noncoding RNAs in HCC tissues that may be clinically useful as prognostic/diagnostic markers will be described herein. The landmark paper that initially addressed this issue focused on *miR26* expression levels in HCC tissues and was published in the New England Journal of Medicine[26]. In this study, HCC showed frequently reduced levels of *miR26*, and patients exhibited low *miR26* expression with a shorter overall survival but a better response to interferon therapy, indicating that miRNA expression status is associated with survival and response to therapy.

Expression levels of miRNAs have tissue specificities. In the liver, *miR122*, *miR192*, and *miR199a/b-3p* are highly expressed miRNAs of all mRNAs in the liver[27]. The role of *miR122* loss in hepatocarcinogenesis was confirmed in a mouse model[28,29], and its expression is decreased in HCCs, especially non-viral HCCs[27]. Decreased expression of *miR122* is also linked with poor prognosis of HCC[30]. Although *miR192* was not deregulated in HCCs in previous studies, *miR199a/b-3p*

Table 1 Representative noncoding RNAs in sera for Hepatocellular carcinoma diagnosis

MiRNA	Expression levels in HCC	Possible targets	Ref.
MiR21	Upregulated	PTEN, AKT, C/EBPb	[32,39,58]
MiR222	Upregulated	PP2A, p27, DDIT4	[42,43,59]
MiR223	Upregulated	Stathmin	[44]
HULC	Upregulated	IGF2BP1	[45-47]

HCC: Hepatocellular carcinoma; HULC: Highly up-regulated in liver cancer; PTEN: Phosphatase and tensin-like protein; AKT: V-akt murine thymoma viral oncogene homolog; C/EBPb: CCAAT/enhancer-binding protein beta; PP2A: Protein phosphatase 2A; IGF2BP1: Insulin-like growth factor 2 MRNA binding protein 1.

is frequently decreased in HCCs[27]. In contrast, *miR21*, whose expression is increased when rat hepatectomy[31], is upregulated as an onco-miRNA, resulting in the promotion of HCC[32]. *MiR21* expression in HCC tissues confers resistance to the antitumor effect of interferon-α and 5FU combination therapy[33].

Similar to miRNAs, expression levels of lncRNAs are also dysregulated in HCC tissues[18]. Among them, HOTAIR is overexpressed in HCC tissues and may confer chemoresistance[34]. Metastasis-associated lung adenocarcinoma transcript 1, which was initially discovered as an lncRNA associated with metastasis[35], is also upregulated in HCC tissues and may be useful as a biomarker for tumor recurrence. Recently, *HOXA* transcript at the distal tip (HOTTIP) was discovered to be located in physical contiguity with the *HOXA13* gene and upregulated in HCC tissues, and this was also associated with metastasis formation and poor patient survival[36]. These results show the functional importance of lncRNA dysregulation in HCC tissues and indicate their possible use as novel prognostic and diagnostic biomarkers.

Noncoding RNAs in the sera of patients with HCC as diagnostic markers

Although α-fetoprotein (AFP), AFP-L3, and des-gamma-carboxy prothrombin are useful noninvasive biomarkers for HCC surveillance[37], novel and sensitive biomarkers that can detect early HCC are needed. The identification of tumor-specific alterations in circulating nucleic acids of patients with cancer as noninvasive methods of cancer diagnosis is encouraging[38]. Although RNAs are generally considered unstable, they are actually quite stable and readily detected in patient serum and plasma. Microarrays, polymerase chain reaction methods, and next-generation sequencing technologies are generally utilized to detect circulating noncoding RNAs.

Although many reports have described circulating miRNA levels in patients with HCC, only a few tests have been reproducible. For example, data regarding upregulation of circulating *miR21*, *miR222*, and *miR223* in patients with HCC are inconsistent[32,33,38-44]. Highly upregulated in liver cancer, a 1.6-kb lncRNA, is also upregulated in HCC tissues[45-47] and is detected in the plasma of patients with HCC[18,48]. Although these results are encouraging, more work is needed to make the usability of circulating noncoding RNAs as novel biomarkers more reliable (Table 1). Specificity and sensitivity, as well as methods to quantitate small amounts of RNAs in sera with high reproducibility and the universal control to adjust the obtained data from differing times and samples, need to be urgently determined[49].

NONCODING RNAS AS NOVEL THERAPEUTICS AGAINST HCC

Ongoing clinical trials

Mounting evidence suggests that noncoding RNAs are frequently dysregulated in HCCs and possibly involved in oncogenesis and may therefore provide novel molecular targets as a therapeutic intervention. However, due to the complexity associated with pleiotropic miRNA functions and lncRNAs, the number of clinical trials is presently limited[50]. The leading nucleotide-targeting therapy, Miravirsen, an LNA-based *anti-miR122* against hepatitis C virus replication, has been successful in a Phase II a study[51]. In addition, MRX34, a liposome-formulated *miR-34* mimic developed by Mirna Therapeutics, produced complete HCC regression in mouse models[52], and a Phase I study is currently recruiting patients with advanced liver cancer for HCC therapeutic intervention (NCT01829971).

While siRNAs are not endogenous noncoding RNAs, they can be described as noncoding RNAs that have been tried as novel therapeutics against HCC. ALN-VSP (Alnylam Pharmaceuticals), an RNAi therapeutic targeting vascular endothelial growth factor and kinesin spindle protein, has been shown to be well tolerated in Phase I studies (NCT008822180 and NCT01158079) for the treatment of primary and metastatic liver cancer. The results demonstrated disease control lasting more than 6 mo in the majority of patients, including a complete response in a patient with endometrial cancer who had multiple liver metastases. TMK-polo-like kinase 1 (PLK1) (Tekmira Pharmaceuticals), an RNAi targeting PLK1, is also under a Phase I / II trial (NCT01437007). Early results show that TKM-PLK is well tolerated and demonstrates clinical benefits. Although primary results from these potential therapeutics are encouraging, the benefits and unexpected side effects need to be determined, especially under long-term use.

Preclinical trials

Anti-miR21 and *anti-miR221* are under development for clinical use (Regulus Therapeutics). *MiR21* is one of the most validated microRNA targets, with numerous scientific publications suggesting that *miR21* plays an important role in the initiation and progression of cancers, including liver cancer[32,53,54]. Similarly, *miR221* has been identified to be upregulated in multiple cancers including liver cancer[54-56]. *Anti-miR21* and *anti-miR221* prolonged survival time in a preclinical mouse model

Table 2 Representative noncoding RNAs under clinical and preclinical trials for hepatocellular carcinoma therapeutics

Target	Name	Content	Vendor	Current status
MiR34	MRX34	Liposome-formulated miR-34 mimic	Mirna Therapeutics	Phase I
VEGF/KSP	ALN-VSP	RNAi targeting VEGF/KSP	Alnylam Pharmaceuticals	Phase I
PLK1	TMK-PLK1	RNAi targeting PLK1	Tekmira Pharmaceuticals	Phase I / II
MiR21	Anti-miR21	Antisense against miR21	Regulus Therapeutics	Preclinical
MiR221	Anti-miR221	Antisense against miR221	Regulus Therapeutics	Preclinical
MiR7	MiR7 mimic	MiR7 mimic	MiReven	Preclinical

VEGF: Vascular endothelial growth factor; KSP: Kidney-specific cadherin; PLK1: Polo-like kinase 1.

that genetically develops HCC. An *miR7* mimic is also under development (MiReven). Mir7 targets the phosphoinositide 3-kinase (PI3K) pathway and decreases tumor growth both *in vitro* and *in vivo*[57]. These results are summarized in Table 2.

CHALLENGES FOR BETTER CLINICAL TRANSLATION

Several other miRNAs, including lncRNAs, which are dysregulated in HCCs, can be attractive therapeutic targets by RNA mimics, antisense RNA, or siRNA. In fact, many publications have reported their efficacy. However, obstacles remain to be addressed[24]: (1) The more reproducibility of the results should be achieved to make the data more reliable; (2) Identification of driver miRNAs in oncogenesis is important to develop therapeutics targeting such miRNAs, although we may be able to use passive miRNAs as prognostic and diagnostic bio-markers; and (3) The delivery methods of oligonucleotides into specific tissues with improved oligonucleotide modification, and safety need to be seriously considered for utilizing miRNAs in clinical applications. Because miRNAs generally target multiple mRNAs, unexpected outcomes, "off-target effects," may occur, even when targeting a single miRNA.

More research to solve these issues is definitely needed for the improved translational application utilizing the data about miRNAs in HCCs.

CONCLUSION

The discovery of miRNAs and lncRNAs has opened up new possibilities for novel diagnostic and therapeutic tools against HCCs. However, several important issues remain to be resolved. We must conduct continuous research to develop innovative and useful applications of the miRNA data in the clinical management of HCCs.

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

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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

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

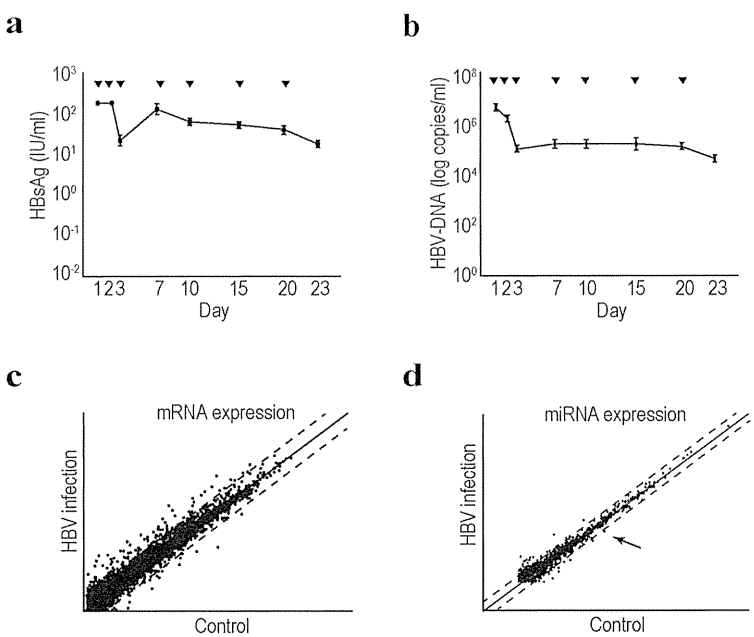

Figure 1: Comprehensive transcriptome and miRNA analyses in HBV-replicating human primary hepatocytes. a, b, Efficient HBV replication in human primary hepatocytes isolated from chimeric mice. Primary human hepatocytes isolated from chimeric mice were seeded into the wells of a 24-well plate. Serum from 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

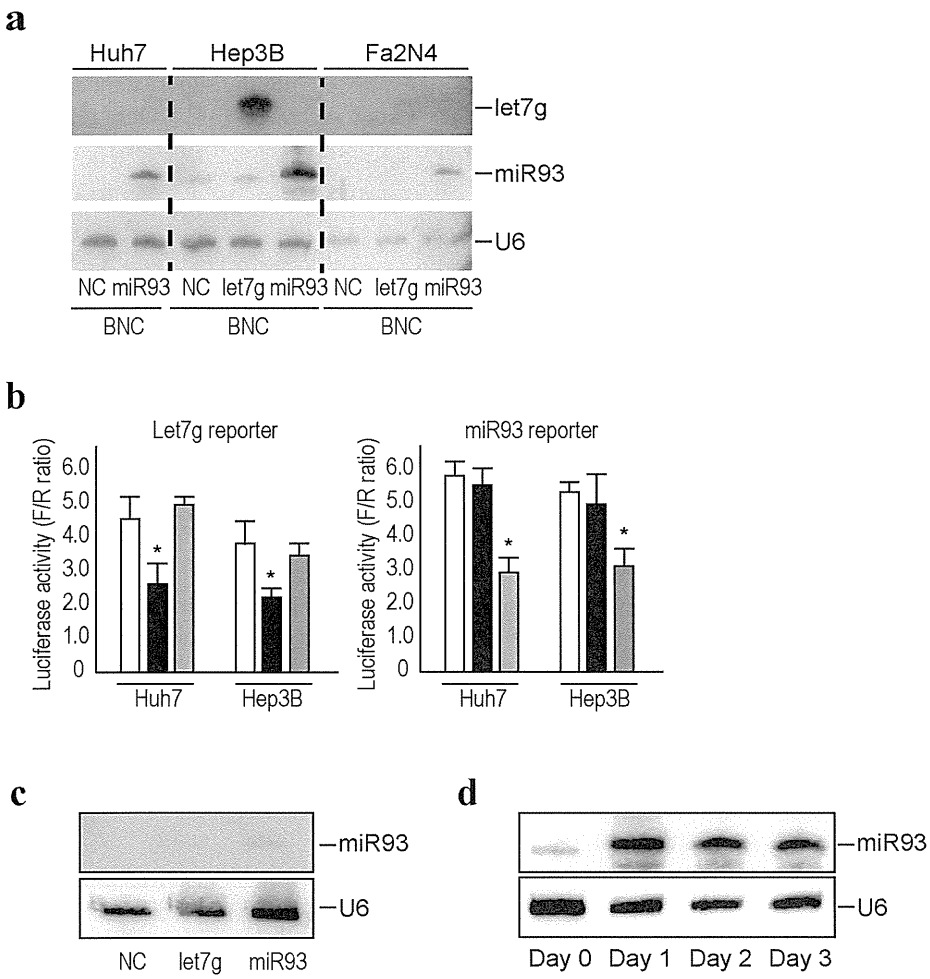

Figure 2: Efficient delivery of miRNAs into liver cell lines using BNCs. a, Northern blotting of miRNAs delivered into liver cells by BNCs. Liver cancer cell lines, Huh7 and Hep3B, and primary hepatocytes immortalized by SV40, Fa2N4, were incubated with BNCs harboring the indicated miRNAs (miRNA93 or 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.

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

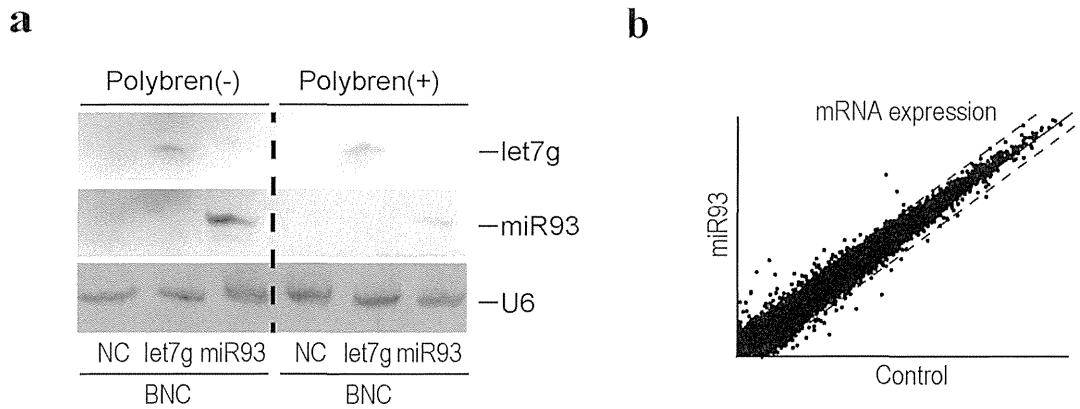

Figure 3: Efficient delivery of functional miRNAs into human primary hepatocytes using BNCs. a, Northern blotting for miRNAs delivered into cells using BNCs. Human primary hepatocytes isolated from chimeric mice were incubated with BNCs containing the indicated miRNAs (miRNA93 or let7g) or BNCs without 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.

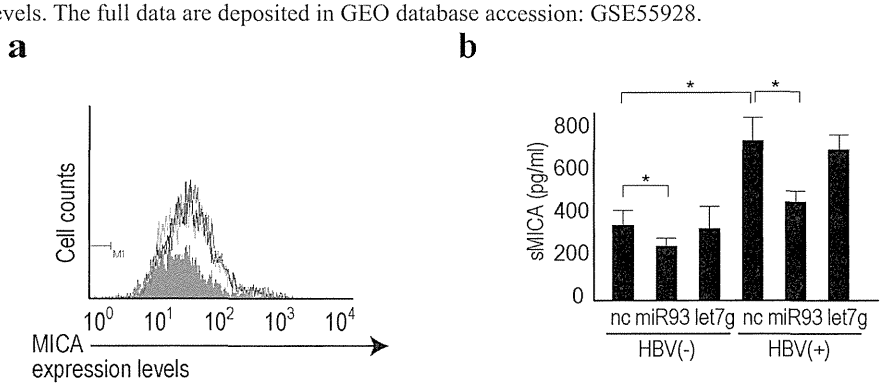

Figure 4: Soluble MICA protein levels were regulated by miRNA93 in human primary hepatocytes. a, Membrane-bound MICA protein expression was not affected by miRNA delivery into human primary hepatocytes. Flow cytometric analysis of membrane-bound MICA protein expression in cells delivered BNC-mediated 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).