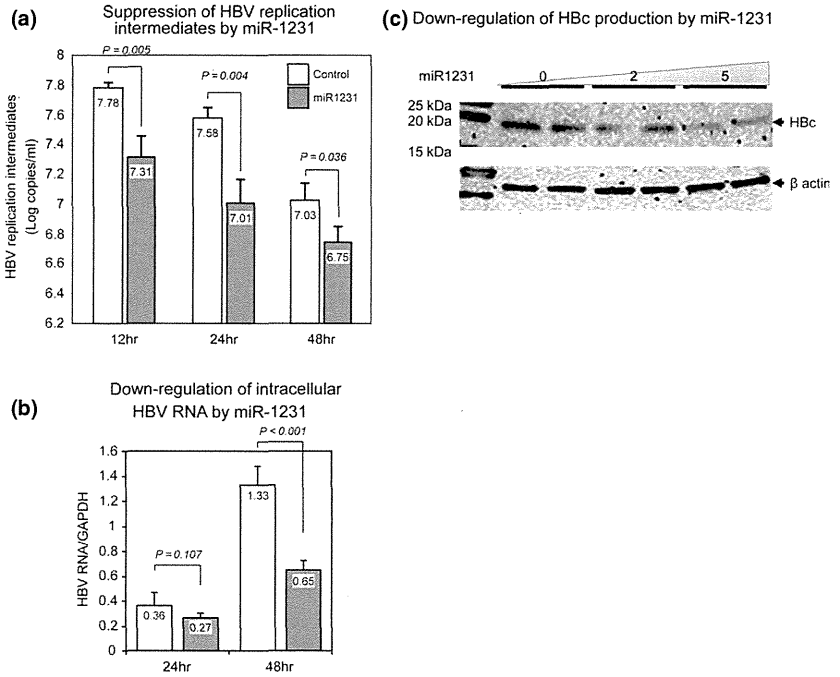

Fig. 4 Suppression of HBV replication by miR-1231. HBV replication intermediates were measured using an *in vitro* HBV replication model. **(a)** Production of HBV replication intermediates was significantly suppressed in cells transfected with both HBV and miR-1231 expression plasmids. **(b, c)** The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

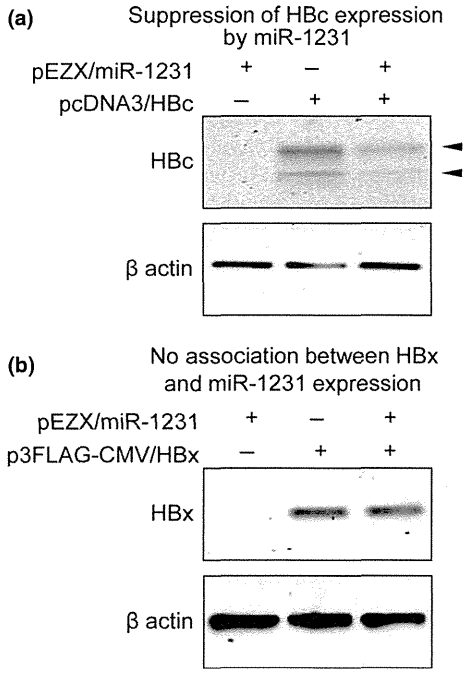

Fig. 5 Identification of miR-1231 target region in HBV genome. To determine the target for miR-1231, HBc or HBx expression plasmid was transfected into HepG2 cells with miR-1231 expression plasmid, and changes in protein levels were analysed by Western blot. HBc protein levels were reduced by miR-1231 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.

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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.

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Original article

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

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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

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

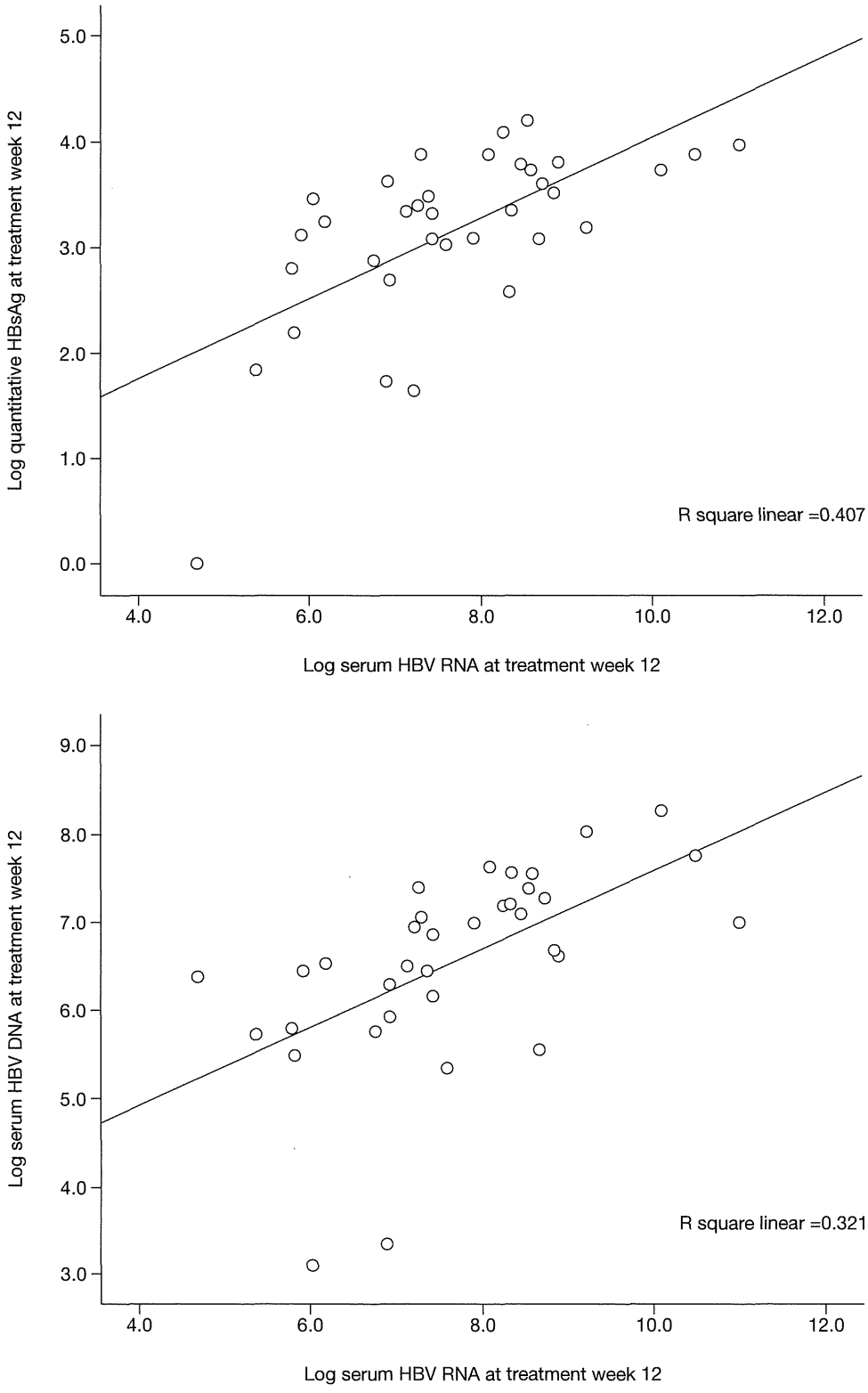
CHB patients with interval from detectable to undetectable serum HBV DNA level <16 weeks (*n*=23) had a significantly lower serum HBV RNA level at week 12 of NA therapy than those with interval ≥16 weeks (*n*=21; 3.8 ±3.8 versus 6.6 ±3.5 log copies/ml [*P*=0.013]; Figure 2A). The time interval based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

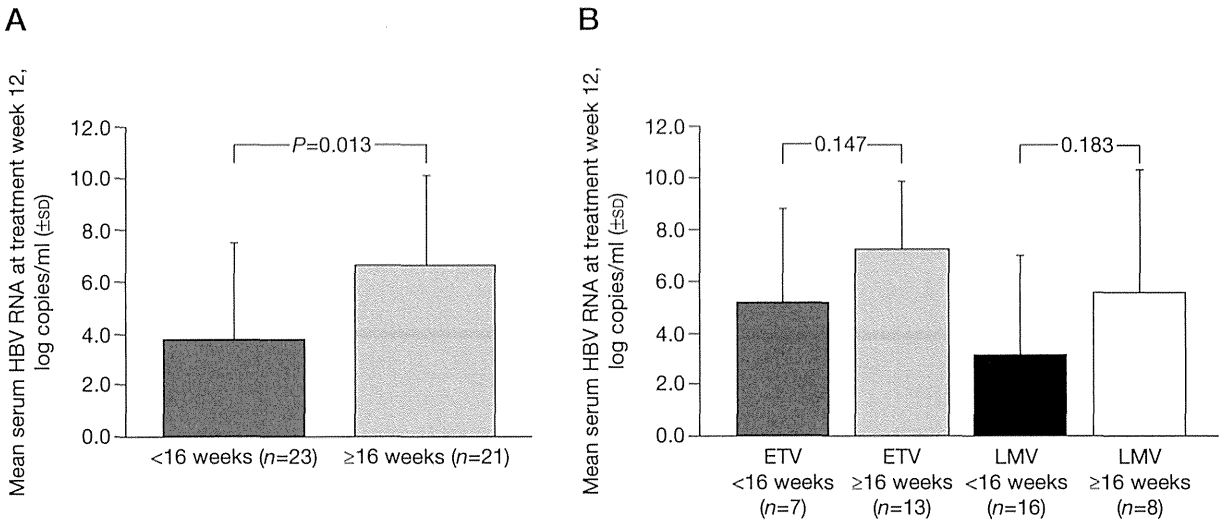
Variable	Lamivudine	Entecavir	*P*-value
HBV RNA detectability			
Pre-treatment, *n*/total *n* (%)	9/26 (34.6)	12/26 (46.1)	0.396
At 12 weeks, *n*/total *n* (%)	13/26 (50)	22/26 (84.6)	0.008
At 24 weeks, *n*/total *n* (%)	10/26 (38.5)	20/26 (76.9)	0.005
Log HBV RNA			
Mean pre-treatment, copies/ml (±SD)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

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.

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.

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.

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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.

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Original Article

Efficacy and safety of the anticoagulant drug, danaparoid sodium, in the treatment of portal vein thrombosis in patients with liver cirrhosis

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Aim: To assess the efficacy and safety of the anticoagulant drug, danaparoid sodium, in the treatment of portal vein thrombosis (PVT) in patients with liver cirrhosis.

Methods: A consecutive 26 cirrhotic patients with PVT were enrolled in this retrospective cohort study. The etiologies of cirrhosis were hepatitis B virus-related, hepatitis C virus-related, alcoholic and cryptogenic in five, 14, three and four patients, respectively. Child–Pugh grade A, B and C was noted in 13, eight and five patients, respectively. Patients were treated with 2 weeks' administration of danaparoid sodium followed by the evaluation of PVT reduction and adverse events.

Results: All patients experienced reduction of PVT through the treatment. The median volume of PVT before and after treatment was 2.40 cm^3 (range, 0.18–16.63) and 0.37 cm^3 (range, 0–5.74), respectively. The median reduction rate of PVT volume was 77.3% (range, 18–100%). According to the reduction rate, complete reduction (CR), partial reduction (PR, ≥50%) and stable disease (SD, <50%) were observed in four (15%), 16 (62%) and six patients (23%), respectively. The median volume of PVT before treatment was significantly different between CR + PR and SD (2.09 vs 4.35 cm^3, $P = 0.045$). No severe adverse events such as bleeding symptoms (e.g. gastrointestinal bleeding and cerebral hemorrhage) and thrombocytopenia were encountered.

Conclusion: Danaparoid sodium for the treatment of PVT in patients with liver cirrhosis was safe and effective. Therefore, anticoagulation therapy with danaparoid sodium could have potential as one of the treatment options in PVT accompanied by cirrhosis.

Key words: anticoagulation, danaparoid sodium, liver cirrhosis, portal vein thrombosis

INTRODUCTION

PORTAL VENOUS THROMBOSIS (PVT) is caused by a combination of general and local prothrombotic risk factors. General risk factors are observed in approximately 70% of patients and local risk factors in 30%.[1–6] General risk factors are known to include myeloproliferative disorders, antiphospholipid syndrome, hereditary anticoagulation factor deficiency (such as protein C/S deficiency and antithrombin deficiency), pregnancy and oral contraceptive use.[3,4,6–12] Local risk factors are known to be cancer of any abdominal organs, focal inflammatory lesions, injury to the portal venous system and liver cirrhosis.[2] PVT is classified as either of chronic phase or acute. PVT accompanied by cirrhosis is considered to be of almost chronic phase. In patients with well-compensated cirrhosis, the incidence of PVT is reported to be between 0.6% and 16%.[13–15] Because cirrhosis is one of the important risk factors of PVT, the management of this is important. However, once the PVT becomes complicated, this is one of the important factors for the prognosis. Accordingly, the management of PVT would be necessary in these patients.

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Although various treatment modalities such as pharmacotherapy for anticoagulation and interventional radiologic treatment (including transjugular intrahepatic portosystemic shunt, percutaneous transhepatic portal vein cannulation[16–18] and surgery)[19,20] for venous thromboembolism (VTE) are widely performed at present, the standard treatments for PVT have yet to be established. Pharmacotherapies such as heparin, unfractionated heparin (UFH), low-molecular weight heparin (LMWH) and warfarin have been reported to treat VTE, including deep vein thrombosis and pulmonary embolism.[21–23] Interventional radiologic treatment and surgery are useful treatment options, however, these are invasive and have limited indication due to unfavorable hepatic functional reserve in cirrhotic patients.

Currently, there is no evidence to support the use of chronic anticoagulant therapy in PVT patients with cirrhosis. Because the risk of bleeding in cirrhotic patients due to the reduced synthesis of coagulation factors and the presence of varices is high, treatment with reduced or lesser risk of bleeding is preferred.

Danaparoid sodium used to treat pulmonary embolism and deep vein thrombosis in the cardiovascular area is a heparinoid glycosaminoglycuronan antithrombotic agent. The risk of bleeding with the use of danaparoid has been reported to be low in comparison with heparin. The anticoagulation effect of danaparoid, which is highly selective for anti-Xa activity, is superior to heparin.[24–25] It has been reported that danaparoid is unlikely to cause gastrointestinal hemorrhage. Danaparoid appeared more effective and safer than heparin in terms of bleeding complications.[24] To our knowledge, only case reports of anticoagulation therapy with danaparoid in PVT have been documented at present; however, the efficacy and safety of danaparoid in PVT, especially in cirrhosis, have not yet been fully demonstrated.

In the present study, we examine the efficacy and safety of chronic anticoagulation therapy with danaparoid sodium in PVT in patients with liver cirrhosis.

METHODS

Patients

IN THIS RETROSPECTIVE cohort study, 26 consecutive cirrhotic patients with PVT treated with danaparoid sodium were enrolled between December 2011 and April 2013. Clinical characteristics of cirrhotic patients with PVT before treatment are shown in Table 1. In brief, all patients had liver cirrhosis. The causes were viral liver cirrhosis in 19 patients (positive for hepatitis B surface antigen in five patients and positive for anti-hepatitis C virus antibody in 14 patients), heavy alcohol abuse in three patients and cryptogenic in four patients. Child–Pugh[26] grade A was noted in 13 patients, B in eight and C in five. Nine patients were complicated with hepatocellular carcinoma (HCC). The median diameter of HCC was 30 mm (range; 22–65). The median number of HCC was two (range, 1–10). No invasion to the bile duct, hepatic vein and portal vein

Table 1 Clinical characteristics of cirrhotic patients with PVT

Parameters	Patients ($n = 26$)
Age (range)†	71 (23–83)
Sex (male/female)	13/13
Etiology of (HBV/HCV/alcohol/cryptogenic)	5/14/3/4
Child–Pugh grade (A/B/C)	13/8/5
Total bilirubin (mg/dL)†	1.0 (0.5–5.5)
Aspartate aminotransferase (IU/L)†	35 (16–242)
Alanine aminotransferase (IU/L)†	27 (9–138)
Albumin (g/dL)†	3.3 (2.2–4.5)
Platelet count ($\times 10^4/\mu L$)†	9.7 (3.0–41.6)
Prothrombin time international normalized ratio†	1.20 (1.01–2.40)
Ammonia (μM)†	47 (21–226)
Activated partial thromboplastin time (s)†	30.3 (23.3–46.8)
Antithrombin III (%)†	65 (30–102)
Fibrinogen (mg/dL)†	211.4 (144.1–418.3)
Fibrinogen degradation products (μg/mL)†	7.1 (1.2–56.1)
D-dimer (μg/mL)†	4.7 (0.8–40.8)
Ascites (with/without)	11/15
Encephalopathy (with/without)	3/23
Esophageal varix (with/without)	23/3
Hepatocellular carcinoma (with/without)	9/17
Previous history of EIS (yes/no)	9/17
Previous history of Hassab's operation (yes/no)	3/23
Development of PV collaterals (with/without)	21/5
Greatest dimension of PV collaterals (mm)†	7 (3–26)
Hepatofugal blood flow (with/without)	19/7

†Data are median value (range).
EIS, endoscopic injection sclerotherapy; HBV, hepatitis B virus; HCV, hepatitis C virus; PV, portal vein; PVT, portal vein thrombosis.

was observed in these patients. Portal vein (PV) collaterals developed in 21 patients. The median of greatest dimension of PV collaterals was 7 mm (range, 3–26). Hepatofugal blood flow was observed in 19 patients.

The study was approved by the institutional review board of the participating clinical sites, the Ethical Committee for Epidemiology of Hiroshima University (Epi-969), and the study was conducted according to the tenets of the Declaration of Seoul, 2008. Written informed consent was obtained from all patients at the time of enrollment.

Protocol for the treatment of portal vein thrombosis

Cirrhotic patients with PVT were treated with a danaparoid sodium (Orgaran; MSD, Tokyo, Japan), 2500 units/day for 2 weeks by i.v. drip infusion. When antithrombin III activity decreased by less than 70%, an antithrombin III (Anthrobin P; CSL Behring, Tokyo, Japan; Japan Blood Products Organization, KENKETSU Nonthron, Tokyo Japan; Nihon Pharmaceutical, Tokyo, Japan), 1500 units/day, was added for 3 days i.v.

Esophageal and gastric varices were examined endoscopically. When varices with F2 or F3 and/or RC1 or RC2/3[27] were observed, these were treated by endoscopic injection sclerotherapy or endoscopic variceal ligation before anticoagulation therapy. The imaging studies, blood tests (hepatic reserve test, platelet count and coagulation/fibrinolytic system) and complications were carefully followed up before and after treatment.

Evaluation of PVT

All patients underwent contrast-enhanced computed tomography (CT) to evaluate the presence of PVT. With the use of the paintbrush tool of the Advantage Workstation (AW ver. 4.2; GE Healthcare, Tokyo, Japan), an image processing apparatus, we traced a shape around PVT in an axial view of contrast-enhanced CT. The volume of PVT was calculated with the AW. This was confirmed by a radiology technologist and physician.

By comparing the reduction rate before and after treatment, we evaluated the responses as follows: complete response (CR), partial response (PR), stable disease (SD), and progressive disease (PD). CR was defined as the complete disappearance of thrombus, PR as 50% or more reduction of thrombus, SD as less than 50% reduction of thrombus, and PD as enlargement of thrombus in comparison with pretreatment volume of thrombus in this study.

Statistical analysis

For categorical variables, χ^2-tests were performed. Factors influencing reduction ratio of thrombus was assessed by Mann–Whitney *U*-test. All analyses were carried out in SPSS for Windows version 16.0 (SPSS, Chicago, IL, USA). Results are expressed as medians and range. All *P*-values are two-tailed, and $P < 0.05$ was considered statistically significant.

RESULTS

Effects of danaparoid treatment on PVT

THE CHARACTERISTICS OF PVT are shown in Table 2. The median volume of PVT was 2.40 cm^3 (range, 0.18–16.63). The median period from the detection of PVT to the start of treatment was 40 days (range, 0–1800). PVT was localized in the portal branch ($n = 2$), main trunk of PV (MPV) to portal branch ($n = 4$), MPV ($n = 9$), MPV to superior mesenteric vein ($n = 9$) and splenic vein ($n = 2$).

All patients showed reduction of PVT through treatment. The median volume of PVT before and after treatment was significantly different at 2.40 cm^3 (range, 0.18–16.63) and 0.37 cm^3 (range, 0–5.74), respectively ($P < 0.001$) (Fig. 1). The median reduction rate of PVT was 77.3% (range, 18–100%). CR was obtained in four patients (15%), PR in 16 (62%), SD in six (23%) and PD in zero (0%). Representative cases of CR, PR and SD are shown in Figure 2. Figure 2(a) shows a CR case of PVT in the main branch of the PV. The volume of PVT was 2.41 and 0 cm^3 before and after treatment, respectively. Figure 2(b) shows a PR case of PVT in the main branch of the PV. The volume of PVT was 2.25 and 0.41 cm^3 before and after treatment, respectively. The

Table 2 Characteristics of portal vein thrombosis

Volume of PVT before treatment (cm^3)†	2.40 (0.18–16.63)
Period from the detection of PVT to the start of treatment (days)†	40 (0–1800)
Localization of PVT	**No. of patients**
Portal branch	2
MPV portal branch	4
MPV	9
MPV SMV	9
SV	2

†Data are median value (range).
MPV, main trunk of PV; PV, portal vein; PVT, portal vein thrombosis; SMV, superior mesenteric vein; SV, splenic vein.

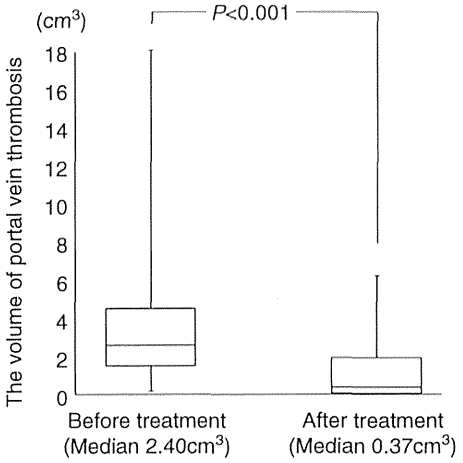

Figure 1 Volume of portal vein thrombosis before and after treatment was 2.40 cm^3 (0.18–16.63) and 0.37 cm^3 (0–5.74), respectively.

reduction rate was 82%. Figure 2(c) shows an SD case of PVT in the main branch of the PV. The volume of PVT was 7.48 and 4.96 cm^3 before and after treatment, respectively. The rate of reduction was 34%. During the 2 weeks of treatment, no major or minor bleeding event, thrombocytopenia, liver dysfunction and/or mortality were observed.

Comparison of clinical profiles between patients with CR + PR and SD

Clinical profiles were compared between the reduction rate of 50% or more (CR + PR) and that of less than 50% (SD) (Table 3). The volume of PVT before treatment ($P = 0.045$) and encephalopathy ($P = 0.009$) were significantly different. The PVT volume before treatment of CR + PR (median, 2.09 cm^3) was smaller than SD (median, 4.35 cm^3). While the period from the detection of PVT to the start of treatment was not different between CR + PR and SD; the median days of CR + PR and SD were 36 and 56 days, respectively.

Changes of hepatic reserve and fibrinolytic system

Hepatic reserve (albumin, total bilirubin, aspartate aminotransferase, alanine aminotransferase and prothrombin time international normalized ratio [PT-INR]) was not significantly different between before and after treatment. The values of D-dimer before and treatment were 4.7 μg/mL (range, 0.8–40.8) and 1.1 μg/mL (range, 0.3–13.7), respectively. The changes of D-dimer were significantly different between before and after treatment ($P = 0.001$) (Fig. 3a). The values of PT-INR before and after treatment were not significantly different ($P = 0.920$) (Fig. 3b).

DISCUSSION

IN THE PRESENT study, we assessed the efficacy and safety of danaparoid sodium for the treatment of PVT in patients with liver cirrhosis. As a result, all patients

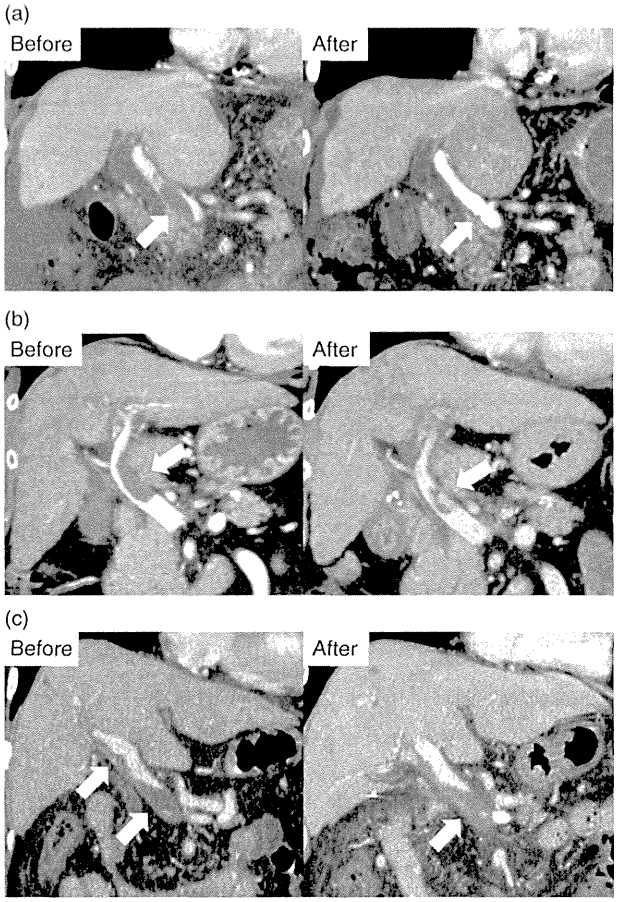

Figure 2 Representative cases of complete reduction (CR), partial reduction (PR) and stable disease (SD). (a) For a representative case of CR, the volume of portal vein thrombosis (PVT) in the main branch of the portal vein (PV) before and after treatment was 2.41 and 0 cm^3, respectively. The rate of reduction was 100%. (b) For a representative case of PR, the volume of PVT in the main branch of the PV before and after treatment was 2.25 and 0.41 cm^3, respectively. The rate of reduction was 82%. (c) For a representative case of SD, the volume of PVT in the main branch of the PV before and after treatment was 7.48 and 4.96 cm^3, respectively. The rate of reduction was 34%.

Table 3 Comparison of pretreatment clinical profiles between patients with CR + PR and SD

Factor	CR + PR ($n = 20$)	SD ($n = 6$)	P
Age (range)†	69 (23–79)	71 (47–83)	0.879
Sex (male/female)	9/11	4/2	0.361
Etiology (HBV, HCV/alcohol, NBNC)	14/6	4/2	0.879
Child-Pugh (A/B, C)	11/9	2/4	0.361
Total bilirubin (mg/dL)†	1.1 (0.6-2.6)	1.0 (0.5-5.5)	0.714
Aspartate aminotransferase (IU/L)†	37 (16–242)	36 (16–107)	0.670
Alanine aminotransferase (IU/L)†	26 (12–123)	29 (9–138)	0.903
Albumin (g/dL)†	3.4 (2.2–4.1)	3.4 (2.4-4.5)	0.669
Platelet count ($\times 10^4/\mu L$)†	9.5 (6.7–25.2)	9.8 (3.0–41.6)	0.605
Prothrombin time international normalized ratio†	1.19 (1.06-1.56)	1.20 (1.01–2.40)	0.738
Ammonia (μmol/L)†	45 (40–89)	48 (21–216)	0.648
Activated partial thromboplastin time (s)†	28 (27.2–35.4)	30.4 (23.3–46.8)	0.260
Antithrombin III (%)†	58 (31–84)	67 (30–102)	0.273
Fibrinogen (mg/dL)†	206.9 (144.2–244.5)	209.2 (144.1–418.3)	0.761
Fibrinogen degradation products (μg/mL)†	22.3 (3.3–41.3)	7.0 (1.2–56.1)	0.113
D-dimer (μg/mL)†	4.5 (3.6–9.4)	3.1 (0.8–40.8)	0.247
Ascites (with/without)	8/12	1/5	0.302
Encephalopathy (with/without)	1/19	3/3	0.009
Esophageal varix (with/without)	17/3	6/0	0.323
Hepatocellular carcinoma (with/without)	8/12	3/3	0.670
Previous history of EIS (yes/no)	8/12	1/5	0.302
Previous history of Hassab's operation (yes/no)	2/18	1/5	0.660
Development of PV collaterals (with/without)	16/4	5/1	0.859
Greatest dimension of PV collaterals (mm)†	7 (3–26)	7 (7–13)	0.151
Hepatofugal blood flow (with/without)	14/6	5/1	0.527
Volume of PVT before treatment (cm^3)†	2.09 (0.18–16.63)	4.35 (1.66–7.48)	0.045
Period to start of treatment (days)†	36 (0–1800)	56 (12–1099)	0.330

†Data are median value (range).
A value of $P < 0.05$ was considered statistically significant.
CR, complete response; EIS, endoscopic injection sclerotherapy; HBV, hepatitis B virus; HCV, hepatitis C virus; NBNC, non-B, non-C; PR, partial response; SD, stable disease.

showed reduction of PVT with 2 weeks' treatment of danaparoid sodium. The reduction rate of PVT volume was 77.3%. According to the different reduction rate, CR was observed in 15% of patients, PR in 62% and SD in 23%. The median volume of PVT before treatment was the determinant for the reduction rate. No severe adverse events were observed such as bleeding symptoms (e.g. gastrointestinal bleeding and cerebral hemorrhage), thrombocytopenia and liver dysfunction.

There are various pharmacotherapies for anticoagulation of venous thrombosis such as heparin, UFH, LMWH and warfarin.[21–23] The response rates for these treatments were reported to range 5–92%.[28,29] Warfarin inhibits coagulation factor II, VII, IX and X. Heparin, UFH, LMWH and danaparoid sodium inhibits coagulation factor Xa and IIa. Danaparoid sodium, a heparinoid glycosaminoglycuronan antithrombotic agent, is an LMWH consisting of a mixture of heparin sulfate (84%), dermatan sulfate (12%) and small amounts of chondroitin sulfate (4%). It specifically potentiates the inhibition of coagulation factor Xa, with a ratio of anti-factor Xa activity to anti-thrombin (factor IIa) activity greater than 22:1.[24–25] Therefore, danaparoid has higher selectivity with a ratio of anti-factor Xa activity than heparin, UFH and LMWH. Danaparoid is less effective on clotting assays such as prothrombin time (PT), partial thromboplastin time (PTT) and bleeding time. This was also confirmed in this study. Considering the high risk of bleeding due to reduced synthesis of coagulation factors and presence of varices in cirrhotic patients, danaparoid with less bleeding may be a suitable treatment in these patients complicated with PVT.

A newly developed antithrombotic agent, fondaparinux sodium (Arixtra; GlaxoSmithKline, Tokyo,

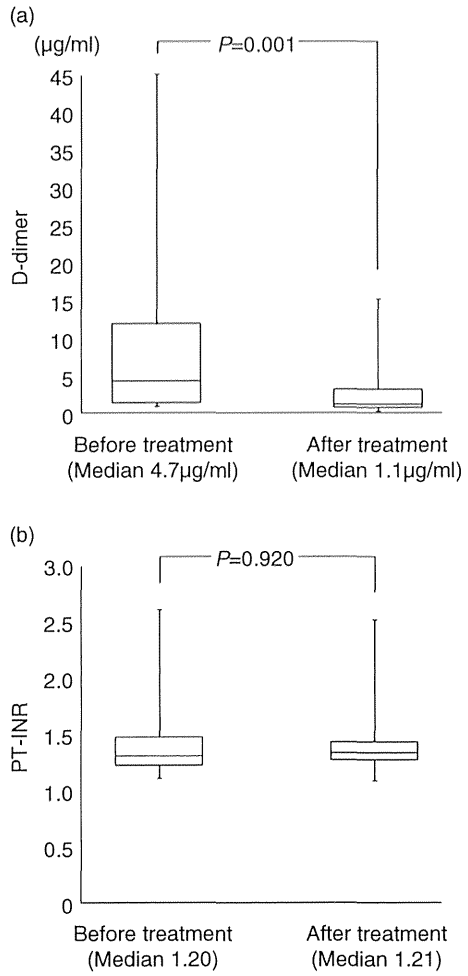

Figure 3 Changes of D-dimer and prothrombin time international normalized ratio (PT-INR) before and after treatment. (a) The value of D-dimer before and after treatment was 4.7 μg/mL (range, 0.8–40.8) and 1.1 μg/mL (range, 0.3–13.7), respectively. (b) The value of PT-INR before and after treatment was 1.20 (range, 1.01–2.40) and 1.21 (range, 0.98–1.87), respectively.

Japan) which is used for the suppression of deep venous thrombosis after surgery, is known to have a considerably stronger effect on inhibition of Xa than danaparoid. Fondaparinux sodium has anti-factor Xa activity to antithrombin activity greater than 7400:1.[30] This agent also may be effective for patients such as ours and awaits further analyses such as what kind of drug selection is suitable.

By comparing the response between CR + PR and SD, significant differences were found in the volume of PVT before treatment ($P = 0.045$) and encephalopathy ($P = 0.009$) (Table 3). The volume of thrombus before treatment was significantly larger in SD and this resulted in the lower efficacy. The existence of encephalopathy resulted in the lower efficacy probably due to the reduced hepatic reserve and blood flow. Other factors might have been the period from the diagnosis to the start of treatment. An earlier diagnosis of PVT would be expected to result in a smaller thrombosis and early intervention would therefore contribute to the higher efficacy of the treatment. For the prediction of the effects, objective evaluation of the volume of PVT on CT would be recommended as shown in our study.

A limitation of our study was that only 2 weeks was allowed to show the efficacy and indication of this treatment. Because the recurrence after disappearance of PVT is another problem in cirrhotic patients with PVT, subsequent maintenance therapy is certainly necessary. Prospective, large and long-scale study is needed for the evaluation of PVT and complications after anticoagulation therapy with danaparoid in the future. The short observation period of this study limits the interpretation of results. However, we believe that this study would be useful and the long-term evaluation including the necessities of subsequent maintenance therapy is expected in future study.

In conclusion, danaparoid sodium therapy for the treatment of PVT in patients with liver cirrhosis was safe and effective. An early diagnosis of PVT along with the evaluation of the volume of PVT on CT and an early intervention would contribute to the higher efficacy of the treatment. Anticoagulation therapy using the anticoagulant, danaparoid sodium, could be a potential treatment option for PVT with cirrhosis.

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The effects of bisphosphonate zoledronic acid in hepatocellular carcinoma, depending on mevalonate pathway.

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Abstract

Background and Aim: Zoledronic acid (ZOL) is a nitrogen-containing bisphosphonate and used to reduce cancer-induced osteolysis. We reported previously that ZOL delayed both the growth and pain progression of bone metastases from hepatocellular carcinoma (HCC). The present study was designed to evaluate the effects of ZOL on hepatoma cell lines and the molecular mechanisms of such effects.

Methods: Cell viability assay, scratch assay, immunohistochemistry, western blotting, and flow cytometry analysis were performed using Huh7 and HepG2 cells treated with and without ZOL.

Results: ZOL reduced cell growth in a dose-dependent manner, and prevented cell migration when used at concentration exceeding 10 μM. Immunohistochemistry showed that the inhibitory effects of ZOL on hepatoma cell progression was not due to the suppression of Ras and RhoA expression but due to inhibition of their translocation from the cytosol to the cell membrane, which terminates mevalonate pathway. Immunoblotting and flow cytometry showed that ZOL inhibited the MAPK pathway and induced apoptosis of hepatoma cells.

Conclusions: Our results indicated that ZOL prevented cell growth and metastasis based on direct antitumor effects in hepatoma cells. The use of ZOL could not only suppress the progression to bone metastatic lesions but also prevented growth of primary HCC.

Key words: apoptosis, zoledronic acid, Ras and RhoA, bisphosphonate

Introduction

Hepatocellular carcinoma (HCC) is one of the most common cancers and is a leading cause of cancer death worldwide.[1-3] Hepatitis C (HCV) and B viruses (HBV) cause chronic infection, and 300 million and 170 million people suffer from chronic hepatitis or liver cirrhosis, respectively. Among these, more than 700,000 people die annually from HCC worldwide. Therefore, the development of new diagnostic and therapeutic modalities is desirable. Recently, the prognosis of such patients and those with HCC has improved.[4-6] However, this improvement has eventually increased the occurrence of extra-hepatic metastases from HCC. This is because, among the patients who survive HCC with treatment, living cancer cells also survive in the peripheral circulation, and thus have a larger chance to form distal metastasis. At present, bone metastasis is the second or third most frequent metastasis from HCC and 5.2 to 10.2% of HCC patients present with bone metastasis at diagnosis.[7-9] Unfortunately, bone metastasis in such patients is associated with severe pain.

Bisphosphonates (BPs) are inhibitors of bone-resorption. Among BPs, zoledronic acid (ZOL) is currently the strongest nitrogen-containing bisphosphonate and is used for cancer-induced osteolysis to prevent skeletal complications associated with bone metastases. We reported previously that ZOL delays both the appearance and worsening of pain associated with bone metastases in patients with HCC.[10] Recent experimental *in vitro* studies have also indicated that ZOL acts directly on cancer cells, such as breast cancer cells, which metastasize easily to bones.[11-16] ZOL inhibits farnesyl pyrophosphate synthase, a key enzyme in the mevalonate pathway.[17,18]