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Please cite this article in press as: Akamatsu S, et al., Differences in serum microRNA profiles in hepatitis B and C virus infection, J Infect (2014), http://dx.doi.org/10.1016/j.jinf.2014.10.017

Antiviral Therapy: in press (doi: 10.3851/IMP2777)

Original article

On-treatment low serum HBV RNA level predicts initial virological response in chronic hepatitis B patients receiving nucleoside analogue therapy

Yi-Wen Huang[1,2,3], Shoichi Takahashi[4,5], Masataka Tsuge[4,5], Chi-Ling Chen[6], Ting-Chuan Wang[7], Hiromi Abe[4,5], Jui-Ting Hu[1,8], Ding-Shinn Chen[3,6,9,10], Sien-Sing Yang[1,8], Kazuaki Chayama[4,5], Jia-Horng Kao[3,6,9,11]**

[1]Liver Center, Cathay General Hospital Medical Center, Taipei, Taiwan
[2]School of Medicine, Taipei Medical University, Taipei, Taiwan
[3]Division of Gastroenterology, Department of Internal Medicine, National Taiwan University College of Medicine and Hospital, Taipei, Taiwan
[4]Department of Gastroenterology and Metabolism, Applied Life Sciences, Institute of Biomedical & Health Sciences, Hiroshima University, Hiroshima, Japan
[5]Liver Research Project Center, Hiroshima University, Hiroshima, Japan
[6]Graduate Institute of Clinical Medicine, National Taiwan University College of Medicine, Taipei, Taiwan
[7]Department of Medical Research, Cathay General Hospital Medical Center, Taipei, Taiwan
[8]School of Medicine, Fu-Jen Catholic University College of Medicine, Taipei, Taiwan
[9]Hepatitis Research Center, National Taiwan University Hospital, Taipei, Taiwan
[10]Genomics Research Center, Academia Sinica, Nankang, Taiwan
[11]Department of Medical Research, National Taiwan University Hospital, Taipei, Taiwan

*Corresponding author e-mails: chayama@mba.ocn.ne.jp; kaojh@ntu.edu.tw

Background: Serum HBV RNA is detectable during nucleoside/nucleotide analogue therapy as a result of unaffected RNA replicative intermediates or interrupted reverse transcription. We studied the predictive value of serum HBV RNA for initial virological response during nucleoside analogue therapy.

Methods: Serum HBV RNA was quantified before and at 12 and 24 weeks of lamivudine or entecavir therapy. Serum HBV DNA was measured every 4–12 weeks during treatment to define initial virological response.

Results: Serum HBV RNA was detectable in 21 of 52 (40%) consecutive patients with a mean of 5.2 log copies/ml (male/female 35/17, mean age of 60 years with a range of 31–82, 44% HBeAg-positive, and 26 with lamivudine and 26 with entecavir) before treatment. Serum HBV RNA level at week 12 in patients with an interval from detectable to undetectable serum HBV DNA level <16 weeks was significantly lower than those with an interval ≥16 weeks (3.8 ±3.8 versus 6.6 ±3.5 log copies/ml, P=0.013). After adjustment for serum HBV DNA level at week 12, serum quantatitive HBsAg level at week 12 and pretreatment ALT level, low serum HBV RNA level at week 12 predicted a shorter interval to undetectable serum HBV DNA level (adjusted hazard ratio =0.908, 95% CI 0.829, 0.993, P=0.035).

Conclusions: Low serum HBV RNA level at week 12 of nucleoside analogue therapy independently predicts initial virological response in treated chronic hepatitis B patients. Serum HBV RNA levels may thus be useful for optimizing treatment of chronic hepatitis B.

Introduction

Although effective vaccines against HBV infection have been available for more than three decades, HBV infection remains a global health problem. It is estimated that more than 350 million people are chronic carriers of HBV worldwide [1,2]. In the United States, 1.2 million individuals have chronic HBV infection [3]. HBV infection causes a wide spectrum of clinical manifestations, ranging from acute or fulminant hepatitis to various forms of chronic liver disease, including inactive carrier state, chronic hepatitis, cirrhosis and even hepatocellular carcinoma [2,4,5].

Nucleoside/nucleotide analogues (NAs) are widely approved for the treatment of chronic hepatitis B (CHB). HBV is a unique DNA virus that replicates via pregenomic RNA. Lamivudine, as well as other NAs, do not affect the HBV cccDNA and its transcripts – the RNA replicative intermediates [6]. Thus, long-term NA therapy is needed for continued viral suppression in CHB patients. Other studies and ours have shown that serum HBV RNA can be detectable during NA therapy as a result of unaffected RNA replicative intermediates or interrupted reverse transcription [7–10].

For CHB patients with NA therapy, the most important determinant of therapeutic outcomes is the degree of on-treatment viral suppression [11]. Although the correlation of baseline parameters and therapeutic outcomes of NA-treated patients has been reported, little is known about the predictive value of on-treatment predictors [12–14]. For example, a roadmap approach by using on-treatment monitoring of serum HBV DNA levels has been proposed [15]; however, the role of on-treatment serum quantitative HBsAg (qHBsAg) levels in predicting outcomes of NA-treated patients is not satisfactory [16]. To seek better on-treatment predictors, we thus evaluated the predictive value of serum HBV RNA for initial virological response in CHB patients receiving NA therapy.

Methods

Subjects

[AU:URLS not permitted in the main body of text. This has been added as reference and all references have been renumbered accordingly, please check carefully and approve]

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We consecutively enrolled 52 CHB patients treated with either lamivudine or entecavir at Hiroshima University Hospital or other hospitals of the Hiroshima Liver Study Group [17]. Serum samples from enrolled patients were obtained just before the initiation of therapy and every 4–12 weeks during therapy. These samples were stored at -80°C until use. Serum HBV RNA was quantified at pretreatment and at treatment weeks 12 and 24. Serum HBV DNA was measured every 4–12 weeks during treatment to record the time of initial undetectable HBV DNA (that is, initial virological response). The lower detection limit of this assay was 2.2 log copies/ml. Informed consent was obtained from each patient.

Extraction of HBV nucleic acid and reverse transcription

Extraction of HBV nucleic acid and reverse transcription with subsequent quantification were performed as previously described [8]. Nucleic acid was extracted from 100 µl serum using SMITEST EX-R&D (Genome Science Laboratories, Tokyo, Japan) and dissolved in 18 µl of ribonuclease-free H_2O. The extract was then divided into two parts with equal amounts. Solution I was mixed with equal amounts of H_2O for DNA quantification. Solution II underwent reverse transcription using random primers (Takara Bio Inc., Shiga, Japan) and M-MLV reverse transcriptase (ReverTra Ace; TOYOBO Co., Osaka, Japan), with subsequent DNA plus cDNA quantification. Low-level pretreatment serum HBV RNA might be masked by serum HBV DNA with this quantification method. This limitation was overcome by treating nucleic acid extracts with deoxyribonuclease digestion before reverse transcription.

The steps in reverse transcription are follows: 25 pM random primer was added and heated at 65°C for 5 min, the mixture was then put on ice for 5 min, 4 µl of 5X reverse transcription buffer, 2 µl of 10 mM dNTPs, 2 µl of 0.1 M dithiothreitol (DTT), 8 units of ribonuclease inhibitor and 100 units of M-MLV reverse transcriptase was then added to each sample and, lastly, the mixture was incubated at 30°C for 10 min, 42°C for 60 min and inactivation was carried out at 99°C for 5 min.

Quantification of HBV DNA and cDNA by real-time PCR

HBV DNA and cDNA quantification were performed as previously described [8]. 1 µl of each solution I and solution II was amplified by real-time PCR with an ABI Prism 7300 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) according to the instructions provided by the manufacturer. Amplification was performed in a 25 µl reaction mixture containing SYBR Green PCR Master Mix (Applied Biosystems), 200 nM of forward primer (5′-TTTGGGGCATGGACATTGAC-3′, nucleotides 1893–1912), 200 nM of reverse primer (5′-GGTGAACAATGGTCCGGAGAC-3′, nucleotides 2029–2049) and 1 µl of solution I or solution II. The steps in real-time PCR are as follows: the mixture was incubated at 50°C for 2 min, denaturation was carried out at 95°C for 10 min, and the PCR cycling programme comprised 40 two-step cycles of 15 s at 95°C and 60 s at 60°C. The HBV RNA quantity was obtained by subtracting the quantification result of solution I from solution II, that is, HBV nucleic acid determined by real-time PCR after reverse transcription reaction minus HBV DNA determined by real-time PCR.

Serological assays

Serum HBeAg and anti-HBe were tested using chemiluminescent immunoassays (Architect HBeAg and Architect HBeAb; Abbott Japan, Tokyo, Japan). Serum HBsAg levels were quantified by Architect HBsAg (Abbott Japan). The dynamic range of the assay was 0.05–250 IU/ml. High HBsAg titre was measured with 1,000-fold diluted serum.

Statistical analyses

Continuous variables were expressed as mean ±SD and evaluated by Student's *t*-test. Categorical variables were expressed as frequencies with proportions and compared using Pearson's χ^2 test, and Fisher's exact test was applied when at least one cell of the table had an expected frequency <5. All of the tests were two-tailed and a *P*-value <0.05 was considered statistically significant. The correlation between serum HBV RNA and serum HBV DNA as well as with serum qHBsAg was analysed by Pearson's correlation using SPSS programme for Windows 10.0 (SPSS Inc., Chicago, IL, USA). Cox regression analysis was applied for predictors of duration to undetectable serum HBV DNA using SAS version 9.2 (SAS Institute, Inc, Cary, NC. USA).

Results

Demographic profiles of patients

Baseline characteristics of CHB patients treated with lamivudine or entecavir are shown in Table 1. There was no significant difference in terms of age, gender ratio, HBeAg status, serum ALT level, serum HBV DNA level and serum qHBsAg level between the two groups.

Table 1. Baseline characteristics of chronic hepatitis B patients treated with lamivudine or entecavir

Variable	Lamivudine	Entecavir	*P*-value
Patients, *n*	26	26	-
Mean age, years (±SD)	61 ±10	59 ±13	0.609
Male, *n*/total *n* (%)	15/26 (57.7)	20/26 (76.9)	0.139
HBeAg positivity, *n*/total *n* (%)	12/26 (46.2)	11/26 (42.3)	0.780
Mean ALT, U/l (±SD)	641 ±1,837	122 ±209	0.158
Mean log HBV DNA, copies/ml (±SD)	9.9 ±2.1	9.7 ±1.8	0.739
Mean quantitative HBsAg, IU/ml (±SD)	4,537.5 ±6,091.3	6,363.7 ±7,064.9	0.323

Serum HBV RNA and qHBsAg levels before and during lamivudine versus entecavir therapy

The detectability and quantification of serum HBV RNA level at baseline, week 12 and 24 of lamivudine versus entecavir therapy are shown in Table 2. The detectability and quantity of serum HBV RNA level was comparable before the initiation of NA therapy. At week 12 and 24 of therapy, entecavir-treated patients had a higher proportion of detectable serum HBV RNA (50% versus 84.6% [*P*=0.008] and 38.5% versus 76.9% [*P*=0.005], respectively) and a higher quantity (3.8 ±4.1 versus 6.5 ±3.1 log copies/ml, [*P*=0.011] and 2.9 ±3.9 versus 6.2 ±3.8 log copies/ml, [*P*=0.003], respectively) when compared with lamivudine-treated patients. In addition, most of them had detectable HBV RNA at 12 weeks of therapy (lamivudine in 13 and entecavir in 22). Serum qHBsAg at week 12 and 24 of therapy as well as the interval to undetectable serum HBV DNA were not different between the two groups (Table 2).

At week 12 of NA therapy, the correlation of serum HBV RNA levels with serum qHBsAg levels and serum HBV DNA levels is shown in Figure 1. Serum HBV RNA levels tended to correlate better with serum qHBsAg levels (R square 0.407) than with serum HBV DNA levels (R square 0.321).

On-treatment predictors of initial virological response

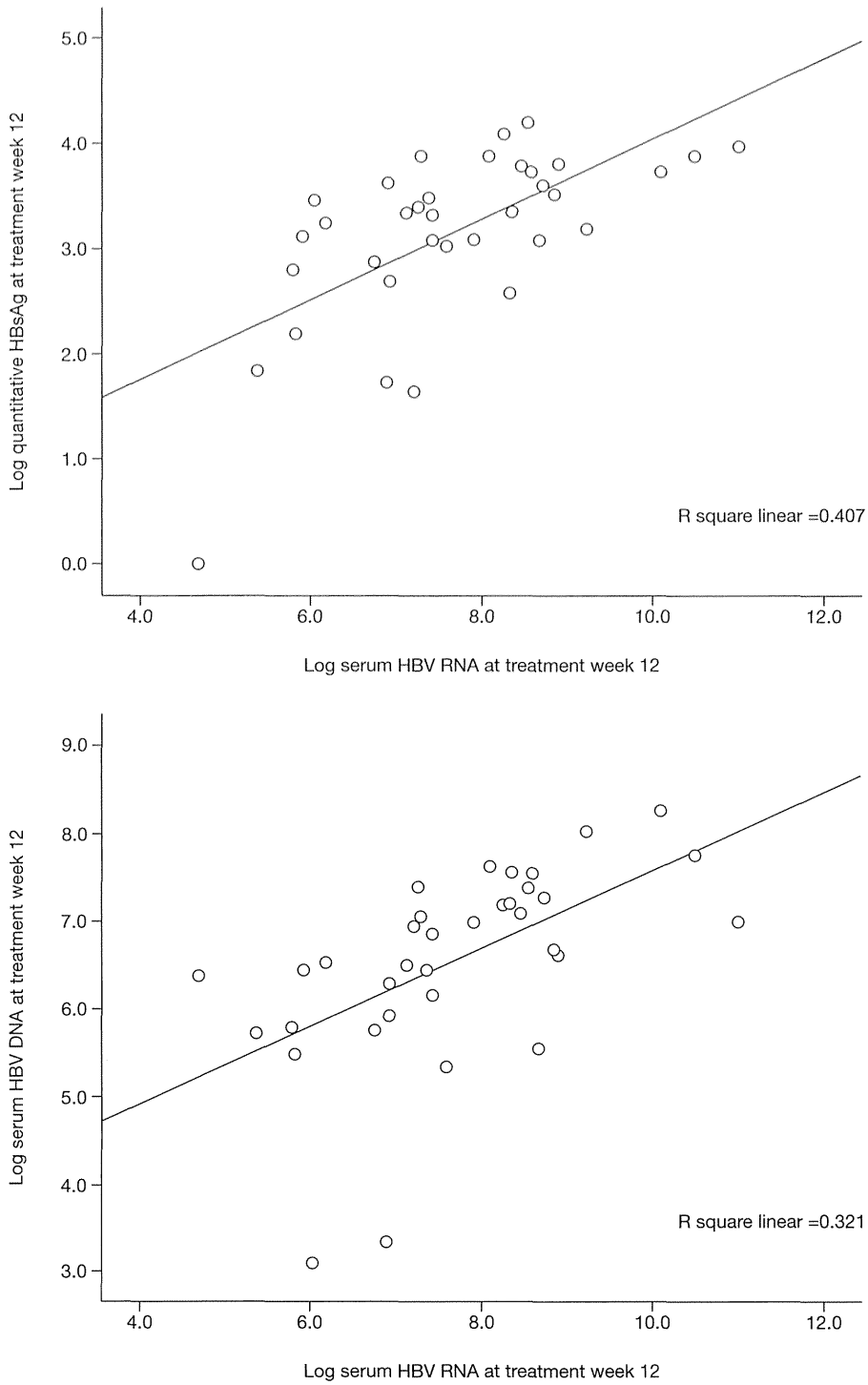
CHB patients with interval from detectable to undetectable serum HBV DNA level <16 weeks (*n*=23) had a significantly lower serum HBV RNA level at week 12 of NA therapy than those with interval ≥16 weeks (*n*=21; 3.8 ±3.8 versus 6.6 ±3.5 log copies/ml [*P*=0.013]; Figure 2A). The time interval based on entecavir and lamivudine therapy is shown in Figure 2B.

Low serum HBV RNA level at week 12 of therapy predicted a shorter interval to undetectable serum HBV DNA (adjusted hazard ratio =0.908, 95% CI 0.829, 0.993, *P*=0.035), after adjustment for pretreatment serum ALT level as well as serum HBV DNA level and

Table 2. Serum HBV RNA and quantitative HBsAg during lamivudine versus entecavir therapy

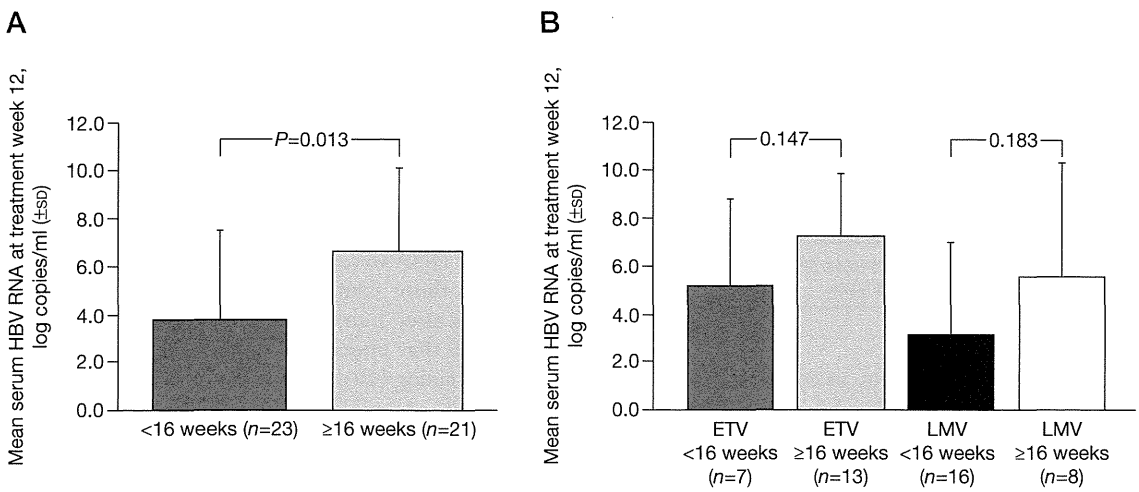
Variable	Lamivudine	Entecavir	*P*-value
HBV RNA detectability			
Pre-treatment, *n*/total *n* (%)	9/26 (34.6)	12/26 (46.1)	0.396
At 12 weeks, *n*/total *n* (%)	13/26 (50)	22/26 (84.6)	0.008
At 24 weeks, *n*/total *n* (%)	10/26 (38.5)	20/26 (76.9)	0.005
Log HBV RNA			
Mean pre-treatment, copies/ml (±SD)	5.2 ±1.1	5.2 ±1.4	0.892
Mean at 12 weeks, copies/ml (±SD)	3.8 ±4.1	6.5 ±3.1	0.011
Mean at 24 weeks, copies/ml (±SD)	2.9 ±3.9	6.2 ±3.8	0.003
Mean quantitative HBsAg at 12 weeks	2,633.8 ±3,423	4,170.9 ±4,599	0.178
Mean quantitative HBsAg at 24 weeks, IU/ml (±SD)	2,566.5 ±3,814.3	3,763.1 ±4,707.6	0.319
Mean duration to undetectable HBV DNA, months (range)	4 (1–28)	5.9 (1–15)	0.232

Figure 1. Correlation of serum HBV RNA with quantitative HBsAg and serum HBV DNA at treatment week 12 of NAs



Serum HBV RNA at treatment week 12 correlates better with **(A)** serum quantitative HBsAg at treatment week 12 (R square 0.407) than **(B)** serum HBV DNA at treatment week 12 (R square 0.321).

Figure 2. Serum HBV RNA levels at week 12 with intervals from detectable to undetectable serum HBV DNA level <16 weeks versus ≥16 weeks



(A) Serum HBV RNA levels at week 12 in chronic hepatitis B patients with intervals from detectable to undetectable serum HBV DNA level <16 weeks was significantly lower than those with interval ≥16 weeks (3.8 ±3.8 versus 6.6 ±3.5 log copies/ml, *P*=0.013). **(B)** Serum HBV RNA level at week 12 in chronic hepatitis B patients based on entecavir (ETV) and lamivudine (LMV) therapy with intervals from detectable to undetectable serum HBV DNA level <16 weeks was comparable to those with interval ≥16 weeks.

serum qHBsAg level at week 12 of therapy in Cox regression analysis (Table 3).

Discussion

In this study, we focused on on-treatment predictors of initial virological response and found serum HBV RNA at week 12 of therapy as a novel predictor, independent of serum HBV DNA level at week 12, qHBsAg level at week 12 or pretreatment serum ALT level. In the Cox regression models of on-treatment predictors, we included on-treatment HBV DNA and qHBsAg instead of their pre-treatment counterparts. Furthermore, we avoided concomitant inclusion of both pre- and on-treatment week 12 HBV DNA and qHBsAg levels due to the issue of multicollinearity, which may generate inaccurate individual predictors.

CHB patients with interval from detectable to undetectable serum HBV DNA level <16 weeks had a significantly lower serum HBV RNA level at week 12 of NA therapy than those with interval ≥16 weeks (Figure 2A). Furthermore, a low serum HBV RNA level at week 12 independently predicted a shorter interval to undetectable HBV DNA level (Table 3). Apart from serum HBV DNA level, serum HBV RNA was the only independent on-treatment predictor of initial virological response in such patients.

The AASLD guidelines for lamivudine-treated CHB patients recommend measurement of serum HBV DNA every 3–6 months (12–24 weeks) [12]. With a roadmap approach, primary non-response in NA-treated CHB patients was assessed at week 12 of therapy [16]. In addition, primary treatment failure is defined by changes in serum HBV DNA levels at week 12 on monitoring for the development of resistance [18]. Furthermore, our previous study suggested serum HBV RNA at week 12 of lamivudine therapy could predict early emergence of YMDD mutation [8]. This present study showed serum HBV RNA level at treatment week 12 predicted time to undetectable serum HBV DNA, supporting the usefulness of on-treatment week 12 monitoring of NA-treated patients.

Serum HBV RNA levels tend to correlate better with serum qHBsAg than with serum HBV DNA levels (Figure 1). Serum qHBsAg poorly predicts NA treatment outcomes; however, HBeAg-positive patients with elevated ALT are likely to experience a decrease in qHBsAg during NA therapy [15]. This decrease is parallel with the gradual decrease in serum HBV RNA during NA therapy as we previously reported [9]. By contrast, serum HBV DNA usually displays a more rapid decrease and thus does not correlate as well with serum HBV RNA.

[AU:OK]
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This present study showed that the amount and detectability of serum HBV RNA were higher in entecavir as compared with lamivudine-treated patients, which is consistent with our previous report [9]. Entecavir is more potent than lamivudine in the inhibition of

Table 3. On-treatment predictors of initial virological response[a] during nucleoside/nucleotide analogue therapy by Cox regression analysis[b]

Variable	Adjusted hazard ratios	95% CI	*P*-value
Serum HBV RNA level at week 12	0.908	0.829, 0.993	0.035
Serum HBV DNA level at week 12	0.717	0.563, 0.913	0.007
Quantitative HBsAg level at week 12	1.524	0.981, 2.368	0.061
Pre-treatment ALT level	1.820	0.919, 3.606	0.086

[a]Duration to undetectable HBV DNA. [b]*P*=0.048. All variables were logarithm transformed before included into the analysis.

serum HBV DNA [12]. Thus, as compared with lamivudine, entecavir may potently inhibit reverse transcriptase more, leading to a higher level of serum HBV RNA. By contrast, entecavir or lamivudine does not have direct effect on serum qHBsAg as reflected by the poor predictive value of serum qHBsAg levels in therapeutic outcomes of NA treatments [16] and the comparable serum qHBsAg levels between entecavir- or lamivudine-treated patients as shown in this study. These findings confirm that serum HBV RNA level, but not qHBsAg, may reflect the antiviral potency of NAs. Furthermore, serum HBV RNA, but not qHBsAg, independently predicts initial virological response in both entecavir- and lamivudine-treated patients.

In contrast to a rapid decrease in serum HBV RNA observed in individuals treated with combination of NA and interferon [9], our previous study showed a gradual decrease of serum HBV RNA in NA-treated patients. Thus, the inhibitory effect of interferon on HBV RNA replicative intermediates may potentiate the suppression of HBV replication [9]. The findings presented in this study suggest that low on-treatment serum HBV RNA could predict earlier HBV suppression and response to NA therapy. Taken together, serum HBV RNA might be useful for optimizing treatment outcomes in patients with CHB, including a shift to more effective oral antiviral drugs or to immunomodulatory interferon.

[AU: OK?]

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Randomized double-blind trials have shown that the mean log HBV DNA difference between lamivudine and entecavir therapy was approximately 0.5 to 0.8 copies/ml at treatment weeks 12 and 24 [19,20]. In the present study, the mean log HBV RNA difference between lamivudine and entecavir therapy was 2.7 and 3.3 copies/ml at treatment weeks 12 and 24, respectively. This difference could not merely be explained by the stronger suppression of HBV DNA by entecavir as compared to lamivudine, instead, suggesting the presence of higher level of serum HBV RNA under entecavir therapy.

[AU:Please reword for clarity]

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The specific presence of serum HBV RNA in CHB patients treated with NA was validated in our previous study using ribonuclease digestion [8]. We have also previously reported persistently detectable serum HBV RNA during NA therapy, although it was inhibited under sequential lamivudine and interferon therapy [9]. Rokuhara *et al.* [21] have shown that HBV RNA was detectable before lamivudine therapy in serum samples of 24 patients; however, the detection rate was not specified. Their results of sucrose density gradient fractionation studies indicated that viral particles containing HBV DNA were dominant at the start of treatment, whereas those containing HBV RNA became more prevalent after 1 and 2 months of treatment. They also suggested that under untreated conditions, viral particles containing HBV RNA accounted for only approximately 1% of total HBV virions. These specific particles became the major component under lamivudine treatment [7]. Furthermore, Rokuhara *et al.* [21] reported a more significant decrease of serum HBV DNA than HBV RNA levels during lamivudine therapy, which support our findings on the poor immediate inhibition of serum viral particles containing HBV RNA by NAs [9].

There were several limitations in this study. First, the enrolled number of patients was relatively small; however, we were able to report that serum HBV RNA is a suitable independent on-treatment predictor. In daily clinical practice, complete collection of samples at several time points (pre- and on-treatment) and maintenance of good quality easily degradable RNA samples by timely handling as well as storage in -80°C remain a daunting challenge. Second, the predictive role of serum HBV RNA in long-term outcomes of these NA-treated patients was unclear. The evaluation of long-term outcomes of such patients was difficult due to the variable duration of NA therapy and the shift to interferon therapy in some.

In conclusion, on-treatment low serum HBV RNA level at treatment week 12 independently predicts initial virological response in NA-treated patients with CHB and further large studies are needed to confirm these observations.

Acknowledgements

This study was funded in part by Cathay General Hospital Medical Center, National Taiwan University Hospital, and National Science Council of Taiwan (grant number NSC 99-2314-B-281-002-MY3). Part of this study had been presented in the *63rd Annual Meeting of the American Association for the Study of Liver Diseases* in Boston, MA, USA (abstract number 470) and was selected as a Presidential Poster of Distinction. The authors thank Yao-Jen Lee and Cheng-Kai Hsu for their technical assistance.

Disclosure statement

Y-WH has served as a speaker for GlaxoSmithKline and Bristol–Myers Squibb. KC has served as a speaker and a received grant from Bristol–Myers Squibb. D-SC, S-SY and J-HK have served as a speaker, a consultant and an advisory board member for GlaxoSmithKline and Bristol–Myers Squibb. All other authors declare no competing interests.

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Accepted 16 February 2014; published online 16 April 2014

JOURNAL OF VIRAL HEPATITIS
JVH

Journal of Viral Hepatitis, 2014, **21**, e89–e97 doi:10.1111/jvh.12240

Human microRNA hsa-miR-1231 suppresses hepatitis B virus replication by targeting core mRNA

T. Kohno,[1,2*] M. Tsuge,[1,2,3*] E. Murakami,[1,2] N. Hiraga,[1,2] H. Abe,[1,2] D. Miki,[1,2,4] M. Imamura,[1,2] H. Ochi,[1,2,4] C. N. Hayes[1,2] and K. Chayama[1,2,4] [1]*Department of Gastroenterology and Metabolism, Applied life sciences, Institute of Biomedical and Health sciences, Hiroshima University, Hiroshima, Japan;* [2]*Liver Research Project Center, Hiroshima University, Hiroshima, Japan;* [3]*Natural Science Center for Basic Research and Development, Hiroshima University, Hiroshima, Japan; and* [4]*Laboratory for Liver Diseases, SNP Research Center, The Institute of Physical and Chemical Research (RIKEN), Hiroshima, Japan*

Received November 2013; accepted for publication February 2014

SUMMARY. Pathogen-specific miRNA profiles might reveal potential new avenues for therapy. To identify miRNAs directly associated with hepatitis B virus (HBV) in hepatocytes, we performed a miRNA array analysis using urokinase-type plasminogen activator (uPA)–severe combined immunodeficiency (SCID) mice where the livers were highly repopulated with human hepatocytes and human immune cells are absent. Mice were inoculated with HBV-infected patient serum samples. Eight weeks after HBV infection, human hepatocytes were collected from liver tissues, and miRNAs were analysed using the Toray 3D array system. The effect of miRNAs on HBV replication was analysed using HBV-transfected HepG2 cells. Four miRNAs, hsa-miR-486-3p, hsa-miR-1908, hsa-miR-675 and hsa-miR-1231 were upregulated in mouse and human livers with HBV infection. These miRNAs were associated with immune response pathways such as inflammation mediated by chemokine and cytokine signalling. Of these miRNAs, hsa-miR-1231, which showed high homology with HBV core and HBx sequences, was most highly upregulated. In HBV-transfected HepG2 cells, overexpression of hsa-miR-1231 resulted in suppression of HBV replication with HBV core reduction. In conclusion, a novel interaction between hsa-miR-1231 and HBV replication was identified. This interaction might be useful in developing new therapeutic strategies against HBV.

Keywords: HB core, hepatitis B virus, hsa-miR-1231, human hepatocyte chimeric mouse, microRNA.

INTRODUCTION

Hepatitis B virus (HBV) is a member of the *Hepadnaviridae* family, which contains a group of hepatotropic small DNA viruses that infect their respective animal hosts [1–3]. Once HBV infects human hepatocytes, the HBV genome translocates into the nucleus. Some genome copies are converted into a covalently closed circular DNA (cccDNA) form and organized into a minichromosome with histone and nonhistone proteins [4–8]. HBV cccDNA utilizes the cellular transcriptional machinery to produce all viral RNAs including the pregenomic RNA [9], and these gene products regulate viral replication and pathogenesis by regulating host gene expression [10,11].

MicroRNAs (miRNAs) are small noncoding RNAs of 21-25 nucleotides in length, processed from hairpin-shaped transcripts [12]. MiRNAs can bind the 3′-untranslated regions (UTRs) of messenger RNAs and downregulate gene expression by cleaving messenger RNA or inhibiting translation. Several miRNAs associated with HBV infection, HBV replication and hepatocarcinogenesis have recently been identified [13–19]. However, the direct influence of HBV infection on miRNA expression is still unclear.

MicroRNAs are currently being investigated for their therapeutic potential in antiviral therapy. As several studies have demonstrated that hsa-miR-122, which is specifically and abundantly expressed in hepatocytes, supported hepatitis C virus (HCV) replication by improving RNA

Abbreviations: HBc, hepatitis B core; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; miRNA, microRNA; RI, replication intermediates.

Correspondence: Kazuaki Chayama, Department of Gastroenterology and Metabolism, Applied life sciences, Institute of Biomedical and Health sciences, Hiroshima University, 1-2-3 Kasumi, Minami-ku, Hiroshima-shi, Hiroshima, 734-8551, Japan.
E-mail: chayama@hiroshima-u.ac.jp
*These authors contributed equally to this work.
This study was supported in part by a Grant-in-Aid for Scientific Research from the Japanese Ministry of Labor and Health and Welfare.

stability [20–24], small molecules or siRNAs which are able to knock down miR-122 expression have been explored as a new therapeutic agent for HCV eradication.

A similar microRNA-based antiviral approach is also sought for the treatment of chronic hepatitis B, as it is difficult to eradicate HBV genomes converted into cccDNA or minichromosomes under present antiviral therapies. To develop new strategies for complete eradication of the viral genome from hepatocytes, it is important to clarify the direct associations between hepatic miRNAs and HBV infection.

In this study, miRNA microarray analysis was performed using human hepatocyte chimeric mouse livers to assess the direct impact of HBV infection on miRNA expression. We successfully demonstrated that HBV infection attenuated the expression of miRNAs under immunodeficient conditions to protect early viral propagation. A novel interaction between hsa-miR-1231 and HBV replication was identified.

MATERIALS AND METHODS

Human serum inoculum

Serum samples were obtained from a carrier infected with HBV genotype C after obtaining written informed consent for the donation and evaluation of blood samples. Inoculum was positive for HBs and HBe antigens with high-level viremia (HBV DNA: 7.1 log copies/mL). The experimental protocol conformed to the ethical guidelines of the Declaration of Helsinki and was approved by the Hiroshima University Hospital ethical committee (Approval ID: D08-9).

Human hepatocyte chimeric mice experiments

Human hepatocyte chimeric mice (PXB mice), in which human hepatocytes were transplanted into $uPA^{+/+}/SCID^{+/+}$ mice, were purchased from Phoenix Bio (Hiroshima, Japan). Mouse experiments were performed in accordance with the guidelines of the local committee for animal experiments at Hiroshima University.

Six chimeric mice, in which more than 90% of the liver tissue was replaced with human hepatocytes, were divided into two experimental groups. Group A contained three uninfected mice. Group B consisted of three mice that were inoculated via the mouse tail vein with human serum containing 6×10^6 copies of HBV. Serum HBV DNA titres were quantified every 2 weeks by real-time PCR, and human albumin levels were measured using the Human Albumin ELISA Quantitation kit (Bethyl Laboratories Inc., Montgomery, TX, USA) as described previously [25]. Eight weeks after inoculation, all three infected mice were sacrificed. Infection, extraction of serum samples and sacrifice were performed under ether anaesthesia as described previously [26].

miRNA microarray analysis

Human hepatocytes were finely dissected from the mouse livers and stored in liquid nitrogen after submerging in RNA*later*® solution (Applied Biosystems, Foster City, CA, USA). Experimental sample RNAs were isolated using RNeasy Mini Kit (Qiagen, Valencia, CA, USA) and analysed using TORAY 3-D Gene Chip human miRNA ver. 12.1 (TORAY, Chiba, Japan).

Data analysis

Gene expression profiles were analysed using GeneSpring GX 10.0.2 software (Tomy Digital Biology, Tokyo, Japan). Expression ratios were normalized per chip to the 50th percentile. To determine whether there were miRNAs differentially expressed among samples, we performed two Welch's *t*-tests ($P < 0.01$) on this prescreened list of miRNAs with Benjamini and Hochberg's correction. Complete linkage hierarchical clustering analysis was applied using Euclidean distance.

Pathway analysis

The miRNA target genes were predicted by the online database miRWalk (http://www.umm.uni-heidelberg.de/apps/zmf/mirwalk/index.html). Target prediction was performed using 3′-UTR sequences of mRNAs, and the probability distributions were calculated using the Poisson distribution [27]. The mRNAs with *P* values <0.01 were considered significant. To improve the accuracy of target gene selection, the predicted genes were screened using other prediction programs, including miRanda (August 2010 release), miRDB (April 2009 release) and TargetScan version 5.1 (Whitehead Institute for Biomedical Research, Cambridge, MA, USA). Genes that were predicted by at least two alternate programs were selected. Pathway analysis was performed by PANTHER version 8.1 (http://www.pantherdb.org/) to determine the effects of the predicted target genes on pathways.

Quantification of miRNAs

Small RNAs were extracted from liver tissues or HepG2 cells with mirVana™ miRNA Isolation Kit (Applied Biosystems) and reverse-transcribed according to the manufacturer's instructions. The selected miRNAs were quantified with TaqMan® MicroRNA Assays (Applied Biosystems) using the 7300 Real-Time PCR System (Applied Biosystems), and the expression of RNU6B served as a control.

Quantification of mRNAs

Total RNA was extracted from HepG2 cells transfected with control miRNA or miR-1231 expression plasmid using

RNeasy Mini Kit and reverse-transcribed (RT) using ReverTra Ace (TOYOBO, Osaka, Japan) with random primer according to the manufacturer's instructions. The selected cDNAs were quantified by real-time PCR. Differences between groups were examined for statistical significance using Student's *t*-test. The primer sequences were as follows: GAPDH forward 5′-ACAACAGCCTCAAGATCATCAG-3′ and reverse 5′-GGTCCACCACTGACACGTTG-3′; Mx1 forward 5′- TTCGGCTGTTTACCAGACTCC-3′ and reverse 5′- CAAAGCCTGGCAGCTCTCTAC-3′; 2′–5′ oligoadenylate synthetase 1 (OAS1) forward 5′- ACCTGGTTGTCTTCCTCA GTCC-3′ and reverse 5′- GAGCCTGGACCTCAAACTTCAC-3′; double stranded RNA dependent protein kinase (PKR) forward 5′- TGGCCGCTAAACTTGCATATC-3′ and reverse 5′- AGTTGCTTTGGGACTCACACG-3′; and SOCS1 forward 5′-ACGAGCATCCGCGTGCACTT-3′ and reverse 5′-AAGAGG CAGTCGAAGCTCTC-3′.

Plasmid construction

The construction of wild-type HBV 1.4 genome length, pTRE-HB-wt, was described previously [25]. The nucleotide sequence of the cloned HBV genome was deposited into GenBank AB206817. The HBc and HBx genes, amplified from pTRE-HB-wt, were cloned into pcDNA3 and p3xFLAG-CMV10 vectors and designated pcDNA-HBc and p3FLAG-HBx, respectively. The human miR-1231 precursor expression plasmid (HmiR0554-MR04) and the control miRNA plasmid (CmiR0001-MR01), which was a miRNA-scrambled control clone, were commercially produced (GeneCopoeia™, Rockville, MD, USA).

Transfection of HepG2 cell lines with the plasmids

The HBV expression plasmid was transfected into HepG2 cells with control miRNA or miR-1231 expression plasmid using *Trans*IT-LT1 (Mirus, Madison, WI, USA) reagent according to the manufacturer's instructions. 24–48 h after transfection, core-associated HBV DNA and HBV RNA were extracted and quantified by real-time PCR or RT real-time PCR, respectively [28]. For identifying targets within the HBV genome, HBc or HBx expression plasmids were transiently transfected with miR-1231 expression plasmid into HepG2 cells. Twenty-four hours after transfection, the cells were harvested to perform Western blot analysis.

Analysis of HBV replication intermediates

Quantitative analysis of HBV replication intermediates was performed as described previously [29]. The HBV-specific primers used for amplification were 5′-TTTGGGCATGGAC-ATTGAC-3′ and 5′-GGTGAACAATGTTCCGGAGAC-3′. The lower detection limit of this assay was 300 copies.

Western blot analysis

Cell lysates, prepared with RIPA like buffer [50 mM Tris-HCl (pH 8.0), 0.1% SDS, 1% NP-40, 150 mM sodium chloride, and 0.5% sodium deoxycholate] containing protease inhibitor cocktail (Sigma-Aldrich, Tokyo, Japan), were separated on 5–20% (wt/v) SDS-polyacrylamide gels (Bio-Rad Laboratories, Inc., Tokyo, Japan). Immunoblotting was performed with anti-FLAG M2 monoclonal antibody (Sigma-Aldrich) or anti-HBV core monoclonal antibody HB91 (Advanced Life Science Institute Inc., Saitama, Japan) or anti β-actin monoclonal antibody (Sigma-Aldrich) followed by incubation with horseradish peroxidase-conjugated sheep anti-mouse immunoglobulin (GE Healthcare, Buckinghamshire, UK). Expression of HBc protein was quantified based on the densities of the immunoblot signals by Quantity One® software (Bio-Rad Laboratories, Inc.).

RESULTS

miRNA expression alterations associated with HBV infection

To analyse the influence of HBV infection on human hepatocytes, miRNA microarray expression profiles were compared between groups A (mice without HBV infection) and B (mice with HBV infection). Among the 900 miRNAs on the microarray, 10 miRNAs showed a more than 2.0-fold change with HBV infection. Five of the 10 miRNAs were upregulated, and the remaining five were downregulated (Fig. S1). Because immunity was severely suppressed in the chimeric mice, changes in miRNA expression are thought to be closely associated with HBV infection, and the upregulated miRNAs might play a protective role against HBV infection. Thus, we focused on these 5 upregulated miRNAs.

Comparison of expression of the 5 upregulated miRNAs in human liver tissues

To verify the microarray results, quantitative analysis of miRNAs was performed using liver tissues from the chimeric mice. Three of the 5 miRNAs were significantly upregulated by HBV infection (Fig. 1). Expression changes in the other 2 miRNAs (hsa-miR-675 and hsa-miR-1908) showed a similar trend but were not significant due to individual variation. Therefore, further quantitative analysis was performed using human liver tissues. Nine liver tissue samples were obtained from patients with chronic hepatitis B ($N = 3$), chronic hepatitis C ($N = 2$) or alcoholic liver dysfunction ($N = 4$), and miRNA expression levels were compared. Expressions of all miRNAs except for miR-886-5p were significantly higher in liver tissues with chronic hepatitis B than in those with other liver diseases (Fig. 2).

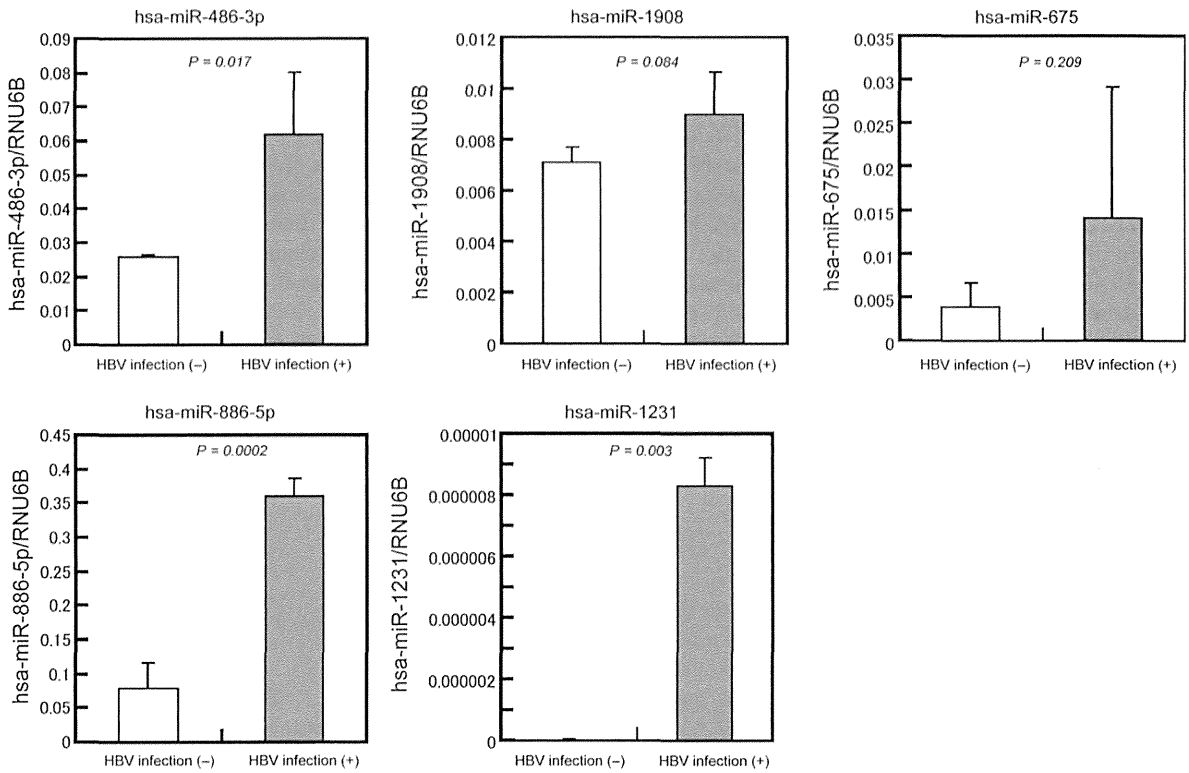

Fig. 1 Upregulation of microRNA by HBV infection. Signal intensities of five upregulated miRNAs were compared between HBV-infected and noninfected mouse livers. All 5 miRNAs were significantly upregulated by HBV infection. *P* values were calculated by the Mann–Whitney *U*-test.

Associations between signalling pathways and the upregulated miRNAs

To analyse the influence of miRNA upregulation on signalling pathways, pathway analysis was performed. However, there are several obstacles in analysing the association between miRNAs and pathways, such as the lack of reliable miRNA target prediction algorithms, differences in the results among target prediction systems, and the small number of validated target genes. To improve the reliability of the targets, we performed the pathway analysis in combination with four prediction tools (miRWalk, TargetScan, miRanda and miRDB). After this operation, 482 targets were predicted (hsa-miR-1231: 203 targets, hsa-miR-1908: 3 targets, hsa-miR-486-3p: 251 targets, hsa-miR-675: 25 targets), and these 482 targets were submitted to the PANTHER classification system for pathway analysis. As shown in Table 1, several immunological pathways such as inflammation mediated by chemokine and cytokine signalling pathway, and the interleukin signalling pathway were identified, but it was difficult to identify characteristic pathways.

Suppression of HBV replication with miR-1231 overexpression

Because hsa-miR-1231 was most the highly upregulated among these four miRNAs and had a high homology with the HBV genome, we focused on hsa-miR-1231. Using GENETYX ver. 8.2.1 (GENETYX, Tokyo, Japan), the hsa-miR-1231 sequence was predicted to hybridize at the HB core and X regions of the HBV genome (Fig. 3). To analyse the influence of hsa-miR-1231 on HBV replication, changes in HBV replication intermediates were evaluated using an *in vitro* HBV replication model. As shown in Fig. 4a, HBV replication intermediates were significantly reduced by hsa-miR-1231 overexpression, and the suppression of HBV RNA and HBc proteins were also observed by hsa-miR-1231 overexpression (Figs 4b,c). Thus, HBV replication was concluded to be inhibited by hsa-miR-1231 at the post-transcriptional level.

Specific regulation of HBV-related protein levels with hsa-miR-1231 overexpression

As the preceding results indicated an association between the production of HBV-related protein or HBV particles and

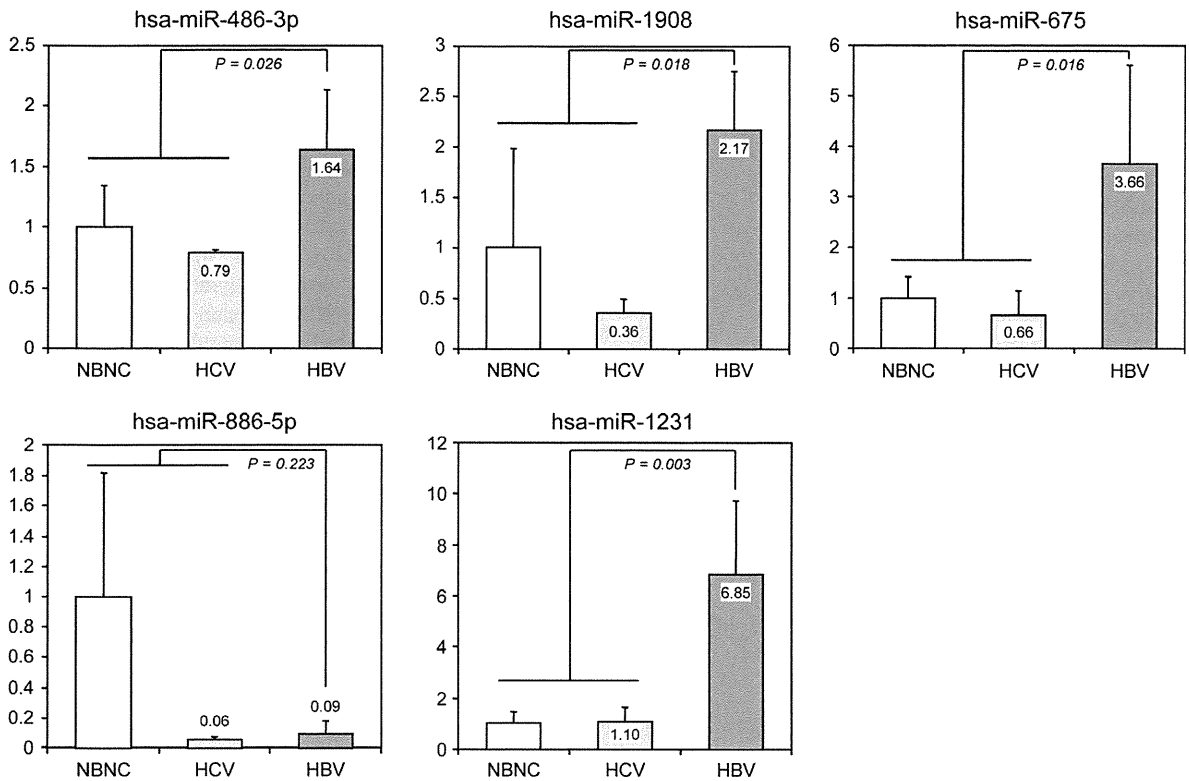

Fig. 2 Comparison of microRNA expression in clinical liver tissues. Quantification of miRNAs was performed by real-time PCR using nine human liver tissues obtained from the patients who had chronic hepatitis B (N = 3), C (N = 2) or alcoholic liver dysfunction (N = 4). Expression levels of four miRNA were significantly higher in the chronic hepatitis B patients than in those of other liver diseases. The results of miR-886-5p levels were not statistically significant. P values were assessed by Mann–Whitney U-test.

hsa-miR-1231 expression, further analysis was performed to identify the region hybridized by hsa-miR-1231. As shown in Fig. 5, HBc protein expression was remarkably reduced by hsa-miR-1231 expression, but no reduction in HBx protein was observed. These results indicate that hsa-miR-1231 might interact with HBV core mRNA and suppress HBV replication by inhibiting HBV core protein production.

The effects of hsa-miR-1231 on the expression of interferon-stimulated genes

Alternatively, hsa-miR-1231 might suppress HBV replication through activation of the interferon signalling pathway. We thus evaluated mRNA expression of interferon-stimulated genes (ISGs) with or without hsa-miR-1231 overexpression. None of the examined ISGs (MxA, PKR, OAS-1 and SOCS1) were regulated by hsa-miR-1231 expression (Fig. S3). These results suggest that hsa-miR-1231 suppresses HBV replication at the post-transcriptional level but not through the activation of interferon signalling.

DISCUSSION

Previously, we have demonstrated that human hepatocyte chimeric mice can be chronically infected with hepatitis B and C viruses [25,30,31]. This mouse model facilitates analysis of the effect of viral infection under immunodeficient conditions. In the present study, we performed miRNA array analysis using this mouse model and obtained miRNA expression profiles reflecting the direct influence of HBV infection on human hepatocytes. Furthermore, we found a novel mechanism for HBV replication mediated by hsa-miR-1231.

To avoid contamination with mouse tissue, human hepatocyte chimeric mice were used in which liver tissue was largely (>90%) replaced by human hepatocytes. Although it is feasible to use microarray analysis in this chimeric mouse model [32], signals from miRNA array analysis may be influenced by cross-hybridization with mouse miRNA from a small amount of contaminated mouse-derived cells because of the high homology between the human and mouse genomes. To compensate

Table 1 Pathways associated with the 4 miRNAs upregulated by HBV infection

Pathway	Number of gene hits	Ratio of genes %
Inflammation mediated by chemokine and cytokine signalling pathway (P00031)	11	2.60
Angiogenesis (P00005)	10	2.30
Integrin signalling pathway (P00034)	9	2.10
Gonadotropin releasing hormone receptor pathway (P06664)	7	1.60
Wnt signalling pathway (P00057)	7	1.60
Parkinson disease (P00049)	7	1.60
EGF receptor signalling pathway (P00018)	7	1.60
Alzheimer's disease–presenilin pathway (P00004)	6	1.40
PDGF signalling pathway (P00047)	6	1.40
B-cell activation (P00010)	6	1.40
Interleukin signalling pathway (P00036)	5	1.20
Huntington disease (P00029)	5	1.20
FGF signalling pathway (P00021)	5	1.20
Cadherin signalling pathway (P00012)	5	1.20
VEGF signalling pathway (P00056)	4	0.90
Toll receptor signalling pathway (P00054)	4	0.90
T-cell activation (P00053)	4	0.90
Ras pathway (P04393)	4	0.90
Heterotrimeric G-protein signalling pathway-Gi alpha and Gs alpha-mediated pathway (P00026)	4	0.90
Endothelin signalling pathway (P00019)	4	0.90

for contamination, mice that were negative for HBV infection were set up as negative controls.

Only 5 miRNAs showed more than 2.0-fold upregulation with HBV infection under miRNA array analysis using chimeric mouse livers (Fig. S1). Comparing these results with our previous study using patient sera, only hsa-miR-486-3p showed a similar change in sera from chronic hepatitis B patients, but no upregulation of the other 4 miRNAs was observed [15]. These results suggest that miRNA expression in sera from chronic hepatitis B patients might be regulated not only by HBV infection but also by human immune responses. In addition, it might be difficult to analyse changes in expression of miRNAs that are expressed at low levels in human hepatocytes, including hsa-miR-1231, using human serum.

To identify targets of miR-1231, we searched using four prediction systems. Although 632 target genes were identified (data not shown), and involvement of a number of pathways was indicated (Table S1), critical targets associated with human immunity or HBV replication could not be identified. Interferon signalling was also a potential mechanism of HBV suppression, but several ISG mRNAs were not induced by hsa-miR-1231 overexpression *in vitro* (Fig. S2). Therefore, we concluded that hsa-miR-1231 does not suppress HBV replication via interferon signalling.

To examine the possibility that miR-1231 directly regulates HBV replication by interacting with HBV-related mRNAs, we searched for hsa-miR-1231-binding motifs and found two candidate sequences in the HBV core and X genes (Fig. 3). As shown in Fig. 5, one target in the HBV core region could hybridize with hsa-miR-1231, and HBc expression was found to be suppressed by hsa-miR-1231 overexpression. The hsa-miR-1231-binding motif in the HBV core region was conserved in more than 90% of the HBV sequences in GenBank, regardless of HBV genotype (data not shown). Thus, we speculate that hsa-miR-1231 binds to the HBc target region and suppresses HBc production to inhibit HBV replication.

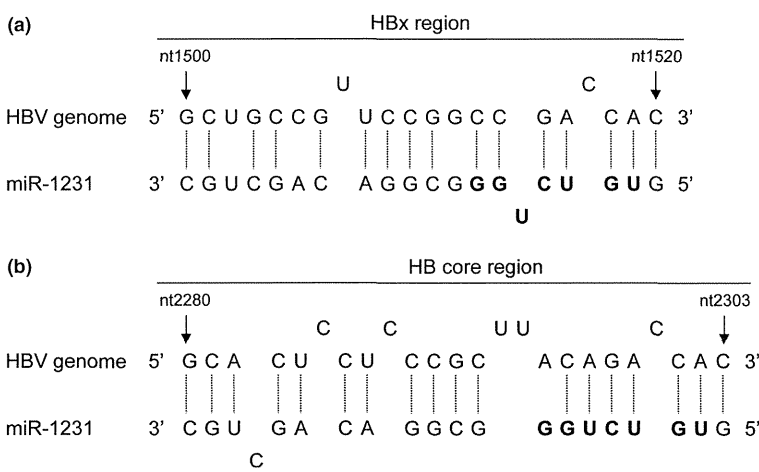

Fig. 3 Alignment of hsa-miR-1231 to HBV genome. Alignment of hsa-miR-1231 to the HBV genome was performed. MiR-1231 sequence was predicted to hybridize at the HBV core **(a)** and HBV X region **(b)**.

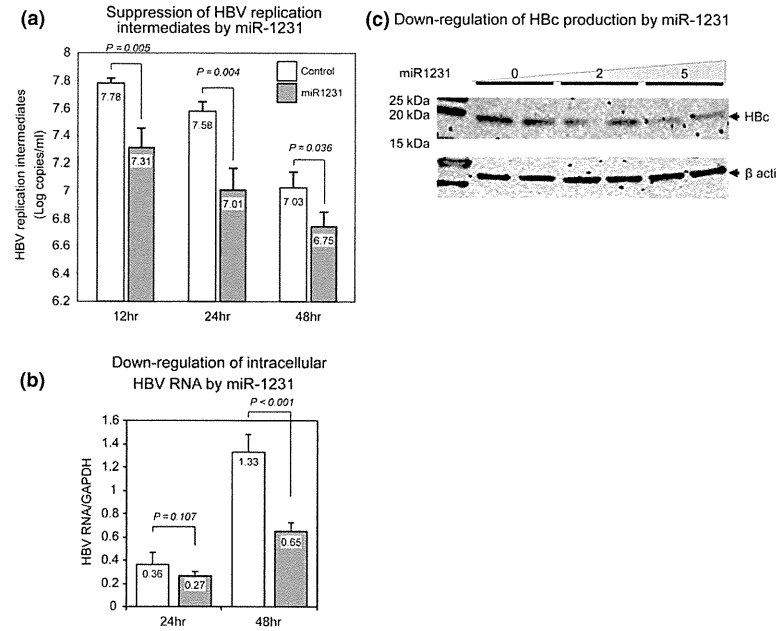

Fig. 4 Suppression of HBV replication by miR-1231. HBV replication intermediates were measured using an *in vitro* HBV replication model. **(a)** Production of HBV replication intermediates was significantly suppressed in cells transfected with both HBV and miR-1231 expression plasmids. **(b, c)** The levels of HBV RNA and HBc protein were also reduced by miR-1231 expression at 24 and 48 h after transfection.

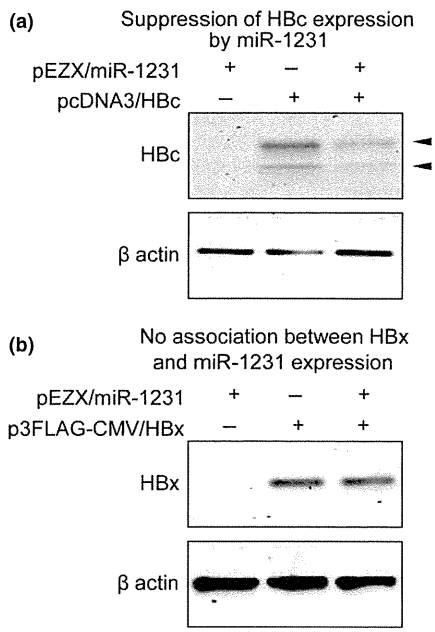

Fig. 5 Identification of miR-1231 target region in HBV genome. To determine the target for miR-1231, HBc or HBx expression plasmid was transfected into HepG2 cells with miR-1231 expression plasmid, and changes in protein levels were analysed by Western blot. HBc protein levels were reduced by miR-1231 expression **(a)**, but HBx protein levels were not reduced **(b)**.

To confirm the association between hsa-miR-1231 and HBV replication, we also tried to suppress hsa-miR-1231 expression using a miRNA inhibitor *in vitro*. However, no significant effects of miR-1231 inhibition on HBV replication were observed *in vitro*. As mentioned previously, expression levels of hsa-miR-1231 are quite low in HepG2 cells and human hepatocytes, and therefore, significant effects of hsa-miR-1231 inhibition could not be observed. The level of hsa-miR-1231 activity was also a factor. As shown in Fig. 4, HBV replication intermediates and HBc expression were significantly suppressed by hsa-miR-1231 overexpression, but the reduction rate was quite small even when 5-fold volume of hsa-miR-1231 plasmid and a volume of HBV expression plasmid were transfected into HepG2 cells. Therefore, it was difficult to observe changes in HBV replication by miRNA inhibition when HBV was replicating vigorously.

In conclusion, we performed miRNA array analysis using human hepatocyte chimeric mice and were able to analyse the direct effects of HBV infection without the confounding effects of the lymphocyte immunological response. We obtained evidence that hsa-miR-1231 was upregulated in response to HBV infection in human hepatocytes, whereupon hsa-miR-1231 suppressed replication of HBV.

ACKNOWLEDGEMENTS

This work was carried out at the Research Center for Molecular Medicine, Faculty of Medicine, Hiroshima University and the Analysis Center of Life Science,

Hiroshima University. The authors thank Rie Akiyama for her excellent technical assistance and Akemi Sada and Emi Nishio for clerical assistance. This study was supported in part by a Grant-in-Aid for Scientific Research from the Japanese Ministry of Labor and Health and Welfare.

FINANCIAL DISCLOSURE

Kohno T, Tsuge M, Murakami E, Hiraga N, Abe H, Miki D, Imamura M, Takahashi S, Ochi H, Hayes CN, Chayama K: None to declare.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1: HBV infection regulated expression of several microRNAs. Complete linkage hierarchical clustering analysis was performed using Euclidean distance. Among the 900 miRNAs, 10 miRNAs showed more than 2.0-fold change between groups. Five of the 10 miRNAs were upregulated by HBV, and the other five were downregulated.

Figure S2: No effect of miR-1231 expression on IFN signalling. To analyse the influence of miR-1231 expression on interferon signalling, four interferon-stimulated genes (ISGs) were quantified by real-time PCR. None of the four ISGs (MxA, PKR, OAS-1 and SOCS1) were suppressed by miR-1231 expression.

Table S1: Pathway analysis of miR-1231 target genes.

J Gastroenterol (2013) 48:779–780
DOI 10.1007/s00535-013-0801-6

LETTER TO THE EDITOR

Availability of monitoring serum HBV DNA plus RNA during nucleot(s)ide analogue therapy

Masataka Tsuge · Kazuaki Chayama

Received: 10 March 2013 / Accepted: 10 March 2013 / Published online: 3 April 2013

We appreciate the comments by Kurosaki et al. on the article entitled "Serum HBV RNA and HBeAg are useful markers for the safe discontinuation of nucleot(s)ide analogue (NUC) treatments in chronic hepatitis B patients" [1]. They raised three important questions: (1) whether HBV DNA levels measured by transcription-mediated amplification and hybridization (TMA-HPA) can be used as an alternative to HBV DNA plus RNA levels measured by RT-PCR; (2) whether post-treatment monitoring of serum HBV DNA plus RNA might serve as a predictor of safe discontinuation after long term NUC; and (3) whether serum HBV DNA plus RNA titer is a predictor of favorable response to sequential interferon therapy.

The presence of HBV RNA in serum is an indicator of ongoing transcription of the HBV pregenome from cccDNA in hepatocytes and may occur even when production of mature HBV particles is effectively suppressed by inhibition of reverse transcription by NUC. As we previously reported, lamivudine resistant strains emerge more easily under such conditions [2], but HBV RNA gradually decreases under continued suppression of reverse transcription and generally becomes undetectable in patients following a year of NUC treatment.

The first question Kurosaki et al. was whether HBV DNA titers measured by TMA-HPA assay, which actually represent HBV DNA plus RNA titers, can be used as an alternative to HBV DNA plus RNA measured by RT-PCR. As we showed in our previous report [2], levels obtained by TMA-PHA assay correlated well with those obtained by RT-PCR during NUC therapy ($r = 0.955$, $P < 0.0001$) [2]. Therefore, measurement of TMA-PHA is a reasonable alternative to RT-PCR. Although the sensitivity of HBV nucleic acids by TMA-HPA assay is lower than RT-PCR, measurement of HBV nucleic acids may provide useful information, especially for those patients who started NUC therapy with high pretreatment HBV DNA levels. RT-PCR is more useful in patients who had relatively lower HBV levels at the beginning of NUC therapy.

The second question was whether monitoring of serum HBV DNA plus RNA at the end of treatment serves as a predictor of safe discontinuation after long term NUC. We found that HBV RNA can be detected in patients who became negative for HBV DNA after long term NUC therapy, and measurement of HBV RNA in patients receiving long term NUC therapy may yield important insight into the risk of reactivation of HBV if NUC therapy is discontinued. However, we have not analyzed enough such patients, and a prospective study is necessary to evaluate the predictive value of HBV RNA plus RNA measurement.

The third question was whether serum HBV DNA plus RNA titer is a predictor of favorable response to sequential NUC and interferon therapy. The mechanisms of these drugs is different, and interferon is not associated with serum HBV RNA because it does not disturb reverse

This author's reply refers to the letter to the editor at doi:10.1007/s00535-013-0800-7.

M. Tsuge · K. Chayama (✉)
Department of Gastroenterology and Metabolism, Applied Life Sciences, Institute of Biomedical and Health Sciences, Hiroshima University, Hiroshima, Japan
e-mail: chayama@hiroshima-u.ac.jp

M. Tsuge · K. Chayama
Liver Research Project Center, Hiroshima University, Hiroshima, Japan

M. Tsuge
Natural Science Center for Basic Research and Development, Hiroshima University, Hiroshima, Japan

transcription but instead suppresses HBV transcription in hepatocytes. In our previous study [3], HBV RNA was negative before administration of NUC and became positive soon after the beginning of NUC therapy, peaking at weeks two to four and then gradually decreasing. We assumed that, after HBV RNA levels have been reduced during long term NUC therapy, HBV RNA should become undetectable during interferon therapy [3]. We tried to assess the predictive effect of HBV RNA titer immediately prior to interferon administration in patients who received sequential therapy, but, incidentally, HBV RNA was undetectable in all patients just before interferon treatment [3]. As we did not show results for sequential therapy in our study in Journal of Gastroenterology [1], results of the 26 patients (20 males, 6 females) who underwent sequential therapy patients in that study are described below. Ten patients were positive for HBeAg at the end of NUC therapy. HBV DNA rebound was observed in 13 patients within 24 weeks after discontinuation of NUC therapy, and ALT rebound occurred in 9 patients. HBV DNA rebound was significantly associated with serum HBV DNA plus RNA titer following 3 months of NUC treatment ($P = 0.029$, Mann–Whitney U test), and ALT rebound was significantly associated with serum HBV DNA titer and DNA plus RNA titer following 3 months of NUC treatment ($P = 0.041$, $P = 0.016$, respectively, Mann–Whitney U test) and the existence of HBeAg at the end of NUC treatment ($P = 0.009$, Fisher's exact test). Although it is necessary to confirm these results in a large, prospective study, we conclude from these results that HBV RNA plus DNA is a predictor for sequential therapy.

Due to the complicated nature of chronic HBV infection and immunological reaction of the host, it is difficult to completely predict the effect of any type of therapy. Further study should be done to identify conditions for safe discontinuation of NUC because otherwise patients must continue lifelong NUC therapy. We thank Kurosaki et al. for their helpful comments and appreciate the opportunity to respond to their questions.

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