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

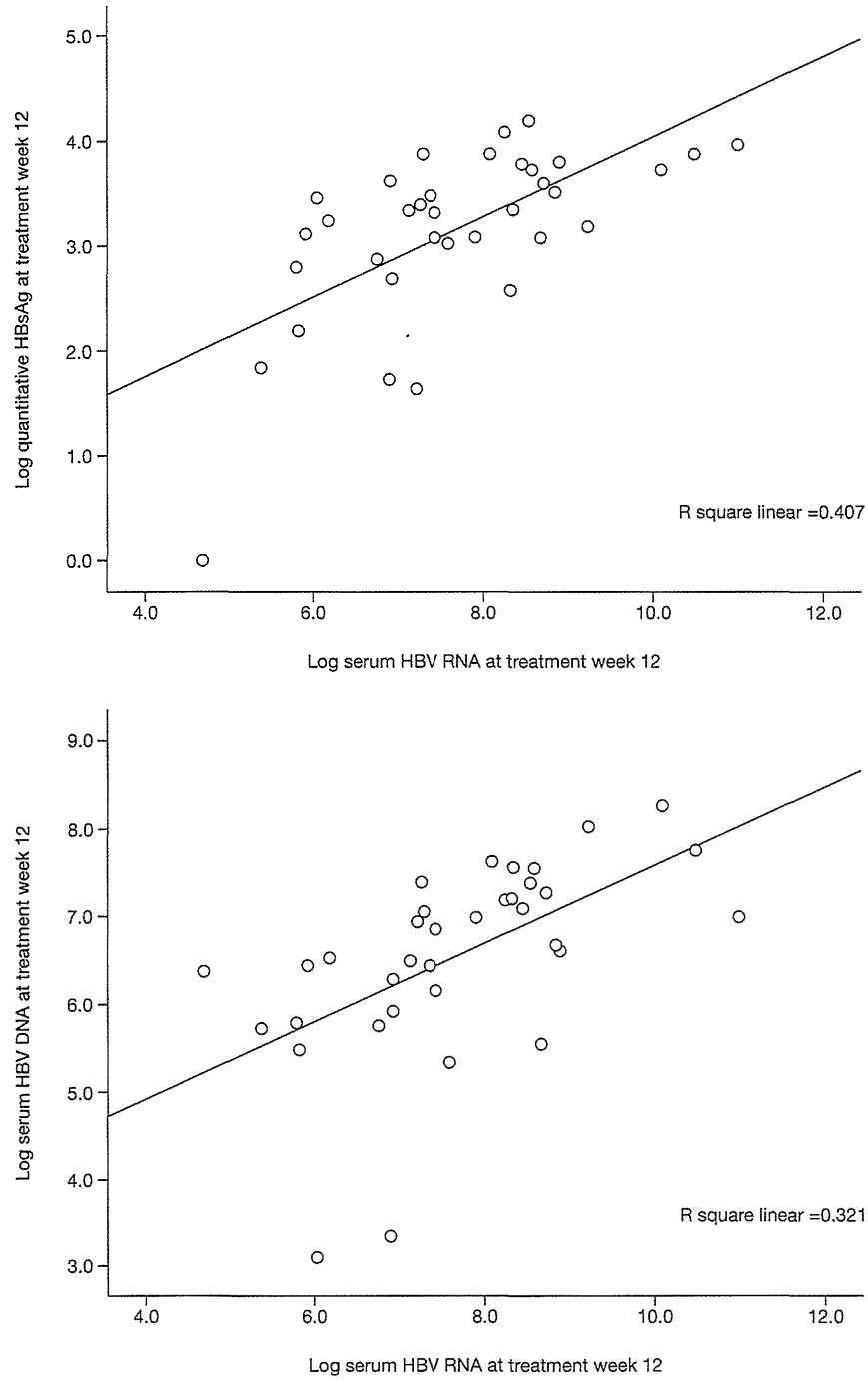
CHB patients with interval from detectable to undetectable serum HBV DNA level <16 weeks (*n*=23) had a significantly lower serum HBV RNA level at week 12 of NA therapy than those with interval ≥16 weeks (*n*=21; 3.8 ±3.8 versus 6.6 ±3.5 log copies/ml [*P*=0.013]; Figure 2A). The time interval based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

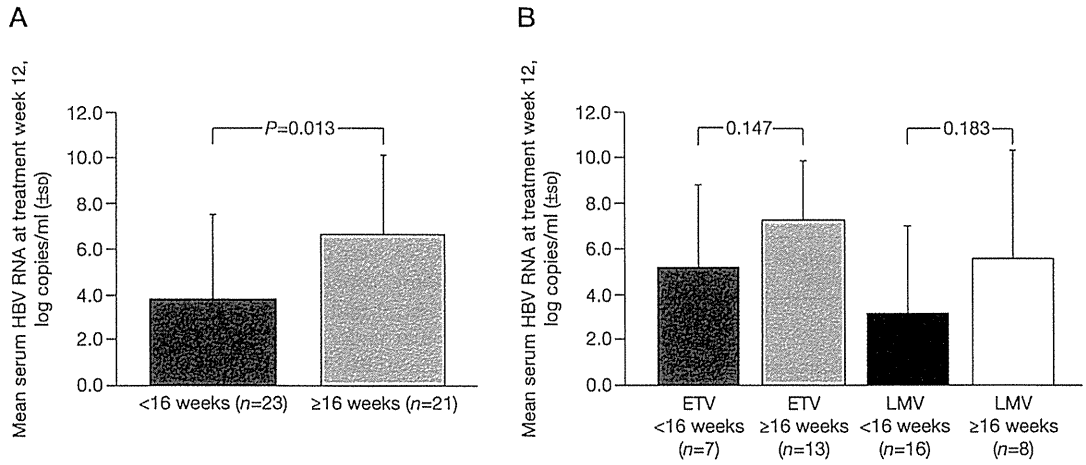
Variable	Lamivudine	Entecavir	*P*-value
HBV RNA detectability			
Pre-treatment, *n*/total *n* (%)	9/26 (34.6)	12/26 (46.1)	0.396
At 12 weeks, *n*/total *n* (%)	13/26 (50)	22/26 (84.6)	0.008
At 24 weeks, *n*/total *n* (%)	10/26 (38.5)	20/26 (76.9)	0.005
Log HBV RNA			
Mean pre-treatment, copies/ml (±SD)	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]
OK

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?]

OK

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]

OK

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

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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.

References

1. Lee WM. Hepatitis B virus infection. *N Engl J Med* 1997; 337:1733–1745.
2. Kao JH, Chen DS. Overview of hepatitis B and C viruses. In JJ Goedert JJ (Editor). *Infectious causes of cancer: targets for intervention*. Totowa: Humana Press 2000; pp. 313–330.
3. McQuillan GM, Townsend TR, Fields HA, Carrol M, Leahy M, Polk BF. Seroepidemiology of hepatitis B virus infection in the United States. *Am J Med* 1989; 87 Suppl 3A:5S–10S.
4. Chen DS. From hepatitis to hepatoma: lessons from type B viral hepatitis. *Science* 1993; 262:369–370.
5. Chen DS. Fighting against viral hepatitis: lessons from Taiwan. *Hepatology* 2011; 54:381–392.
6. Doong SL, Tsai CH, Schinazi RF, Liotta DC, Cheng YC. Inhibition of the replication of hepatitis B virus in vitro by 2′,3′-dideoxy-3′-thiacytidine and related analogues. *Proc Natl Acad Sci U S A* 1991; 88:8495–8499.
7. Zhang W, Hacker HJ, Tokus M, Bock T, Schroder CH. Patterns of circulating hepatitis B virus serum nucleic acids during lamivudine therapy. *J Med Virol* 2003; 71:24–30.
8. Hatakeyama T, Noguchi C, Hiraga N, *et al.* Serum HBV RNA is a predictor of early emergence of the YMDD mutant in patients treated with lamivudine. *Hepatology* 2007; 45:1179–1186.
9. Huang YW, Chayama K, Tsuge M, *et al.* Differential effects of interferon and lamivudine on serum HBV RNA inhibition in patients with chronic hepatitis B. *Antivir Ther* 2010; 15:177–184.
10. Tsuge M, Murakami E, Imamura M, *et al.* Serum HBV RNA and HBeAg are useful markers for the safe discontinuation of nucleotide analogue treatments in chronic hepatitis B patients. *J Gastroenterol* 2013; 48:1188–1204.
11. Liaw YF. Hepatitis B virus replication and liver disease progression: the impact of antiviral therapy. *Antivir Ther* 2006; 11:669–679.
12. Lok ASF, McMahon BJ. Chronic hepatitis B: Update 2009. *Hepatology* 2009; 50:661–662.
13. European Association For The Study Of The Liver. EASL clinical practice guidelines: Management of chronic hepatitis B virus infection. *J Hepatol* 2012; 57:167–185.
14. Liaw YF, Kao JH, Piratvisuth T, *et al.* Asian-Pacific consensus statement on the management of chronic hepatitis B: a 2012 update. *Hepatol Int* 2012; 6:531–561.
15. Janssen HL, Sonneveld MJ, Brunetto MR. Quantification of serum hepatitis B surface antigen: is it useful for the management of chronic hepatitis B? *Gut* 2012; 61:641–645.
16. Keeffe EB, Zeuzem S, Koff RS, *et al.* Report of an international workshop: roadmap for management of patients receiving oral therapy for chronic hepatitis B. *Clin Gastroenterol Hepatol* 2007; 5:890–897.
17. Hiroshima Study Group. (Updated 29 August 2012. Accessed 1 January 2014.) Available from http://home.hiroshima-u.ac.jp/naika1/research_profile/pdf/liver_study_group_e.pdf
18. Locarnini S, Hatzakis A, Heathcote J, *et al.* Management of antiviral resistance in patients with chronic hepatitis B. *Antivir Ther* 2004; 9:679–693.
19. Lai CL, Shouval D, Lok AS, *et al.* Entecavir versus lamivudine for patients with HBeAg-negative chronic hepatitis B. *N Engl J Med* 2006; 354:1011–1020.
20. Yao G, Chen C, Lu W, *et al.* Efficacy and safety of entecavir compared to lamivudine in nucleoside-naïve patients with chronic hepatitis B: a randomized double-blind trial in China. *Hepatol Int* 2007; 1:365–372.
21. Rokuhara A, Matsumoto A, Tanaka E, *et al.* Hepatitis B virus RNA is measurable in serum and can be a new marker for monitoring lamivudine therapy. *J Gastroenterol* 2006; 41:785–790.

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Differences in serum microRNA profiles in hepatitis B and C virus infection

Sakura Akamatsu [a,b,c,1], C. Nelson Hayes [a,b,c,1], Masataka Tsuge [c,d], Daiki Miki [a,b,c], Rie Akiyama [a,b,c], Hiromi Abe [a,b,c], Hidenori Ochi [a,b,c], Nobuhiko Hiraga [a,b,c], Michio Imamura [a,b,c], Shoichi Takahashi [e], Hiroshi Aikata [a,c], Tomokazu Kawaoka [a,b,c], Yoshiiku Kawakami [a,b,c], Waka Ohishi [f], Kazuaki Chayama [a,b,c,*]

[a] *Department of Gastroenterology and Metabolism, Applied Life Sciences, Institute of Biomedical & Health Sciences, Hiroshima University, Hiroshima, Japan*
[b] *Laboratory for Digestive Diseases, Center for Genomic Medicine, RIKEN, Hiroshima, Japan*
[c] *Liver Research Project Center, Hiroshima University, Hiroshima, Japan*
[d] *Natural Science Center for Basic Research and Development, Hiroshima University, Hiroshima, Japan*
[e] *Koyo New Town Hospital, Hiroshima, Japan*
[f] *Department of Clinical Studies, Radiation Effects Research Foundation, Hiroshima, Japan*

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KEYWORDS
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HBeAg;
Microarray

Summary *Objectives:* Patients infected with chronic hepatitis B virus (HBV) or hepatitis C virus (HCV) are at greater risk of cirrhosis and hepatocellular carcinoma. The objective of this study was to identify virus-specific serum microRNA profiles associated with liver function and disease progression. Microarray analysis of serum microRNAs was performed using the Toray 3D array system in 22 healthy subjects, 42 HBV patients, and 30 HCV patients. Selected microRNAs were then validated by qRT-PCR in 186 HBV patients, 107 HCV patients, and 22 healthy subjects.

Results: Microarray analysis showed up-regulation of a number of microRNAs in serum of both HBV and HCV patients. In qRT-PCR analysis, miR-122, miR-99a, miR-125b, miR-720, miR-22, and miR-1275 were up-regulated both in HBV patients relative to healthy subjects, and all except

List of abbreviations: HBV, Hepatitis B virus; HCV, Hepatitis C virus; HCC, hepatocellular carcinoma; qRT-PCR, quantitative real-time polymerase chain reaction; HBsAg, HBV surface antigen; HBeAg, HBe antigen; HBeAb, HBe antibody; HBcAg, HBV core antigen; γGTP, γ-glutamyl transpeptidase.

* Corresponding author. Department of Gastroenterology and Metabolism, Applied Life Sciences, Institute of Biomedical & Health Sciences, Hiroshima University, 1-2-3 Kasumi, Minami-ku, Hiroshima 734-8551, Japan. Tel.: +81 82 257 5190; fax: +81 82 255 6220.
E-mail address: chayama@hiroshima-u.ac.jp (K. Chayama).
[1] Co-first authors.

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miR-1275 were up-regulated in HBeAg-positive patients relative to HBeAg-negative patients. Specific microRNAs were independently associated with different aspects of HBV infection. MiR-122 was independently associated with HBV DNA level, whereas miR-125b was independently associated with levels of HBV DNA, HBsAg, and HBeAg. MiR-22 and miR-1275 were independently associated with serum γ-glutamyl transpeptidase levels.

Conclusions: Serum microRNA levels reflect differences in the etiology and stage of viral hepatitis.

Introduction

Chronic infection with hepatitis B virus (HBV), a partially double-stranded DNA virus, and hepatitis C virus (HCV), a single stranded RNA virus, increases the risk of cirrhosis and hepatocellular carcinoma (HCC). Despite improvements in antiviral therapy, many patients fail to respond to current therapies.[1–3] Therefore, non-invasive methods are needed for early detection of changes in liver function. One such approach is to measure changes in levels of small RNAs present in the serum of infected patients. In addition to messenger RNA, transfer RNA, and ribosomal RNA, there are many other classes of RNAs, many of which act to fine-tune gene expression and may play a role in disease pathogenesis. MicroRNAs are among the most important classes of non-coding RNA and consist of short linear RNA sequences that range in size from 19 to 24 nucleotides. MicroRNAs may influence gene expression by binding to a partially complementary region in the 3′ untranslated region of a targeted messenger RNA, thereby inhibiting translation or promoting degradation of the transcript. Because a single microRNA may regulate multiple genes, and a single gene may be regulated by multiple microRNAs, microRNAs may form complex regulatory networks.[4] Viral pathogenesis and inflammation may disrupt these intricate networks, resulting in changes in microRNA levels inside and outside of the cell. Given the liver's dual blood supply and central role in circulation, pathogenic changes in gene expression in the liver are likely to be reflected in changes in microRNA profiles in the serum.

Understanding the origin and function of serum microRNAs is important in the development of strategies to eradicate HCV and HBV and to monitor the degree of liver damage. Analysis of differential microRNA expression in liver tissues has revealed HCV- and HBV-specific microRNAs as well as microRNAs associated with the stage of liver disease.[5–9] MicroRNA levels in the liver have been found to be correlated with serum levels for a number of microRNAs,[10,11] suggesting that serum microRNAs might act as a surrogate measure of microRNA activity in the liver. While RNA typically has a short-half life and is quickly degraded by RNases, microRNAs tend to exist stably in serum when bound to argonaute proteins such as AGO2 as part of the RNA-induced silencing complex, the molecular scaffold that facilitates interaction of a microRNA with its target sequence.[12] Circulating microRNAs may exist in this form as vesicle-free ribonucleoprotein complexes, or they may be transported within HBV surface antigen (HBsAg) particles or contained within exosomes/microvesicles.[12–14] However, serum microRNAs are typically concentrated in exosomes.[15]

Exosomes are 30–150 nm endosome-derived microvesicles that are released from multiple cell types and are detectable in blood, urine, saliva, and other body fluids. Exosomes are involved in removal of cellular waste products as well as cell–cell communication and immune activation but may also be exploited by pathogens and contribute to tumor proliferation. Exosomes contain characteristic RNA transcripts, including microRNAs, transfer RNAs and other types of non-coding RNAs[16] and have been shown to affect gene expression in recipient cells. MiR-99a, miR128, miR-124, miR-22, and miR-99b account for 49% of identified exosome-associated microRNAs.[16] While exosomal RNA profiles vary by cell type, they do not completely mirror the RNA profile of the parent cell due to selective sorting and may change in response to cellular conditions.[16] Hepatocyte-derived exosomes are enriched for gene products involved in lipoprotein metabolism and xenobiotic processing and therefore have potential as a diagnostic tool by reflecting hepatic changes linked to disease.[17] Interferon-stimulated release of exosomes containing antiviral products and internalization by HBV-infected hepatocytes may also play a role in antiviral defense by bypassing viral interference in interferon signal transduction.[18] It is likely that analysis of serum microRNA profiles will provide insight into disease progression and antiviral activity in the liver, particularly in the case of HBV infection.

In order to investigate the relationship between serum microRNA profiles and viral hepatitis, we performed microarray and quantitative real-time polymerase chain reaction (qRT-PCR) analysis to identify host microRNAs that differ between healthy subjects and patients with chronic HBV or HCV infection as well as between HBeAg-positive and negative patients.

Methods

Study subjects

All patients had either chronic hepatitis B or C infection and were negative for HIV and HCC. No patients were co-infected with both HBV and HCV. All healthy subjects were negative for HBsAg and HCV antibody. Patient profiles are shown in Table 1. Histopathological diagnosis was determined as in Desmet et al.[19] The study was approved *a priori* by the ethical committee of Hiroshima University and conforms to the ethical guidelines of the 1975 Declaration of Helsinki. All patients provided written informed consent.

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Microarray analysis of serum microRNA expression levels

Host microRNA expression in serum samples was measured using the Toray Industries microRNA analysis system, in which serum microRNA samples were hybridized to 3D-Gene human microRNA ver17.1 chips containing 1200 microRNAs (Toray Industries, Inc., Tokyo, Japan). Serum from 42 patients with chronic HBV infection and 30 patients with chronic HCV infection were compared with serum from 12 healthy males and 10 healthy females using a separate microarray for each sample.

Quantitative RT-PCR microRNA analysis

A subset of microRNAs was selected for validation using qRT-PCR based on preliminary microarray results and a search of the literature. Expression of 7 microRNAs was measured in serum from 186 HBV patients, 107 HCV patients, and 22 healthy subjects. Circulating microRNA was extracted from 300 μl of serum samples using the mirVana PARIS Kit (Ambion Inc., Austin, TX) according to the manufacturer's instructions. RNA was eluted in 80 μl of nuclease free water and reverse transcribed using TaqMan MicroRNA Reverse Transcription Kit (Life technologies Japan Ltd, Tokyo, Japan). Each sample was spiked with *Caenorhabditis elegans* miR-238 (cel-miR-238) as a control for extraction and amplification. The reaction mixture contained 5 μl of RNA solution, 2 μl of 10x reverse transcription buffer, 0.2 μl of 100 mM dNTP mixture, 4 μl of 5x RT primer, 0.25 μl of RNase inhibitor and 7.22 μl of nuclease free water in a total volume of 20 μl. The reaction was performed at 16 °C for 30 min followed by 42 °C for 30 min. The reaction was terminated by heating the solution at 85 °C for 5 min. MicroRNAs were amplified using primers and probes provided by Applied Biosystems Inc. using TaqMan MicroRNA assays according to the manufacturer's instructions. The reaction mixture contained 12.5 μl of 2x Universal PCR Master Mix, 1.25 μl of 20x TaqMan Assay solution, 1 μl of reverse transcription product and 10.25 μl of nuclease free water in a total volume of 25 μl. Amplification conditions were 95 °C for 10 min followed by 50 denaturing cycles for 15 s at 95 °C and annealing and extension for 60 s at 60 °C in an ABI7300 thermal cycler. For the cel-miR-238 assay, a dilution series using chemically synthesized microRNA was used to generate a standard curve that permitted absolute quantification of molecules. A separate internal normalization factor was not used.

Statistical analysis

MicroRNA microarray expression data was normalized using cyclic loess and analyzed using moderated *t*-tests using the limma package in the R statistical framework (http://www.r-project.org). *P*-values were adjusted for multiple testing using the false discovery rate (P_{FDR}). qRT-PCR expression levels were compared between healthy subjects and HBV or HCV using the non-parametric Mann–Whitney *U* test. Association between qRT-PCR microRNA levels and clinical parameters such as HBsAg, HBV DNA, HBeAg, HBeAb, AST, and ALT were evaluated using multiple linear regression. Factors that were significant at 0.05 in univariate analysis were included as candidates in the multivariate model, and forward-backward stepwise selection based on Akaike information criterion (AIC) was used to identify independently associated factors.

Pathway analysis

Target genes of differentially expressed microRNAs were predicted using the miRWalk database (http://www.umm.

Table 1 Clinical characteristics of healthy controls and patients with chronic viral HBV or HCV infection. Continuous variables are shown as median and range, and categorical variables are shown as counts.

Factor	Healthy(N = 22)	Hepatitis B virus (N = 186)	Hepatitis C virus (N = 107)
Age	33 (27–45)	48 (22–79)	64 (24–85)
Sex (male/female)	12/10	122/64	47/60
Alanine aminotransferase (IU/l)	18.5 (15–22)	73.5 (10–1867)	30.5 (18–145)
Aspartate aminotransferase (IU/l)	13.5 (6–44)	47.5 (15–982)	33.5 (11–141)
γ-glutamyl transpeptidase (IU/l)	20 (11–52)	41.5 (9–459)	22 (8–161)
rs8099917 genotype (TT/GT/GG/unknown)	5/0/0/17	89/76/3/18	–
Liver fibrosis (1/2/3/4/unknown)	–	65/76/28/3/14	39/35/11/4/18
Necroinflammatory activity (1/2/3/unknown)	–	58/80/34/14	32/48/9/18
Alpha-fetoprotein (ug/l)	–	6.1 (<5.0–2510.0)	5.0 (<5.0–104.8)
Promthrombin time (s)	–	95 (35–123)	98 (71–116)
Albumin (g/dl)	–	4.4 (2.8–4.9)	4.3 (3.5–5.0)
Platelets ($x10^4/mm^3$)	–	17.4 (5.0–35.7)	17.6 (5.3–29.8)
rs8099917 genotype (TT/GT/GG/unknown)	5/0/0/17	89/76/3/18	–
HBV DNA (IU/ml)	–	6.7 (<2.1–≥9.1)	–
HBsAg (IU/l)	–	3650 (1.2–239000)	–
HBeAg (−/+)	–	82/104	–
HBeAb (−/+)	–	88/98	–
HBV genotype (A/B/C/unknown)	–	3/14/129/40	–
HCV RNA (Log IU/ml)	–	–	6.5 (1.7–7.3)
HCV genotype (1a/1b/2a/2b/3a)	–	–	5/42/18/9/1/32

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