Our theory may also give an account of the non-metastatic and multicentric de novo occurrence characteristics of HCC, which would be the result of persistent HCV infection.

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Short Communication

RNA-dependent RNA polymerase of hepatitis C virus binds to its coding region RNA stem–loop structure, 5BSL3.2, and its negative strand

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The hepatitis C virus NS5B RNA-dependent RNA polymerase (RdRp) is a key enzyme involved in viral replication. Interaction between NS5B RdRp and the viral RNA sequence is likely to be an important step in viral RNA replication. The C-terminal half of the NS5B-coding sequence, which contains the important *cis*-acting replication element, has been identified as an NS5B-binding sequence. In the present study, we confirm the specific binding of NS5B to one of the RNA stem–loop structures in the region, 5BSL3.2. In addition, we show that NS5B binds to the complementary strand of 5BSL3.2 (5BSL3.2N). The bulge structure of 5BSL3.2N was shown to be indispensable for tight binding to NS5B. *In vitro* RdRp activity was inhibited by 5BSL3.2N, indicating the importance of the RNA element in the polymerization by RdRp. These results suggest the involvement of the RNA stem–loop structure of the negative strand in the replication process.

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The hepatitis C virus (HCV) is a positive-strand RNA virus belonging to the family *Flaviviridae* (Miller & Purcell, 1990). HCV NS5B RNA-dependent RNA polymerase (RdRp) is known to play a pivotal role in the viral replication process (Behrens *et al.*, 1996). Although HCV replication is regulated by host cellular factors, the initial replication complex formation requires an interaction between NS5B and viral RNA (Hamamoto *et al.*, 2005; Tu *et al.*, 1999; Wang *et al.*, 2005; Watashi *et al.*, 2005). Interestingly, many of the RNA molecules appear to have the potential to be substrates of NS5B RdRp in an *in vitro* RdRp assay system (Behrens *et al.*, 1996; De Francesco *et al.*, 1996; Ferrari *et al.*, 1999). However, NS5B appears to exhibit a binding preference for certain select RNA molecules (Biroccio *et al.*, 2002; Kanamori *et al.*, 2009; Lohmann *et al.*, 1997; Vo *et al.*, 2003). Because of the high error rate of the viral RdRp (Holland *et al.*, 1982), variability in the viral sequence is observed not only between the different genotypes, but also within the same genotype or subgenotype (Simmonds *et al.*, 1993). Among the HCV genome sequence variants, the well-conserved RNA sequences are located at the 5′-end (Bukh *et al.*, 1992; Smith *et al.*, 1995), 3′-end (Kolykhalov *et al.*, 1996; Tanaka *et al.*, 1995; Yamada *et al.*, 1996) and within a portion of the NS5B-coding region (Walewski *et al.*, 2001). The RNA elements that interact with NS5B have been located mainly in these conserved sequence areas. NS5B was shown to bind to a highly conserved 98 nt 3′-terminal segment, designated 3′-X, as well as to its upstream poly U/UC tract in the 3′-non-coding region (NCR) (Cheng *et al.*, 1999; Oh *et al.*, 2000). Recent studies have revealed that the C-terminal half of the NS5B-coding RNA exhibits tighter binding to NS5B (Kim *et al.*, 2002; Lee *et al.*, 2004). This region contains certain conserved RNA stem–loop structures (Walewski *et al.*, 2001; You *et al.*, 2004). Among these, the 5BSL3 stem–loop structures were candidates for the NS5B-binding site (Lee *et al.*, 2004), of which 5BSL3.2 was shown to contain the *cis*-acting replication element (Friebe *et al.*, 2005; Lee *et al.*, 2004; You *et al.*, 2004). We and others have demonstrated the binding of NS5B to 5BSL3.2 (Kanamori *et al.*, 2009; Zhang *et al.*, 2005), although the binding specificity of NS5B to 5BSL3.2 remains to be determined.

Following the synthesis of the negative-strand viral RNA, the positive-strand viral RNA is synthesized using the replication intermediate as a template. A part of the 3′-end structure of the negative-strand HCV RNA was shown to bind to NS5B (Astier-Gin *et al.*, 2005; Oh *et al.*, 1999) while the corresponding positive-strand (5′NCR) RNA appeared not to bind to NS5B (Lee *et al.*, 2004). The interaction of NS5B with the 3′-end negative-strand RNA should be key for the initiation of the positive-strand RNA synthesis (Astier-Gin *et al.*, 2005), but there have been only a few studies on NS5B binding to other RNA regions on the

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negative strand. In the present study, we show that NS5B binds not only to the 5BSL3.2 RNA but also to its complementary strand, suggesting the importance of the negative-strand viral RNA complementary to the *cis*-acting replication element in the formation of the viral replication complex.

Among the HCV genome variants, NS5B SL3 is a well-conserved region that is key for viral replication because of the presence of a *cis*-acting replication element in this region (Friebe *et al.*, 2005; You *et al.*, 2004). In addition, NS5B appears to bind to RNA elements in this area (Lee *et al.*, 2004). Thus, we employed RNA gel mobility shift analysis using three of the RNA stem–loop structures (5BSL3.1, 5BSL3.2 and 5BSL3.3; Fig. 1a) to estimate which structure contributes most to the recruitment of the viral polymerase (Fig. 1c). A C-terminal 21 aa-truncated glutathione *S*-transferase (GST)-NS5B (strain BK, genotype 1b) fusion protein was produced and purified by using *Escherichia coli* BL21 as described previously (Kanamori *et al.*, 2009). Synthetic RNA oligonucleotides were ^{32}P-labelled at the 5′-end by using T4 polynucleotide kinase and [γ-^{32}P]ATP (PerkinElmer). The ^{32}P-labelled RNA oligonucleotides (5 nM, final concentration) were incubated with NS5B protein (100 nM) in a total of 10 µl binding buffer [8 mM HEPES, pH 7.9, 40 mM NaCl, 5 mM $MgCl_2$, 2 mM EDTA, 0.2 mM dithiothreitol, 0.2 mM PMSF and 1.6 % glycerol (v/v)] containing 50 µg tRNA ml^{-1} for 15 min at 22 °C, then loaded onto 4 % polyacrylamide gels (80 : 1 acrylamide-bisacrylamide electrophoresis in 0.25 × Tris borate/EDTA buffer) and run at 300 V at 4 °C. The 5BSL3.2 RNA exhibited substantial binding to NS5B, while the other stem–loop structures (5BSL3.1 and 5BSL3.3) did not exhibit binding. The binding specificity of 5BSL3.2 RNA to NS5B was confirmed by a cold competition experiment (Fig. 2a). Competition

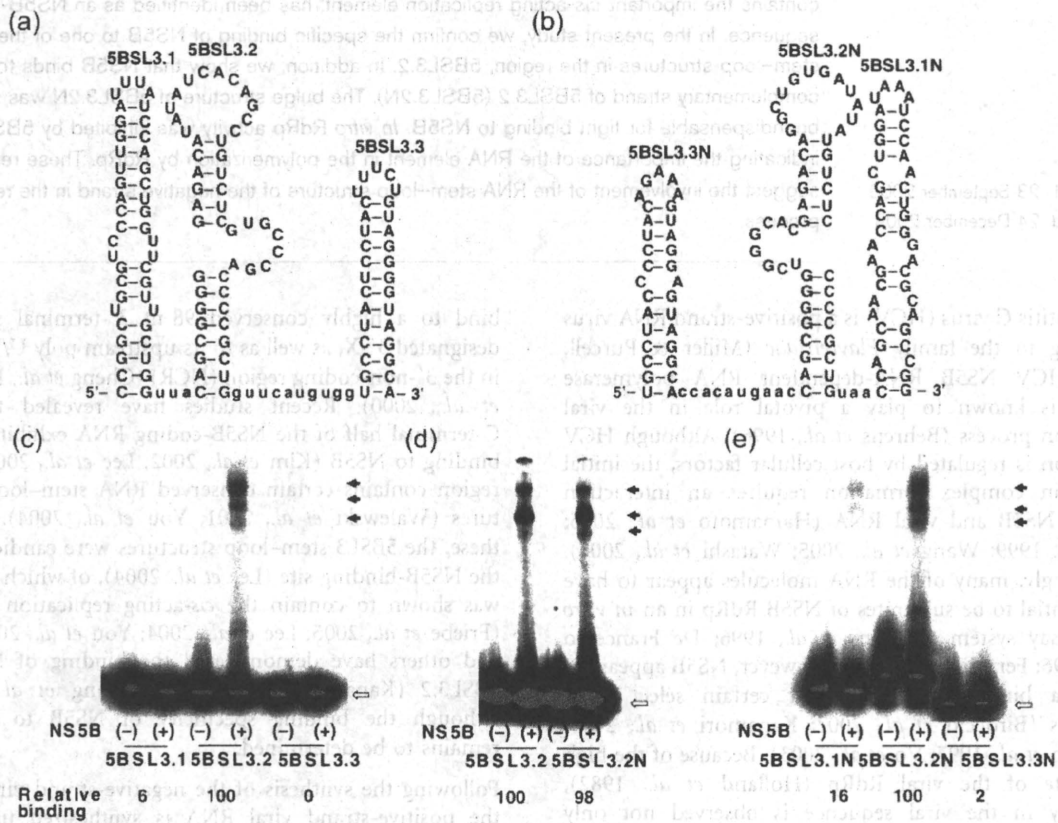

Fig. 1. Evaluation of the RNA–NS5B binding by RNA gel electrophoretic mobility shift assays. The predicted secondary structure of the HCV NS5B-coding region RNA is shown. The probes used in the experiments are shown in upper case. (a) The sequence of the positive-strand 5BSL3.1 (44 nt), 5BSL3.2 (48 nt) and 5BSL3.3 (31 nt) is from the Con1 clone, and the predicted secondary structure was described by Friebe *et al.* (2005). (b) The RNA secondary structure of the negative-strand RNA was predicted by using Zuker's Mfold program and is shown (Zuker, 2003). RNA gel mobility shift analysis using (c) 5BSL3.1, 5BSL3.2 and 5BSL3.3, (d) 5BSL3.2 and 5BSL3.2N, (e) 5BSL3.1N, 5BSL3.2N and 5BSL3.3N. Open arrows indicate RNA probes. Solid arrows indicate the positions of the RNA–protein complexes. The relative binding was calculated and is shown at the bottom of the gels.

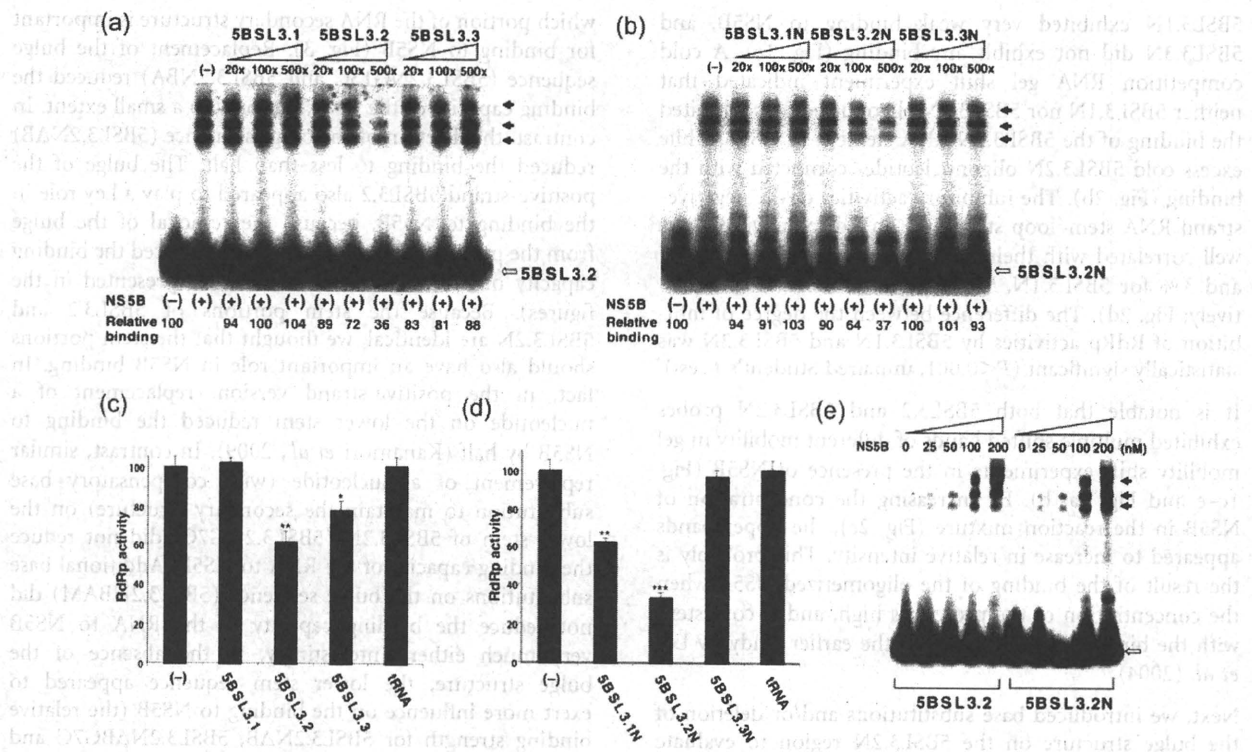

Fig. 2. Evaluation of the RNA–NS5B binding by RNA gel electrophoretic mobility shift competition assays and *in vitro* RdRp assays. (a) RNA gel mobility shift analysis by using 5BSL3.2 as the probe. Cold competitor oligonucleotides (20-, 100- or 500-fold excess of 5BSL3.1, 5BSL3.2 or 5BSL3.3) were added to the reaction mixture and analysed. (b) RNA gel mobility shift analysis by using 5BSL3.2N as the probe, the cold competitor oligonucleotides were added (a 20-, 100- or 500-fold excess of 5BSL3.1N, 5BSL3.2N or 5BSL3.3N) and analysed. The relative RdRp activity in the presence of each RNA stem–loop structure [(c) 50 nM of 5BSL3.1, 5BSL3.2, 5BSL3.3 or tRNA, (d) 5BSL3.1N, 5BSL3.2N, 5BSL3.3N or tRNA] is shown by a bar graph. Experiments were performed in triplicate and the standard deviations are shown in the figure. ***, $P<0.001$; **, $P<0.01$; *, $P<0.05$ (unpaired Student's *t*-test) compared with (−). (e) Either the 5BSL3.2 or 5BSL3.2N RNA probe was incubated with different concentrations of NS5B (0–200 nM, final concentration) and analysed by RNA gel mobility shift analysis.

experiments were performed by adding excess unlabelled oligonucleotides to the binding reaction (a 20–500-fold excess) 5 min prior to adding the probe. Binding was competed by excess cold 5BSL3.2, but not by excess cold 5BSL3.1 or 5BSL3.3 oligonucleotides.

Although it is not possible to estimate the effect of all cellular factors, the *in vitro* RdRp system is a versatile assay to evaluate the ability of inhibitory factors, including oligonucleotides against RdRp activity. We used a primer-dependent RdRp assay system to evaluate the inhibitory effects of the RNA stem–loop structures on the RdRp activity of NS5B (Fig. 2c). RdRp activity was measured by using the poly(C)-oligo(G) system, as described previously (Uchiyama *et al.*, 2002). The 5BSL3.2 RNA efficiently inhibited the RdRp activity (39 %), while 5BSL3.1 did not exert any influence on the RdRp activity. The 5BSL3.3 RNA inhibited the RdRp activity, but to a lesser extent (22 %). The difference in the degree of inhibition of RdRp activities by 5BSL3.2 and 5BSL3.3 was statistically significant ($P<0.05$, unpaired Student's *t*-test). This suggests that the 5BSL3.3 stem–loop structure may inhibit RdRp activity via a different mechanism than 5BSL3.2, which binds to NS5B.

The predicted RNA secondary structure of the negative strand RNA corresponding to 5BSL3 is shown on Fig. 1(b). Each RNA element (5BSL3.1N, 5BSL3.2N and 5BSL3.3N) appears to form a mirror image structure of the positive-strand RNA. It is notable that 5BSL3.2 and its negative strand (5BSL3.2N) share the identical 6 bp upper- and 8 bp lower-stem sequences. Both the 5BSL3.2 positive- and negative-strand stem–loop structures contain 12-base terminal loops and 8-base bulges, the nucleotide sequences of which are unique to each strand. Because of the similarity between the secondary structures of 5BSL3.2 and its negative strand, we thought that 5BSL3.2N might bind to NS5B. In fact, 5BSL3.2N bound to NS5B with a binding strength similar to 5BSL3.2 (Fig. 1d). In contrast,

5BSL3.1N exhibited very weak binding to NS5B, and 5BSL3.3N did not exhibit any binding (Fig. 1e). A cold competition RNA gel shift experiment indicated that neither 5BSL3.1N nor 5BSL3.3N oligonucleotides inhibited the binding of the 5BSL3.2N RNA element to NS5B, while excess cold 5BSL3.2N oligonucleotides competed with the binding (Fig. 2b). The inhibitory activities of the negative-strand RNA stem–loop structures on RdRp activities were well correlated with their binding ability to NS5B (38, 67 and 3 % for 5BSL3.1N, 5BSL3.2N and 5BSL3.3N, respectively; Fig. 2d). The difference between the degree of inhibition of RdRp activities by 5BSL3.1N and 5BSL3.2N was statistically significant ($P<0.001$, unpaired Student's *t*-test).

It is notable that both 5BSL3.2 and 5BSL3.2N probes exhibited multiple shifted bands of different mobility in gel mobility shift experiments in the presence of NS5B (Fig. 1c–e and Fig. 2a, b). By increasing the concentration of NS5B in the reaction mixture (Fig. 2e), the upper bands appeared to increase in relative intensity. This probably is the result of the binding of the oligomerized NS5B when the concentration of the protein is high, and is consistent with the binding data obtained in the earlier study by Lee *et al.* (2004).

Next, we introduced base substitutions and/or deletion of the bulge structure on the 5BSL3.2N region to evaluate which portion of the RNA secondary structure is important for binding to NS5B (Fig. 3). Replacement of the bulge sequence (5BSL3.2NG13C and 5BSL3.2NBA) reduced the binding capacity of the RNA, but only to a small extent. In contrast, the deletion of the bulge sequence (5BSL3.2NΔB) reduced the binding to less than half. The bulge of the positive-strand 5BSL3.2 also appeared to play a key role in the binding to NS5B, because the removal of the bulge from the positive-strand 5BSL3.2 RNA reduced the binding capacity of the RNA to 23 % (data not presented in the figures). Because the stem portions of 5BSL3.2 and 5BSL3.2N are identical, we thought that the stem portions should also have an important role in NS5B binding. In fact, in the positive-strand version, replacement of a nucleotide on the lower stem reduced the binding to NS5B by half (Kanamori *et al.*, 2009). In contrast, similar replacement of a nucleotide (with compensatory base substitution to maintain the secondary structure) on the lower stem of 5BSL3.2N (5BSL3.2NG7C) did not reduce the binding capacity of the RNA to NS5B. Additional base substitutions on the bulge sequence (5BSL3.2NBAM) did not reduce the binding capacity of the RNA to NS5B very much either. Interestingly, in the absence of the bulge structure, the lower stem sequence appeared to exert more influence on the binding to NS5B (the relative binding strength for 5BSL3.2NΔB, 5BSL3.2NΔBG7C and

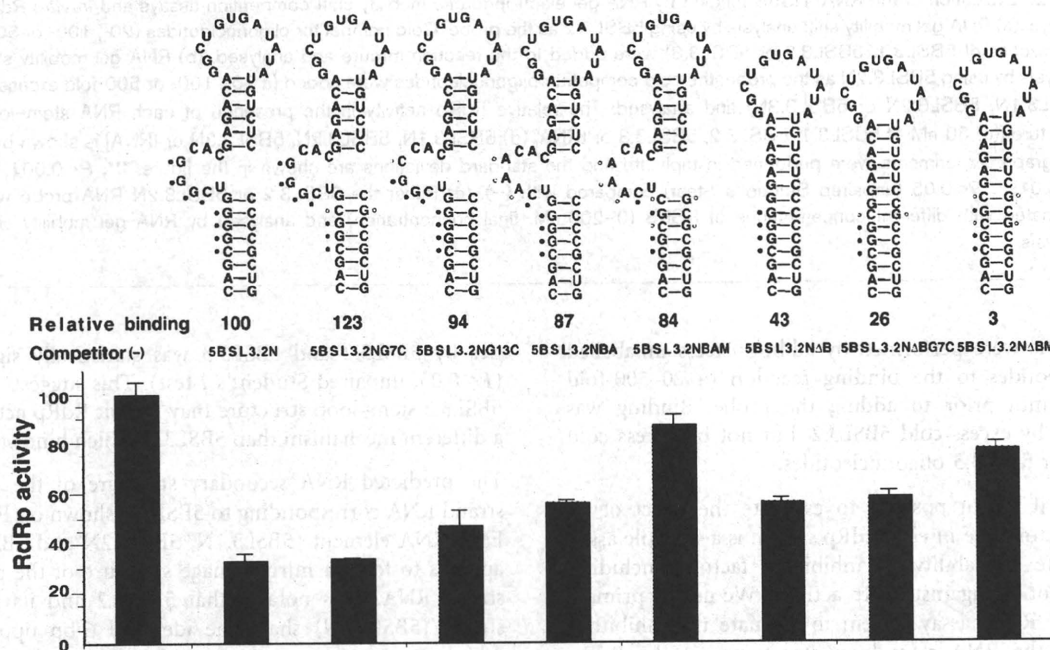

Fig. 3. Secondary structures and binding strengths of mutated and/or deleted RNA clones. The predicted secondary structures of the tested RNA oligonucleotides by Mfold analysis (Zuker, 2003) are shown. The filled circles near each nucleotide indicate the CGGG motifs. Open circles indicate substituted nucleotides. The relative binding capacity of each RNA oligonucleotide to NS5B was determined by RNA gel mobility shift analysis, and is shown at the bottom of each RNA secondary structure. RdRp activities in the presence of RNA stem–loop structures are shown by a bar graph in the bottom portion. The RdRp activity in the absence of the competitor RNA was set to 100.

5BSL3.2NΔBM, which were 43, 26 and 3 % of that for 5BSL3.2N, respectively). Inhibition of the RdRp activity was less in the mutants with a higher number of base substitutions in a series of mutants with a bulge (5BSL3.2N, 5BSL3.2NG7C, 5BSL3.2NG13C, 5BSL3.2NBA and 5BSL3.2NBAM; 0, 1, 1, 3 and 5 nt substitutions, plus compensatory base substitutions, respectively). In a series of mutants without the bulge, RdRp inhibition was also less, with a greater number of base substitutions in the GC-rich motif on the stem (5BSL3.2NΔB, 5BSL3.2NΔBG7C and 5BSL3.2NΔBM; 0, 2 and 4 nt substitutions, respectively). These results indicate the importance of the bulge structure, as well as the lower stem sequence, for the tight binding of the 5BSL3.2N RNA stem–loop structures to NS5B.

HCV 5BSL3.2 is one of several RNA stem–loop structures in the NS5B-coding RNA, and is considered to be a highly important *cis*-acting replication element (Friebe *et al.*, 2005; You *et al.*, 2004). The hairpin–loop sequence on 5BSL3.2 and the sequence on the loop of the 3′-X (3′SL2) have the potential to form a pseudoknot, which is regarded as essential for viral replication. In addition, a more recent study by Diviney *et al.* (2008) provided evidence that the long range RNA interaction between 5BSL3.2 and its approximately 200 base upstream CGGG motif is also important for viral replication.

Combined with the results from the analysis of the aptamers against NS5B in our previous study, we thought it likely the CGGG motif, which is found on the lower stem of 5BSL3.2, would play an important role in binding to NS5B (Kanamori *et al.*, 2009). The CGGG motif is also present on the lower stem of the negative-strand 5BSL3.2N stem–loop structure. Furthermore, an additional CGGG sequence appears on the bulge portion. Removal of the bulge structure reduced the binding of 5BSL3.2N. In the cases of RNA structures without the bulge (5BSL3.2NΔBG7C and 5BSL3.2NΔBM), the base substitutions on the CGGG motif reduced the binding, as was reportedly observed in the case of the positive-strand version, 5BSL3.2 (Kanamori *et al.*, 2009).

At the initiation of positive-strand RNA synthesis, NS5B is likely to bind to the 3′-end structures of the negative-strand viral RNA (Astier-Gin *et al.*, 2005). In addition, it is possible that NS5B binds to RNA elements such as 5BSL3.2N on the negative-strand RNA before the synthesis of the positive-strand RNA starts, and this stabilizes the replication complex for more efficient positive-strand viral RNA synthesis. Because 5BSL3.2N appears to form the RNA secondary structure mirror image of the positive-strand 5BSL3.2, 5BSL3.2N may also interact with the distant negative-strand RNA motifs to facilitate viral RNA synthesis.

In the present study, it has been shown that NS5B specifically binds to 5BSL3.2 and its negative-strand structure. NS5B binding to the negative-strand 5BSL3.2N RNA may also be a key step in viral RNA replication.

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Plasma concentration of bioactive lipid mediator sphingosine 1-phosphate is reduced in patients with chronic hepatitis C

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ABSTRACT

Background: Bioactive lipid mediator S1P has been suggested to play pathophysiological roles in various fields of clinical science as a circulating paracrine mediator. We previously established a reliable method of measuring plasma S1P concentration, and reported that the one in healthy subjects has a gender difference and a correlation with red blood cell (RBC)-parameters, however, the reports of S1P measurements in the blood in patients with a specific disease have been scarce. Because our previous evidence suggests that S1P is involved in liver pathophysiology, we examined plasma S1P concentration in chronic hepatitis C patients.

Methods: S1P assay was performed using a high-performance liquid chromatography system.

Results: Plasma S1P concentrations were reduced in chronic hepatitis C patients compared with in healthy subjects with the same hemoglobin concentration, irrespective of gender. Among the blood parameters, serum hyaluronic acid concentration, a surrogate marker for liver fibrosis, was most closely and inversely correlated with plasma S1P concentration. Furthermore, plasma S1P concentration decreased throughout the progression of carbon tetrachloride-induced liver fibrosis in rats.

Conclusions: Plasma S1P concentration was reduced in chronic hepatitis C patients, and liver fibrosis might be involved, at least in part, in the mechanism responsible for this reduction.

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1. Introduction

Sphingolipids are now known as modulators of membrane signal transduction systems, being involved in diverse cellular processes. Sphingosine 1-phosphate (S1P), one of these sphingolipids, first emerged as an intracellular mediator affecting cellular proliferation and survival [1,2]. S1P subsequently gained even more attention after it was shown to act as an extracellular mediator with a wide variety of cellular responses, including proliferation, survival, migration and contraction [3–5]. Indeed, recent investigations have revealed that S1P acts through at least five high-affinity G protein-coupled receptors referred to as $S1P_{1-5}$ [6–10]. Furthermore, several lines of evidence have demonstrated various phenotypes of S1P receptor mutants [11,12]. So far, S1P has been shown to be involved in the regulation of important physiological functions of the vascular system, such as vascular morphogenesis and maturation, cardiac function, vascular permeability, and tumor angiogenesis [13–18]. Furthermore, S1P interaction with $S1P_1$ has been shown to be essential for lymphocyte egress from the secondary lymphoid tissues to the lymph [19–21], and $S1P_1$ downregulation by FTY720 is now attracting much attention as an effective way of immunosuppression in experimental models of transplantation and autoimmunity, causing a marked decrease in the number of circulating lymphocytes in the peripheral blood [22]. Very recently, S1P and $S1P_1$ have been further shown to play a role in bone homeostasis [23]. Thus, these accumulating findings strongly suggest that S1P has normal *in vivo* roles as well as potentially pathophysiological roles as a circulating paracrine mediator.

In order to know these roles of S1P, the S1P concentration in the blood *in vivo* and its regulatory mechanism should be known. It was previously reported that S1P is present in human plasma at a readily detectable concentration [24], and of note, this concentration of S1P is comparable to the concentration of S1P causing various responses in cells *in vitro* [25]. We then recently established a reliable method of measuring the S1P concentration in the blood and reported the plasma S1P concentrations in the healthy subjects [26], however, the reports of S1P measurements in the blood in patients with a specific disease have been scarce.

Abbreviations: LPA, lysophosphatidic acid; RBC, red blood cell; S1P, sphingosine 1-phosphate.

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We have been focusing our attention on the potential role of S1P in liver pathophysiology. We first demonstrated that S1P stimulates proliferation and contractility via $S1P_2$ in rat hepatic stellate cells [27,28]. We also revealed that S1P inhibits proliferation in rat hepatocytes with Rho activation via $S1P_2$ and that the administration of S1P in 70% hepatectomized rats reduces the synthesis of DNA in hepatocytes [29]. We then examined the phenotypes of $S1P_2$ mutants and observed accelerated hepatocyte regeneration and reduced liver fibrosis in those mice [30]. Serriere-Lanneau et al. also reported a reduced wound healing response to liver injury in $S1P_2$-deficient mice [31]. With these findings, we wondered if the S1P concentration in the blood could be modulated in liver injury. In this study, we examined the plasma S1P concentration in patients with chronic hepatitis C, one of the most common liver diseases worldwide.

2. Patients and methods

2.1. Patients

We enrolled 15 patients with chronic hepatitis C who were seen in the Department of Gastroenterology, the University of Tokyo Hospital, Tokyo, Japan. The patients with chronic hepatitis C were diagnosed based on serum positivity for anti-hepatitis C virus antibodies and detectable hepatitis C virus RNA. The exclusion criteria were co-infection with hepatitis B virus and other causes of liver disease. The study was carried out in accordance with the Declaration of Helsinki (2000) of the World Medical Association, and was approved by the Institutional Research Ethics Committee of the Faculty of Medicine of the University of Tokyo. Informed consent from the patients was obtained for the use of samples in this study.

2.2. Plasma sample preparation

Blood samples were collected with ethylenediamine tetraacetic acid dipotassium salt $2H_2O$ and citrate-theophylline-adenosine-dipyridamole (BD Biosciences, Tokyo, Japan), and centrifuged at 2500×g at 4 °C for 30 min to obtain the plasma [26].

2.3. S1P assay

The S1P assay was performed using a high-performance liquid chromatography system as previously described [26]. The value of within-day assay CV for the plasma S1P concentrations was 2.4% ($n = 10$), while between-day assay CV was 7.6% ($n = 5$).

2.4. Animals

Male Sprague–Dawley rats (230–250 g; Shizuoka Laboratory Animal Center, Shizuoka, Japan) were fed a standard pelleted diet and water *ad libitum* and were used in all the experiments. All animals received humane care with the anesthetic procedures used in full and the precautions to ensure that they did not suffer unduly during and after the experimental procedure in compliance with the Institutional Guidelines of the University of Tokyo.

2.5. Carbon tetrachloride (CCl_4) treatment

To induce liver fibrosis, rats were treated with 20% CCl_4 in olive oil at 0.15 ml/kg intraperitoneally twice a week and with phenobarbital (0.4 g/l) in drinking water for 4 and 8 weeks [32]. Control rats received injections of the carrier (olive oil) alone and phenobarbital in drinking water.

2.6. Histological analysis

Excised livers were analyzed by hematoxylin–eosin and Masson's trichrome staining [33]. The analysis of fibrosis development was carried out with the Nikon Digital Camera DXM1200 (NIKON, Japan) using the public domain Scion Image developed by the Scion Corporation. The extent of liver fibrosis was quantitated by calculating the area of fibrosis/area of section.

2.7. Statistical analysis

To compare the means between groups, a Student *t*-test was performed. The relationships between variables were analyzed using the Spearman's correlation coefficient by rank, and a partial correlation coefficient was calculated to remove the influence of confounding variables. Differences were considered significant at a $p < 0.05$.

3. Results

The biological characteristics of patients with chronic hepatitis C are summarized in Table 1. The plasma S1P concentration in these patients with chronic hepatitis C was 280.3 ± 29.6 nmol/l, which was significantly lower than the previously reported plasma S1P concentration in healthy subjects, 386.8 ± 55.5 nmol/l ($p < 0.0001$) [26]. Because the plasma S1P concentration in healthy subjects was significantly higher among men than among women [26], we also analyzed the plasma S1P concentrations in male and female patients with chronic hepatitis C. The plasma S1P concentrations were 280.1 ± 31.4 nmol/l in men and 280.7 ± 27.8 nmol/l in women with chronic hepatitis C, both of which were significantly lower than that in gender-matched healthy subjects ($p < 0.001$), as shown in Fig. 1. These results indicate that the plasma S1P concentration was significantly reduced in patients with chronic hepatitis C, compared with in healthy subjects, irrespective of gender.

A close correlation between the plasma S1P concentration and red blood cell (RBC)-parameters has recently been revealed [26]. Thus, it was possible that the reduced plasma S1P concentration in patients with chronic hepatitis C could be caused by a reduction in RBCs; however, the hemoglobin concentration, indicating the amount of RBCs, in chronic hepatitis C patients was 13.5 ± 1.7 g/dl as shown in Table 1, which is not significantly different from that in healthy subjects in the previous report (14.1 ± 1.9 g/dl) [26]. In this study, there was no difference in the hemoglobin concentration between male (13.8 ± 1.8 g/dl) and female (13.0 ± 1.4 g/dl) patients with chronic hepatitis C.

To elucidate the mechanism responsible for the reduced plasma S1P concentrations in patients with chronic hepatitis C, we analyzed the relation between the plasma S1P concentration and various blood

Table 1
Baseline characteristics of the patients.

Variables	$n = 15$
Male/female	9/6
Age (yr)[a]	68 ± 9 (55–92)
Hemoglobin (g/dl)[a]	13.5 ± 1.7 (10.7–17.2)
Platelet (×10⁴/ml)[a]	13.3 ± 4.9 (6.3–22)
Albumin (g/dl)[a]	3.7 ± 0.5 (2.9–4.2)
Aspartate aminotransferase (IU/l)[a]	43 ± 19 (25–89)
Alanine aminotransferase (IU/l)[a]	38 ± 17 (20–75)
γ-Glutamyltransferase (IU/l)[a]	36 ± 31 (11–136)
Total bilirubin (mg/dl)[a]	1.0 ± 0.6 (0.3–2.6)
Total cholesterol (mg/dl)[a]	170 ± 42 (116–248)
Prothrombin time (%)[a]	80.4 ± 16.4 (55.8–100)
Hyaluronic acid (ng/ml)[a]	355 ± 316 (43–1040)

[a] Expressed as mean ± SD (range).

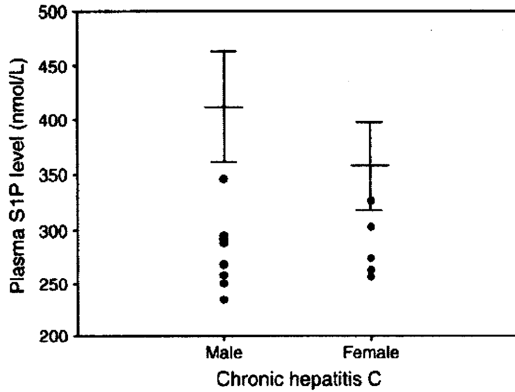

Fig. 1. Plasma S1P concentrations in male and female patients with chronic hepatitis C. The Plasma S1P concentration was measured in 9 male and 6 female patients with chronic hepatitis C. Horizontal bars indicate the means ± SD of healthy male and female subjects. There was a significant difference between male patients with chronic hepatitis C and healthy male subjects ($p<0.0001$) and that between female patients with chronic hepatitis C and healthy female subjects ($p<0.001$).

parameters. As demonstrated in Table 2, the strongest inverse correlation was found between the plasma S1P concentration and the serum hyaluronic acid concentration, followed by the correlation between the serum albumin concentration and the hemoglobin concentration. Fig. 2 depicts the relation between the plasma S1P concentration and the serum hyaluronic acid concentration (A), the serum albumin concentration (B), or the hemoglobin concentration (C).

Table 2
Correlation between plasma S1P level and various blood parameters.

Variables	Spearman's rank correlation coefficient	*p* value
Hemoglobin (g/dl)	0.514	0.0498
Platelet ($\times 10^4$/ml)	0.286	NS
Albumin (g/dl)	0.521	0.0466
Aspartate aminotransferase (IU/l)	−0.104	NS
Alanine aminotransferase (IU/l)	0.173	NS
γ-Glutamyltransferase (IU/l)	−0.054	NS
Total bilirubin (mg/dl)	−0.052	NS
Total cholesterol (mg/dl)	−0.034	NS
Prothrombin time (%)	0.125	NS
Hyaluronic acid (ng/ml)	−0.611	0.0156

In contrast, no significant correlation was found between the plasma S1P concentration and the platelet count (Fig. 2D), serum aspartate aminotransferase concentration, serum alanine aminotransferase concentration, serum γ-glutamyltransferase concentration, serum total bilirubin concentration or prothrombin time. Because the plasma S1P concentration has been shown to strongly correlate with red blood cell (RBC)-parameters in healthy subjects [26], a partial correlation coefficient between the concentrations of plasma S1P and serum hyarulonic acid was calculated to remove the influence of the hemoglobin concentration as a confounding factor in patients with chronic hepatitis C. As a result, the plasma S1P concentration was still significantly and inversely correlated with the serum hyarulonic acid concentration (partial correlation coefficient = −0.636, $p=0.014$).

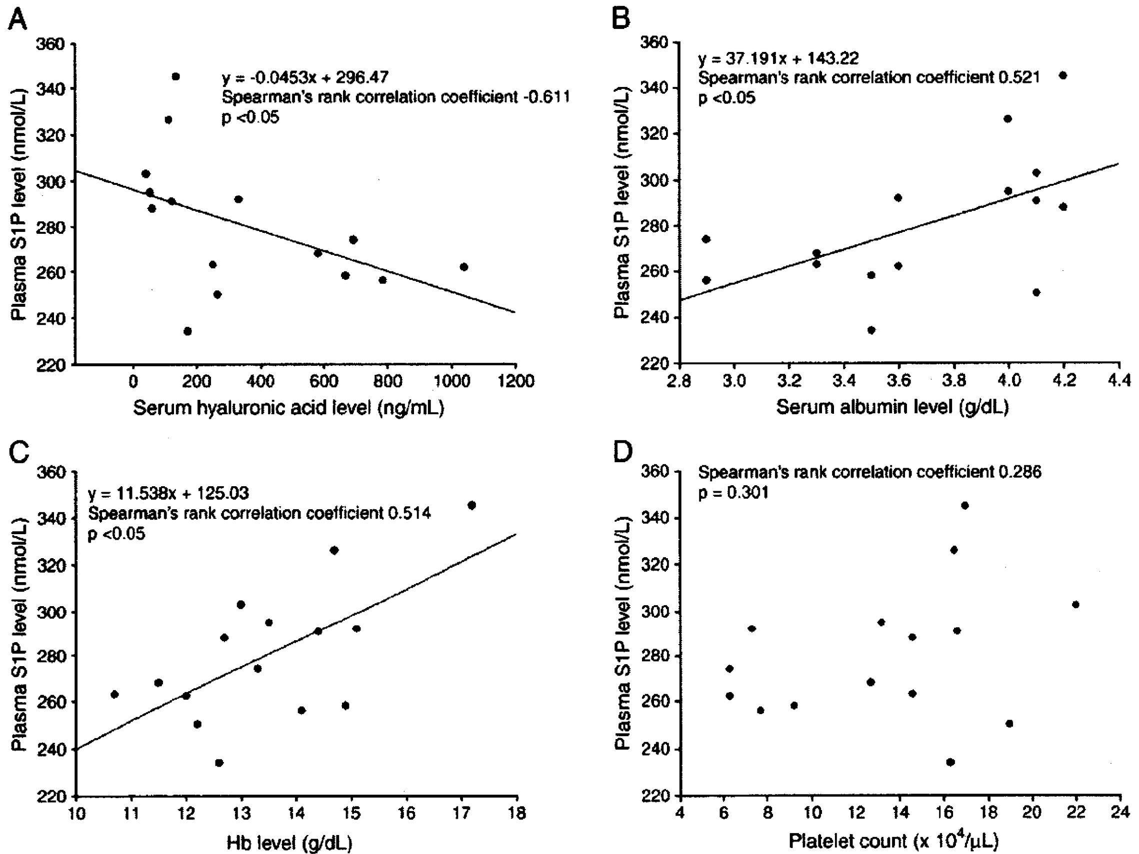

Fig. 2. Relation between plasma S1P concentration and serum hyaluronic acid concentration (A), serum albumin concentration (B), hemoglobin concentration (C) or platelet count (D).

Among the blood parameters that were significantly correlated with the plasma S1P concentration, hyaluronic acid is known to be a surrogate marker for liver fibrosis [34]. Thus, we examined whether the plasma S1P concentration was reduced during liver fibrosis by using a rat model treated with CCl_4. As previously described [35], the severity of liver fibrosis increases according to the duration of CCl_4 treatment from 4 to 8 weeks, in which the significant difference of liver fibrosis was found between no CCl_4 treatment and CCl_4 treatment for 8 weeks ($p<0.05$) but not between no CCl_4 treatment and CCl_4 treatment for 4 weeks (Fig. 3A). In these rats, the plasma S1P concentration was not altered by CCl_4 treatment for 4 weeks but was significantly reduced by CCl_4 treatment for 8 weeks ($p<0.05$) (Fig. 3B).

4. Discussion

As described in the Introduction, the reports of S1P measurements in the blood have been scarce; the serum S1P concentration is reportedly an indicator of obstructive coronary artery disease in human [36]. Furthermore, the serum S1P concentration has been also shown to be enhanced in rats with CCl_4- and bile duct ligation-induced liver fibrosis [37], which is different from the current evidence. However, the recent study has clearly indicated that the S1P concentration measured in the serum samples is largely attributable to the release from platelets during the sample preparation, indicating that S1P in the blood should be measured in plasma but not in serum in order to investigate its clinical or pathological significance [26]. Thus, the current study constitutes the first evidence of the clinical significance of S1P measurements in the blood in patients with a specific disease.

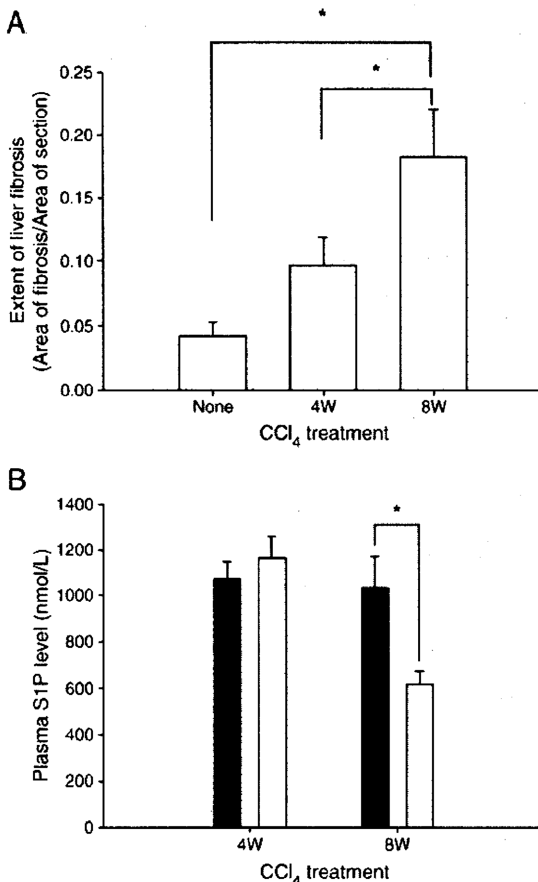

Fig. 3. Extent of liver fibrosis (A) and plasma S1P concentration (B) in rats treated with CCl_4 for 4 and 8 weeks. The extent of liver fibrosis (A) and the plasma S1P concentration (B) were measured in rats treated with CCl_4 for 4 and 8 weeks ($n=5$). The columns and bars represent the means ± SEM. (A) There was a significant difference between rats treated with CCl_4 for 8 weeks and untreated control rats ($n=8$) ($p<0.01$) or between rats treated with CCl_4 for 8 weeks and those for 4 weeks ($p<0.05$). (B) There was a significant difference between untreated control (black column) and CCl_4-treated rats for 8 weeks (white column).

A close inverse correlation between the plasma S1P concentration and the serum hyaluronic acid concentration in chronic hepatitis C patients suggests that the plasma S1P concentration decreases during the progression of liver fibrosis, which was confirmed in rats with liver fibrosis due to CCl_4-treatment. Because the serum albumin concentration is known to decline during the process of liver fibrosis in chronic hepatitis C, the significant correlation between the plasma S1P concentration and the serum albumin concentration found in this study may also be related to the progression of liver fibrosis. Collectively, the reduction in the plasma S1P concentration in chronic hepatitis C patients may be caused by, at least in part, liver fibrosis.

Regarding the regulatory mechanism of plasma S1P concentration, RBCs reportedly store and release S1P in the blood [38,39]. Pappu et al. reported that plasma S1P is mainly hematopoietic in origin, with RBCs being the major contributor, in a study using conditional knockouts of sphingosine kinases [40]. In line with this, the plasma S1P concentration was correlated with RBC-related parameters in healthy subjects and a gender difference in the plasma S1P concentration in healthy subjects may be explained by a difference of RBC-related parameters between male and female subjects: a significant lower concentration of hemoglobin in female healthy subjects than in male healthy subjects [26]. Furthermore, platelets are known to store S1P abundantly and to release S1P into the plasma upon activation [24], suggesting that platelets may be another source of plasma S1P. S1P in those cells is known to be produced by phosphorylation of sphingosine with sphingosine kinase [24,40]. In addition, there might be more unknown source(s) of plasma S1P. On the other hand, it is speculated that plasma S1P may be cleared by dephosphorylation by lipid phosphate phosphohydrolases [41,42].

In line with these previous findings, the plasma S1P concentration was correlated with RBC-related parameters including hemoglobin concentration also in chronic hepatitis C patients in the current study. However, there was no gender difference in the plasma S1P concentration. This may be explained by the finding that there was no difference in hemoglobin concentration between male and female chronic hepatitis C patients in this study.

Although the platelet count and prothrombin time, expressed as a percentage of the value in a control group, are also known to be reduced during liver fibrosis in chronic hepatitis C, neither of these parameters were correlated with the plasma S1P concentration in the current study. Regarding the platelets, they are often activated in chronic liver diseases [43]. Thus, platelets may be activated more in patients with a lower platelet count i.e. in those with more advanced chronic liver diseases, which could lead to the increase in the S1P release from activated platelets. This may be one possible explanation why a significant correlation was not found between the plasma S1P concentration and the platelet count.

Of note, the plasma S1P concentration in chronic hepatitis C patients was significantly lower than that in healthy subjects with the same hemoglobin concentration, suggesting that unknown mechanism in the regulation of plasma S1P may be at work in these patients. This unknown mechanism should involve the reduced production and/or the enhanced clearance of plasma S1P. We previously reported that the plasma concentration of lysophosphatidic acid (LPA), another multi-potential lipid mediator, is increased in chronic hepatitis C patients [44], which is strikingly different from the trend for S1P. Importantly, both S1P and LPA in the plasma are assumed to be dephosphorylated by the same enzyme of a

family of lipid phosphate phosphohydrolases [41,42], suggesting that the mechanism of inactivation and clearance from the plasma may be the same for S1P and LPA. Accordingly, the distinct alteration in the plasma concentrations of S1P and LPA in chronic hepatitis C patients is likely caused by the change in their accumulation in the plasma but not by the alteration in their inactivation and clearance from the plasma. In fact, the increase in the plasma LPA concentration in liver fibrosis was attributable to the enhanced activity of its synthetic enzyme, lysophospholipase D, in the blood [44]. Thus, the reduced plasma S1P concentration in chronic hepatitis C patients may be caused by the reduced accumulation of S1P in the plasma but not by the enhanced clearance of S1P from the plasma. When considering this, it is possible that the release from RBCs may be reduced. Of interest is the fact that the lipid content of the membranes of RBCs is altered in liver fibrosis [45], which may affect the S1P release from those cells. Another possibility is that the release from unknown source(s) of plasma S1P might be reduced. This issue should be further elucidated.

Our previous evidence suggests the involvement of S1P and especially $S1P_2$ in the pathogenesis of liver fibrosis [27–30], raising the possibility that a reduction in the plasma S1P concentration might negatively contribute to the development of liver fibrosis. Whether a reduction in the plasma S1P concentration in liver fibrosis might be a simple result of the disease or might possibly contribute to the pathogenesis should be further evaluated. It is also of interest to determine whether the plasma S1P concentration would be useful as a novel marker of liver fibrosis. To clarify this, the plasma S1P concentration should be measured in a larger number of patients with liver fibrosis caused by various agents. To do so, an easier method other than a high-performance liquid chromatography system is better to be employed.

In conclusion, the plasma S1P concentration was significantly reduced in patients with chronic hepatitis C, and liver fibrosis might possibly be involved, at least in part, in the mechanism responsible for this reduction. We suggest that the liver may be one of the key regulators of plasma S1P concentration.

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Original Article

Effect of nucleoside analog-interferon sequential therapy on patients with acute exacerbation of chronic hepatitis B

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Aim: Nucleoside analog (NA)-interferon (IFN) sequential therapy may enable the long-term control of chronic hepatitis B (CHB) and the withdrawal of the nucleoside analog. We evaluated the efficacy of NA-IFN sequential therapy for acute exacerbation of CHB.

Methods: A total of 12 patients with acute exacerbation of CHB, nine of whom were positive for hepatitis B e antigen (HBeAg), were enrolled in this study. All the patients were treated with lamivudine 100 mg/day alone for 20 weeks, then with both IFN-α 6 megaunits three times per week and lamivudine for 4 weeks, and lastly, with IFN-α alone for 20 weeks. Patients whose serum alanine aminotransferase (ALT) level was normalized, whose serum hepatitis B virus (HBV) DNA level decreased to less than 5 log copies/mL, and HBeAg level was absent 24 weeks after the end of treatment were defined as having sustained virological response (SVR). The other patients were defined as having no response (NR).

Results: Four out of nine (44.4%) HBeAg-positive and all three HBeAg-negative patients achieved SVR. The levels of serum alanine aminotransferase (ALT), HBV DNA and HBV core-related antigen were similar between SVR and NR patients at baseline. Three of four patients (75.0%) whose serum HBeAg became negative at the end of treatment achieved SVR, while one of five (20.0%) whose serum HBeAg remained positive achieved SVR.

Conclusion: NA-IFN sequential therapy for patients with acute exacerbation of CHB enables the withdrawal of treatment and is particularly effective for patients whose serum HBeAg has become undetectable by the end of the IFN treatment.

Key words: chronic hepatitis B, lamivudine, interferon, sequential therapy.

INTRODUCTION

CHRONIC INFECTION WITH hepatitis B virus (HBV) is a major global health problem, affecting more than 400 million people worldwide.[1] Approximately 15–40% of infected patients develop cirrhosis, liver failure or hepatocellular carcinoma (HCC).[2] An appropriate antiviral treatment to prevent advanced liver disease and reduce the number of HBV-related deaths is thus crucial.[3,4]

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Among the few currently approved agents for the treatment of chronic HBV infection, the most commonly used are interferon (IFN)-α and nucleoside analogs (NA), such as lamivudine, adefovir dipivoxil and entecavir.[5,6]

Interferon-α exerts an antiviral effect by degrading viral mRNA and proteins.[7] Additionally, IFN-α upregulates the immunological response to HBV by enhancing human leukocyte antigen class I expression on hepatocytes.[8] Long-term remission of hepatitis after treatment completion may be expected because the immunological effect of IFN-α continues even after discontinuing the treatment. However, infrequent sustained virological response,[9] several adverse effects[10] and high cost are the problems associated with IFN-α treatment.

Nucleoside analogs cause a rapid and strong antiviral effect.[11-13] Their adverse effects are generally mild.[14]

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However, treatment withdrawal is difficult because post-treatment flare with viral proliferation often occurs following the withdrawal.[15] The other problem associated with NA is drug resistance. Prolonged treatment generates a drug-resistant HBV mutant, which often causes breakthrough hepatitis.[16–18] Thus, treatment options that enable long-term remission are necessary.

Nucleotide analog-interferon combination therapy for chronic hepatitis B (CHB) may be more effective than IFN monotherapy because synergistic or additional antiviral effects may be expected. To date, various studies on the combination of IFN and lamivudine have been reported.[19–32]

Sequential therapy switching from lamivudine to IFN may also be effective for the treatment of acute exacerbation of CHB. NA withdrawal is difficult in this situation. IFN treatment overlapping with NA treatment may enable NA withdrawal without generating drug-resistant HBV.[24,25] This treatment is expected to be highly effective because the remission of hepatitis with hepatitis B e (HBe) seroconversion sometimes occurs following the exacerbation of HBV,[33,34] but this needs to be confirmed. We conducted this study to elucidate the efficacy of lamivudine-IFN sequential therapy for those who experienced acute exacerbation of CHB.

METHODS

Patients

A TOTAL OF 12 patients over 20 years old with acute exacerbation of CHB between August 2003 and August 2007, who started a daily dose of 100 mg of lamivudine at St Marianna University School of Medicine, were enrolled in this retrospective cohort study. The diagnosis of acute exacerbation of CHB was made as follows: (i) patients were already diagnosed as having CHB; (ii) patients were positive for hepatitis B surface antigen (HBsAg) and HBV DNA; (iii) other causes of liver damage were excluded; and (iv) serum alanine aminotransferase (ALT) levels in all the patients increased above 300 IU/L within 4 weeks before treatment. As for the serum ALT levels, acute exacerbation of CHB is usually defined as "elevation of ALT over 10 times as upper normal limit".[35] In Japan, 30 IU/L is now regarded as upper normal in many institutions. Therefore, we defined an ALT level above 300 IU/L as exacerbated. The exclusion criteria were as follows: (i) presence of serum antibodies against hepatitis C virus or HIV; (ii) development of liver cirrhosis and/or HCC; (iii) coexistence of other acquired or inherited liver diseases; (iv) coexisting of serious medical or psychiatric illnesses; (v) history of antiviral or immunosuppressive therapy within the preceding 6 months; and (vi) pregnancy.

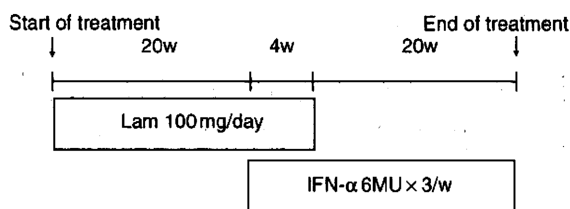

Figure 1 Treatment protocol. All the patients were treated with a daily dose of 100 mg of lamivudine for an initial 20 weeks. Subsequently, 6 megaunits of IFN-α were administrated thrice weekly in combination with lamivudine for 4 weeks, followed by IFN-α monotherapy for an additional 20 weeks. IFN, interferon; Lam, lamivudine; MU, megaunits.

Written informed consent was obtained from all the patients. Our study was approved by the institutional ethics review board of St Marianna University School of Medicine Hospital (no. 1163).

Protocol

All the patients were treated with a daily dose of 100 mg of lamivudine for an initial 20 weeks. Subsequently, 6 megaunits of natural IFN-α (Sumiferon; Dainippon Sumitomo Pharma, Osaka, Japan) were administrated thrice weekly in combination with lamivudine for 4 weeks, followed by IFN-α monotherapy for an additional 20 weeks (Fig. 1). As for the definition of therapeutic effect, antiviral treatment is recommended for the patients whose serum HBV DNA level exceeds 5 log copies/mL because HBV DNA over this level usually accompanies ALT elevation, especially in hepatitis B e-antigen (HBeAg)-positive patients. In addition, one clinical study from Japan adopted this level as a definition of sustained virological response (SVR).[36] Therefore, patients whose serum ALT level was normalized, whose serum HBV DNA level decreased to less than 5 log copies/mL, and whose HBeAg was seroconverted to anti-HBe (in HBeAg-positive cases) 24 weeks after the end of treatment were defined as having SVR. The other patients were defined as having no response (NR).

HBV-related markers

Serum blood samples were frozen at –80 °C until use. The HBsAg level was determined using a commercially available chemiluminescence enzyme immunoassay kit (LUMIPULSE II HBsAg; Fujirebio, Tokyo, Japan). The

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levels of immunoglobulin (Ig)M anti-hepatitis B core (HBc), HBeAg, and anti-HBe (HBe antibody; HBeAb) were determined using other commercial chemiluminescence immunoassay kits (LUMIPULSE II IgM-HBcAb, LUMIPULSE II HBeAg, LUMIPULSE II HBeAb; Fujirebio). The serum HBV DNA levels were determined using a commercial transcription-mediated amplification kit or commercial polymerase chain reaction kits (DNA probe FR-HBV from Fujirebio; Amplicor HBV monitor or TaqMan PCR from Roche Diagnostics, Tokyo, Japan). The lowest detection limits of those assays were 3.7, 2.6 and 1.8 log copies/mL, respectively. The HBV genotype was determined using a commercial enzyme-linked immunosorbent assay kit (SMITEST HBV genotype detection kit; Genome Science Laboratories, Fukushima, Japan). The serum HBV core-related antigen (HBcrAg) levels were determined using a commercial chemiluminescence enzyme immunoassay (LUMIPULSE HBcrAg; Fujirebio).[37,38] The lowest detection limit of this assay was 3.0 log U/mL. As for the detection of the HBV lamivudine-resistant gene, gene mutation in the YMDD motif was analyzed using a commercial polymerase chain reaction enzyme-linked minisequence assay kit (SMITEST HBV YMDD motif mutation detection kit; Medical & Biological Laboratories, Nagoya, Japan).[39] HBV-related markers (HBeAg, anti-HBe, HBcrAg and HBV DNA) and biochemical tests were examined just before treatment, every 4 weeks during the treatment, and every 4 weeks thereafter for 24 weeks after the end of the treatment. The normal serum ALT level was defined as less than 30 IU/L.

Histological evaluation

Nine of the 12 patients underwent liver biopsies within 1 month before the start of the treatment. Two experienced liver pathologists who had no clinical information except for knowledge of the HBV infection histopathologically evaluated the specimens. The histological appearance of the liver specimens was evaluated using the METAVIR histological score.[40]

Statistical analyses

Quantitative variables were expressed as mean ± standard deviation. The collected data were analyzed using SPSS ver. 15.0J. The distribution of continuous variables was analyzed using Mann–Whitney *U*-test. Differences in categorical data were determined using Fisher's exact test. A two-tailed *P*-value of less than 0.05 was considered to indicate statistical significance.

RESULTS

Baseline characteristics of patients treated with lamivudine and IFN-α

THE BASELINE CHARACTERISTICS of the enrolled patients are shown in Table 1. The mean age of the patients was 32.0 ± 7.8 years, and the patients were 10 men and two women. Of the 12 patients, nine were positive for HBeAg. All the patients were infected with HBV genotype C. The mean platelet count and prothrombin time (activity) were 19.3 ± 4.0 × 10^4/μL and 81.0 ± 13.3%, respectively. The mean serum ALT, total bilirubin and albumin levels were 850.8 ± 664.3 IU/L, 1.2 ± 0.5 mg/dL and 3.8 ± 0.3 g/dL, respectively. The mean levels of serum HBV DNA and HBcrAg were 7.4 ± 1.2 log copies/mL and 6.7 ± 0.4 log U/mL, respectively. Histopathologically, seven patients were diagnosed as having activity stage 2 inflammation and two patients were diagnosed as having activity stage 3 inflammation. As for fibrosis, three patients were diagnosed as having stage 2 fibrosis and six patients were diagnosed as having stage 3 fibrosis.

Response to antiviral treatment

Seven of the 12 (58.3%) patients achieved SVR. The SVR rates among the HBeAg-positive and -negative groups

Table 1 Baseline characteristics of the patients treated with lamivudine-interferon sequential therapy

	All cases
Number of patients	12
Age	32.0 ± 7.8
Sex (male/female)	10/2
Platelet (×10^4/μL)	19.3 ± 4.0
Prothrombin time (%)	81.0 ± 13.3
Albumin (g/dL)	3.8 ± 0.3
Total bilirubin (mg/dL)	1.2 ± 0.5
ALT (IU/L)	850.8 ± 664.3
HBV DNA (log copies/mL)	7.4 ± 1.2
HBcrAg (log U/mL)	6.7 ± 0.4
HBeAg positive/negative	9/3
Activity stage 0/1/2/3 (n = 9)	0/0/7/2
Fibrosis stage 0/1/2/3/4 (n = 9)	0/0/3/6/0

Data are shown as mean ± standard deviation.
Fibrosis and activity stage are evaluated on a scale from 0–4 and 0–3.
ALT, alanine aminotransferase; HBcrAg, hepatitis B virus core-related antigen; HBeAg, hepatitis B virus e antigen; HBV DNA, hepatitis B virus DNA.

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Table 2 Comparison of the baseline clinical characteristics between SVR and NR

	SVR	NR	*P*-value
Number of patients	7	5	0.6843
Age	29.7 ± 7.0	35.0 ± 9.2	0.2821
Sex (male/female)	5/2	0/5	0.4697
Platelet ($\times 10^4/\mu L$)	20.1 ± 2.3	18.4 ± 5.7	0.1775
Prothrombin time (%)	75.6 ± 14.1	87.6 ± 9.8	0.1775
Albumin (g/dL)	3.7 ± 0.2	4.0 ± 0.3	0.0519
Total bilirubin (mg/dL)	1.4 ± 0.5	0.9 ± 0.4	0.1190
ALT (IU/L)	931.6 ± 724.9	737.8 ± 570.8	0.8075
HBV DNA (log copies/mL)	7.4 ± 1.4	7.3 ± 1.1	1.0000
HBcrAg (log U/mL)	6.7 ± 0.5	6.8 ± 0.4	0.6905
HBeAg positive/negative	4/3	5/0	0.2045
Activity stage 0/1/2/3 (n = 9)	0/0/3/2	0/0/4/0	0.4444
Fibrosis stage 0/1/2/3/4 (n = 9)	0/0/2/3/0	0/0/1/3/0	1.0000

Data are shown as mean ± standard deviation.
ALT, alanine aminotransferase; HBcrAg, hepatitis B virus core-related antigen; HBeAg, hepatitis B virus e antigen; HBV DNA, hepatitis B virus DNA; NR, no response; SVR, sustained virological response.

were 44.4% (4/9) and 100% (3/3), respectively. On the other hand, all the NR patients were positive for HBeAg.

Comparison of baseline clinical features between SVR and NR patients

As shown in Table 2, there was no difference in the age, platelet count, prothrombin time, ALT level, albumin level and bilirubin level between SVR and NR patients.

As for the serum HBV DNA and HBcrAg levels, there was little difference between SVR and NR patients (HBV DNA 7.4 ± 1.4 vs 7.3 ± 1.1 log copies/mL; HBcrAg 6.7 ± 0.5 vs 6.8 ± 0.4 log U/mL). HBeAg was detected more often in NR than in SVR patients (100% [5/5] vs 57.1% [4/7]).

Histopathological examination showed that the patients with activity grade 3 are more likely to achieve SVR than those with grade 2 (100% [2/2] in grade 3 and 43.0% [3/7] in grade 2). With regard to the fibrosis stage, no significant difference in SVR was observed between patients with stage 2 and stage 3 (66.7% [2/3] for stage 2 and 50.0% [3/6] for stage 3) (Table 2).

Comparison of SVR rate according to age group

In the Japanese national guidelines, IFN is not recommended as a first line of treatment for patients over 35 years old because it is ineffective. On the basis of the guideline, we evaluated SVR rates according to age group. The SVR rate for all the patients aged under 35 years tended to be higher (66.7% [6/9] of patients achieved SVR) than that for the patients aged 35 years or over (33.3% [1/3]), although the difference was not statistically significant.

Time-dependent change in serum ALT, HBV DNA and HBcrAg levels

The serum ALT levels in all the patients normalized shortly after the treatment and remained within the normal range during the treatment (Fig. 2a). The serum HBV DNA levels decreased below the detection limit shortly after the treatment in most patients. The fluctuation of HBV DNA levels was observed in one SVR and two NR patients (Fig. 2b). As for the serum HBcrAg levels, a continuous decrease during and after the treatment was observed in SVR patients. In contrast, the HBcrAg levels in NR patients did not show a continuous decrease during or after the treatment (Fig. 2c).

HBV-related markers before, during and after treatment

In the HBeAg-positive patients, HBeAg was seroconverted to anti-HBe by the end of the treatment in 75.0% (3/4) of SVR and 40.0% (2/5) of NR patients. One SVR patient achieved HBe seroconversion 24 weeks after treatment. Therefore, HBe seroconversion was observed in all the patients who achieved SVR. On the other hand, one of two NR patients, who once achieved HBe seroconversion during treatment, became HBeAg-positive during IFN-α monotherapy and the other patient became HBeAg-positive after the end of the treatment.

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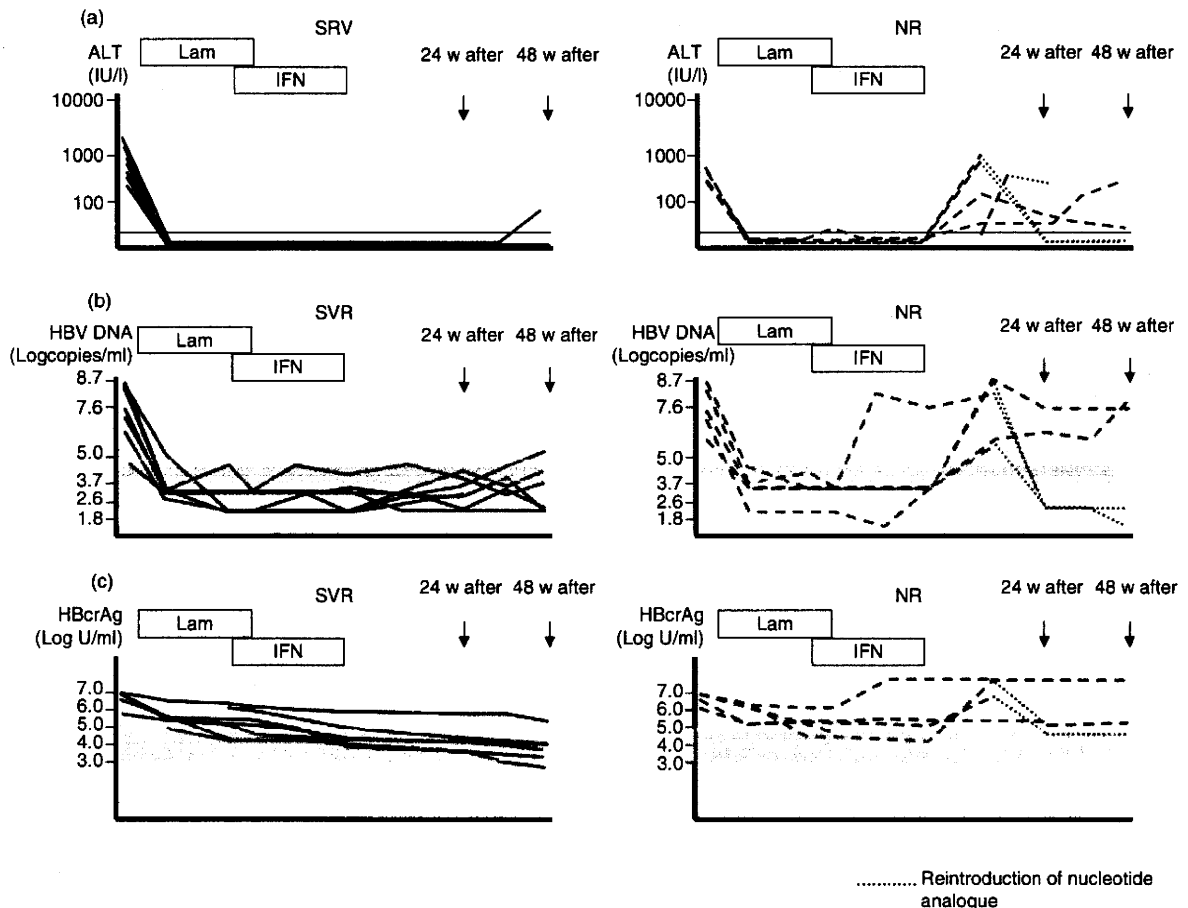

Figure 2 Temporal change in the level of serum ALT and viral markers. The serum ALT levels in all the patients normalized quickly after the start of treatment and remained within the normal range during treatment (a). The serum HBV DNA levels decreased below the detection limit shortly after treatment in most patients. Fluctuation of HBV DNA levels was observed in one SVR and two NR patients (b). The HBcrAg levels decreased continuously during and after treatment in SVR patients. The HBcrAg levels did not decrease continuously in NR patients (c). ALT, alanine aminotransferase; HBV DNA, hepatitis B virus DNA; HBcrAg, HBV core-related antigen; IFN, interferon; Lam, lamivudine; NR, no response; SVR, sustained virological response.

However, all the patients who showed NR remained HBeAg-positive 24 weeks after treatment.

The SVR rate for patients who achieved HBe seroconversion at the end of the treatment was 75.0% (3/4) whereas that for the patients who remained HBeAg-positive was 20.0% (1/5). Hence, HBe seroconversion at the end of the treatment may be predictive of the treatment response. In contrast, the HBV DNA and HBcrAg levels before or at the end of the treatment were not predictive of the outcome (Table 3).

Amino acid sequences of the YMDD motif were determined over time in nine patients. One of the nine patients showed substitution to YIDD during the lamivudine administration period. The substitution was corrected after starting the IFN treatment. The patient showed NR.

Reactivation of CHB was observed in one SVR patient during the observation period of 36 weeks after the end of the treatment, and lamivudine administration was restarted. All the NR patients required retreatment with lamivudine or entecavir for the control of hepatitis. No patient experienced serious side-effects including post-treatment flare after discontinuing the interferon administration.

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