

1. Introduction

Ribavirin, a synthetic guanosine analog, has broad antiviral effects against both DNA and RNA viruses. Although ribavirin monotherapy has minimal efficacy on hepatitis C viral (HCV) eradication [1–3], studies have reported higher sustained response rates following combination therapy with interferon (IFN)-alpha and ribavirin than following IFN-alpha monotherapy [4–7]. Several mechanisms of action of ribavirin have been proposed [8]. In vitro and animal studies, in particular, have demonstrated that the antiviral activity of ribavirin is exerted through its potent mutagenic effects on RNA viruses after being incorporated into newly synthesized genomes by viral RNA-dependent RNA polymerase (RdRp) [9–11]. Still, little information is available regarding the mechanisms responsible for the increased virological efficacy associated with concurrent administration of ribavirin and IFN. No clinical studies to date have determined whether ribavirin induces mutations in the clinical setting nor examined the relationship between the mutagenic effects of ribavirin and viral response to IFN/ribavirin combination therapy.

The present study evaluated a set of patients with chronic hepatitis C. To elucidate whether ribavirin acts as a mutagen in the clinical setting, for each subject the sequential nucleotide mutations occurring during ribavirin monotherapy were compared with mutations occurring in the same patient during the non-treatment observation period immediately preceding the initiation of ribavirin monotherapy as a control. The relationship between mutations observed during ribavirin monotherapy and viral response to subsequent IFN/ribavirin combination therapy was also determined.

2. Methods

2.1. Patients

Among patients with biopsy-proven chronic hepatitis C hospitalized at the Musashino Red Cross Hospital from December 2001 to June 2002, 34 patients of HCV genotype 1b with a high viral load (> 100 kcopies/ml by Amplicor-HCV monitor assay; Roche Molecular Diag. Co., Tokyo, Japan) were included in the present study (Table 1). Patients with liver cirrhosis, autoimmune hepatitis, and alcoholic liver injury were excluded from the study. No patient was positive for hepatitis B virus-associated antigen/antibody or anti-human immunodeficiency virus antibody. No patient received immunomodulatory therapy before enrolment in the study. Written informed consent was obtained from all patients, and this study was approved by the ethical committee of Musashino Red Cross Hospital in accordance with the Helsinki Declaration.

2.2. Treatment protocol and study design

Treatment schedule and time points for sequential genetic analysis are described in the upper part of Fig. 1. Following a non-treatment observation period, all patients received oral ribavirin daily for 4 weeks. Ribavirin dosage was 600 mg daily for patients who weighed less than 60 kg and 800 mg daily for patients who weighed between 60 and 80 kg. All patients subsequently received a 24-week course of treatment consisting of

Table 1
Clinical characteristics of the study patients

No. of patients	34
Age (years)	59 ± 8
Gender (M/F)	14/20
Liver histology	
A1/A2/A3	14/15/5
F1/F2/F3	10/15/9
Number of ISDR mutations (0/1)	22/12
Baseline data	
ALT (IU/L)	81 ± 56
Platelet count (× 10 ³ /ml)	150 ± 52
Viral load (KIU/ml)	572 ± 204
SVR (%)	29.4 (10/34)

Values are expressed as mean ± standard deviation.

intramuscular IFN-alpha 2b (Intron, Schering-Plough, Kenilworth, NJ) at an initial dosage of 6 MU daily in combination plus daily oral ribavirin at the same dosage (600 mg daily or 800 mg daily) as was given during pretreatment monotherapy. After the first 2 weeks of IFN/ribavirin combination therapy, the IFN dosing frequency was reduced to 6 MU three times a week for the remaining 22 weeks.

Nucleotide sequences of the NS5A and NS5B regions of the HCV genome were determined at the following time points: (1) enrolment into the study; (2) end of the non-treatment observation period (immediately before initiation of ribavirin monotherapy); (3) end of the 4-week ribavirin monotherapy (immediately before initiation of IFN/ribavirin combination therapy). For each patient, nucleotide changes between time points 1 and 2 during the non-treatment observation period (mean: 6 months, range: 2–48 months) were used as a control to determine whether mutations observed during the period of ribavirin monotherapy (between time points 2 and 3) represented true effects of ribavirin. Mutation rates for the non-treatment observation period were compared with the mutation rates during the subsequent ribavirin monotherapy period. Moreover, the relationships between observed mutations and the clinical outcome of the subsequent IFN/ribavirin therapy were evaluated.

2.3. Nucleotide sequencing

Nucleotide sequences of the NS5A and NS5B regions of the HCV genome were determined by direct sequencing of polymerase chain reaction (PCR)-amplified DNA, as previously described [12,13]. In brief, after RNA was extracted from sera of the study subjects, NS5A and NS5B regions were amplified by RT-PCR using Taq polymerase, and the sequences corresponding to nucleotides 6703–7320 and 7730–8874 of HCV-J were determined [13]. The sequences of the primers for the NS5A region were the same as described previously [12]. Sequences for the NS5B region, which were amplified with two partially overlapping sets of primers, were as follows: NS5B1-5' outer set, 5'GGTGAATACCTGGAAATCA-AAGAAA3'; NS5B1-3' outer set, 5'AGAAATGAGTCATCAGAATCAT-CCT3'; NS5B1-5' inner set, 5'TGTAAAACGACGGCCAGTATGGGGCTTTCATATGACAC3'; NS5B1-3' inner set, 5'CAGGAAACAGCTAT-GACCCATGATGATGTTGCCCTAGCC3'; NS5B2-5' outer set, 5'GCA-GAAGAAGGTCACCTTTGACAGA3'; NS5B2-3' outer set, 5'TCGGGGGCCAAGTCAACATTGGT3'; NS5B2-5' inner set, 5'TGTAAAA-CGACGGCCAGTTTTGACAGACTGCAAGTCCT3'; NS5B2-3' inner set, 5'CAGG AAACAGCTATGACCTTCTCAGTGACCGTTGAGTC3'. M13-forward and M13-reverse sequences were attached to the 5'-termini of sense and anti-sense nested PCR primers. Both strands of the PCR products were cycle sequenced with the PRISM dye termination kit (CN402069, Applied Biosystems, Chiba, Japan), and nucleotide sequences were determined by an automated DNA sequencer Model 373A (Applied Biosystems).

Mutations resulting from ribavirin therapy were defined as the detection of a new nucleotide which was not detected as even a minority population in the prior specimen from that subject. Electropherograms were read by two independent readers without knowledge of the patients' backgrounds and outcomes. A nucleotide detected in the post-ribavirin monotherapy specimen was considered to be a 'new nucleotide' only when both readers could not identify any tracking peak of this nucleotide in a prior specimen.

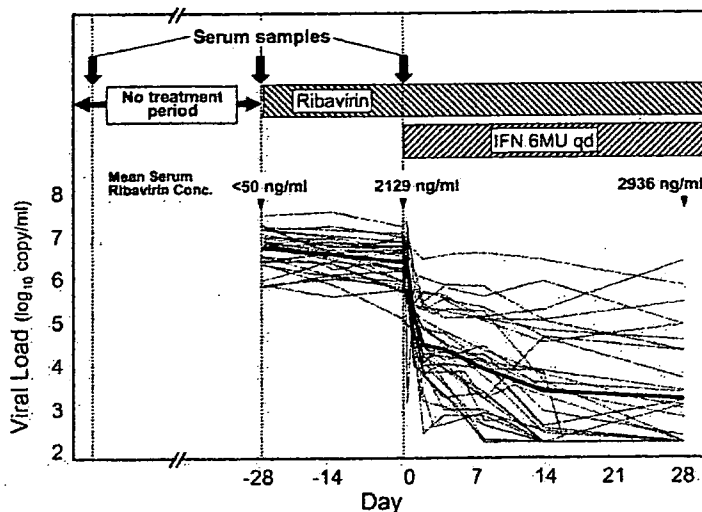


Fig. 1. Treatment schedule and HCV dynamics during ribavirin monotherapy and subsequent IFN/ribavirin combination therapy. After a non-treatment observation period, all patients received oral ribavirin daily for 4 weeks and subsequently received intramuscular IFN- α 2b in combination with daily oral ribavirin. For the first 2 weeks of IFN/ribavirin combination therapy, 6 MU of IFN- α 2b was given daily; the IFN dosing frequency was then reduced to 6 MU three times a week for the remaining 22 weeks of combination therapy. Serum HCV dynamics of individual patients are shown in dotted lines, and the solid line represents the mean of these values. The nucleotide sequences were serially analyzed at the time points indicated by the arrows. Closed triangles indicate the time points of serum ribavirin concentration measurements.

Peaks less than 10% of the dominant peak were considered to be background signals. To calculate values for dN (nonsynonymous substitution), dS (synonymous substitution), dN/dS ratios, and the number of point mutations, analyses of nucleotide sequences were performed using a software program (MEGA version 2.1.). Separate dN/dS ratios were determined for each NS5 region in patients who had nucleotide mutations in the corresponding NS5 regions. To determine the locations of amino acid mutations in the tertiary structure of the NS5B molecule, a crystal structure model of HCV NS5B-RdRp was constructed using 1QUV from the Protein Data Bank. A space-filling representation of each atom was generated using Rasmol 2.7.2.1. The deduced amino acid sequences of the NS5A and NS5B regions were also compared with a prototype HCV 1b strain, HCV-J [14].

2.4. HCV dynamics in serum

To analyze the effect of ribavirin on viral dynamics, HCV-RNA concentrations were quantified just before and at the end of ribavirin monotherapy, and also at 4, 8, 24, 48, 96, 192, and 336 hours after initiating IFN/ribavirin combination therapy, using real-time detection PCR, as reported previously [15–17]. The detection sensitivity of this assay is approximately 10 copies/ml, and the dynamic range is from 10 copies/ml to more than 1×10^8 copies/ml [17]. For each patient, the viral decline curve was plotted on a semilogarithmic scale, and the slopes of the exponential viral declines were calculated for each viral decline phase by a straight-line fit of the data.

2.5. Definitions of response to therapy

A patient negative for serum HCV-RNA during the first six months following the completion of IFN/ribavirin combination therapy was defined as a sustained viral responder (SVR), and a patient positive for HCV-RNA during this time period was defined as a non-responder (NR).

2.6. Statistical analysis

Categorical data were compared by the chi-square test or Fisher's exact test. Distributions of continuous variables were analyzed by the Student's

t -test for two groups. All tests of significance were two-tailed, and P values less than 0.05 were considered statistically significant.

3. Results

3.1. HCV dynamics

During the 4-week period of ribavirin monotherapy, the mean serum HCV-RNA level significantly decreased from 6.90 to 6.56 \log_{10} copy/ml ($P < 0.0001$, paired t test) (Fig. 1). Serum HCV dynamics after the start of subsequent IFN/ribavirin combination therapy demonstrated a biphasic kinetic pattern of HCV-RNA decline. The exponential decay slopes for the first phase and the second phase were $2.00 \pm 0.77 \log_{10}/\text{day}$ and $0.15 \pm 0.14 \log_{10}/\text{day}$, respectively.

3.2. The effect of ribavirin on HCV gene mutation and the relationship between mutations and virological response to IFN/ribavirin combination therapy

In a pairwise comparison of the NS5 sequences before and after ribavirin monotherapy, new HCV gene mutations occurring during ribavirin monotherapy were observed in the NS5A region in 10 patients and in the NS5B region in 8 patients. Mean gene mutation rates in the NS5A and NS5B regions during ribavirin administration were $2.9 \times 10^{-2}/\text{site/year}$ and $1.3 \times 10^{-2}/\text{site/year}$, respectively, rates which were significantly higher compared with the rates observed during the prior non-treatment observation periods in

Table 2
Mutation rates during the non-treatment observation period and during subsequent ribavirin monotherapy

	NS5A	NS5B
During non-treatment observation period		
Total	0.6×10^{-2}	0.24×10^{-2}
SVR	1.4×10^{-2}	0.46×10^{-2}
NR	0.28×10^{-2}	0.14×10^{-2}
During subsequent ribavirin monotherapy		
Total	2.9×10^{-2}	1.3×10^{-2}
SVR	8.8×10^{-2}	2.2×10^{-2}
NR	0.38×10^{-2}	0.96×10^{-2}

Note that the difference in NS5A mutation rate between SVR and NR patients was greater during ribavirin treatment than that during the non-treatment observation (control) period (23-fold vs. 5-fold). * $P=0.02$ (paired *t* test). † $P=0.02$ (paired *t* test). ‡ $P=0.04$ (unpaired *t* test). § $P=0.0005$ (unpaired *t* test).

the same patients (Table 2, $P=0.02$ for both NS5 regions). Of all nucleotide mutations, 72.1% in the NS5A region and 85.5% in the NS5B region were transition mutations. Percentages of transition mutations for all mutations are detailed in Table 3. Mutations from C to T and from G to A were frequent (Table 3). Synonymous mutations occurred more frequently than non-synonymous mutations in both regions, comprising 80.6% of all NS5A mutations and 73.5% of all NS5B mutations. No significant correlation was found between gene mutation rates and the subject's serum ribavirin concentration at the end of four weeks of ribavirin monotherapy.

Next, the relationship between mutations during ribavirin therapy and virological response to combination therapy was evaluated. The sustained viral response rate was 24.9% (10/34) (Table 1). The proportion of patients who had mutations in

Table 3
Transition mutations during the non-treatment observation period and during subsequent ribavirin monotherapy

	C to T	T to C	G to A	A to G	Others ^a
During non-treatment observation period					
NS5A (%)	22.2	33.3	22.2	11.1	11.2
NS5B (%)	28.5	57.1	0	14.3	0
During subsequent ribavirin monotherapy					
NS5A (%)	18.6	9.3	25.6	18.6	27.9
NS5B (%)	38.5	20.5	10.2	16.3	14.5

Data are expressed as a percentage of all mutations observed in each region.

^a Transversion mutations were included in this column.

the NS5A region during ribavirin monotherapy was significantly higher in SVRs than in NRs (8 out of 10 vs. 2 out of 24 patients, respectively, $P<0.0001$, Fisher's exact test). Correspondingly, gene mutation rates in the NS5A region were significantly higher in SVRs than in NRs: 8.8×10^{-2} /site/year vs. 0.38×10^{-2} /site/year, respectively ($P=0.0005$) (Table 2). In the NS5B region, although statistically significant differences were not observed, the gene mutation rate tended to be higher in SVRs than in NRs: 2.2×10^{-2} /site/year vs. 0.96×10^{-2} /site/year, respectively (Table 2). The proportion of patients with mutations in the NS5B region did not significantly differ between SVRs and NRs.

3.3. Non-synonymous mutations and virological response to IFN/ribavirin combination therapy

In the NS5A region, non-synonymous mutations were found in 19.4% of all nucleotide mutations observed during ribavirin monotherapy. Alterations in deduced amino acid residues by non-synonymous mutations are illustrated in Fig. 2, which shows all 10 patients who had nucleotide mutations during ribavirin monotherapy. In a pairwise comparison between pre- and post-ribavirin monotherapy, amino acid alterations were found in 5 of these 10 patients. Non-synonymous mutations were found exclusively in SVRs. In other words, 5 out of the 8 patients who achieved SVR status had 1 or 3 non-synonymous mutations; 2 of these 5 patients had amino acid alterations accompanied with an increase in the number of amino acid mutations in the interferon sensitivity determining region (ISDR). In contrast, all nucleotide mutations detected in NRs were synonymous mutations. dN/dS tended to be higher in SVRs than in NRs ($P=0.25$, Fig. 3).

In the NS5B region, non-synonymous mutations were detected in 3 out of the 10 SVRs and in 2 out of the 24 NRs. The proportion of patients with non-synonymous mutations did not significantly differ between the two response groups. dN/dS also did not significantly differ between the SVRs and the NRs ($P=0.77$, Fig. 3). Of the 9 amino acid mutations detected in the NS5B region, two were located in the functional domain of the RdRp, one was located in domain C (D310N), and one was located in domain D (R345S). As shown by the crystal model of the NS5B-RdRp in Fig. 4, amino acid mutations were primarily located on the molecular surface; none occurred in the nucleotide groove or in the tunnel.

3.4. Genetic changes during the non-treatment observation period

Gene mutation rates in the NS5A and NS5B regions during the non-treatment observation period were calculated as 0.60×10^{-2} /site/year and 0.24×10^{-2} /site/year, respectively, rates which were significantly lower than the mutation rates observed during ribavirin monotherapy (Table 2). Next, the relationships between viral mutation rates during

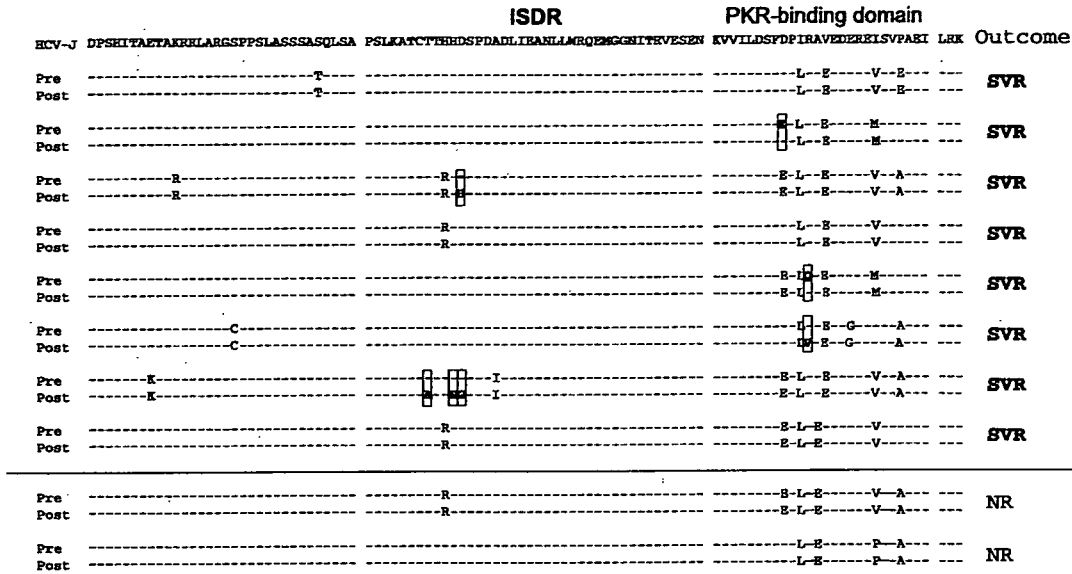


Fig. 2. Amino acid sequence alignments in the NS5A region obtained pre- and post-ribavirin monotherapy. The figure contains data for all 10 patients out of 34 with nucleotide mutations occurring during ribavirin monotherapy. Amino acid residues are indicated by the standard single-letter codes, and dashes indicate identical amino acid residues, with the consensus sequence and HCV-J shown at the top. Outcomes of combination therapy are shown on the right side. In a pairwise comparison between pre- and post-ribavirin monotherapy sera, amino acid alterations were found in 5 of 10 patients. Non-synonymous mutations were exclusively found in SVR patients.

the non-treatment observation period and viral responses to subsequent IFN/ribavirin therapy were evaluated. Interestingly, gene mutation rates in the NS5A region during the non-treatment observation period were significantly higher in SVRs than in NRs: 1.4×10^{-2} /site/year vs. 0.28×10^{-2} /site/year, respectively ($P=0.04$). However, it should be noted that the relative difference in NS5A mutation rates between SVRs and NRs during ribavirin treatment was larger than the relative difference between SVRs and NRs during the non-treatment observation period (23-fold vs. 5-fold, $P=0.01$).

Similarly, gene mutation rates in the NS5B region were higher in SVRs (0.46×10^{-2} /site/year) than in NRs (0.14×10^{-2} /site/year), although these differences were not statistically significant. Seven of the nine patients who had mutations during the non-treatment observation period also had mutations during ribavirin monotherapy. However, 53% (8/15) of the patients with mutations during ribavirin monotherapy had no gene mutations during the non-treatment observation period.

4. Discussion

In the present study, we identified HCV gene mutations occurring during ribavirin monotherapy and found that the mutation rate was associated with the virological response to subsequent IFN/ribavirin combination therapy. Since the mutation rate was significantly higher during ribavirin monotherapy than during non-treatment observation periods

in the same patients, at least some of the mutations observed during ribavirin treatment were likely an effect of ribavirin administration. Therefore, ribavirin appears to act as a mutagen during clinical treatment, and this mutagenic effect correlates with improvements in the virological response rate resulting from the synergistic use of ribavirin with IFN.

Recently, several in vitro and animal studies [9–11, 18, 19] have provided evidence that ribavirin has mutagenic

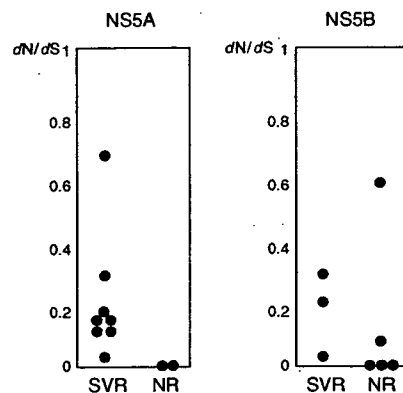


Fig. 3. Ratio of *dN* (nonsynonymous substitution) to *dS* (synonymous substitution) distances for the NS5A and NS5B regions. All 10 patients with nucleotide mutations in the NS5A region and all 8 patients with nucleotide mutations in the NS5B region during ribavirin monotherapy are shown in this figure. All pairwise *dN/dS* ratios were calculated using MEGA ver. 2.1 for each subject. *dN/dS* in the NS5A region tended to be higher in SVR patients than in NR patients (NS5A: $P=0.25$, NS5B: $P=0.77$).

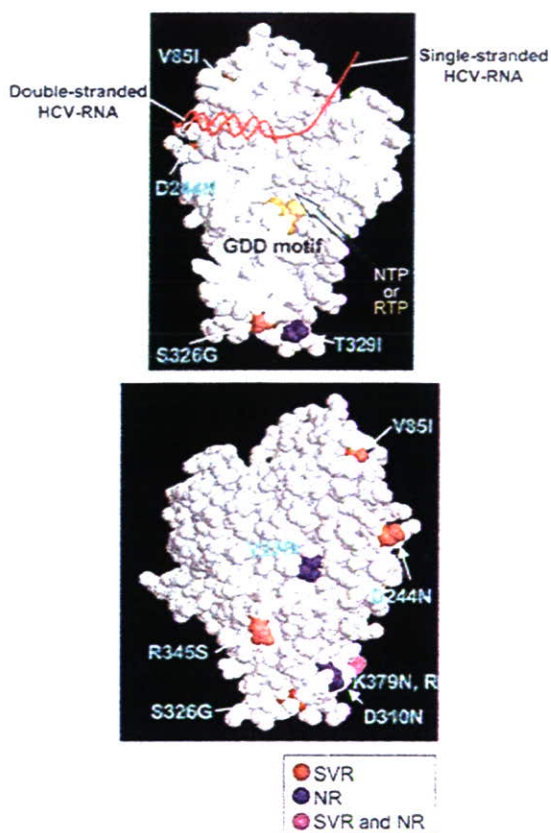


Fig. 4. Crystal structure of HCV NS5B-RNA dependent RNA polymerase. The molecular model of NS5B was constructed using 1QUY from the Protein Data Bank. A space-filling representation of each atom is shown. Graphics were generated using RasMol 2.7.2.1. View of a cross section of the RdRp at level of nucleotide tunnels (A) and the surface (B) are illustrated. Single stranded HCV RNA enters the enzyme through a groove at the top of the finger domain, and NTP (or ribavirin triphosphate; RTP) enters the enzyme through the right lower NTP tunnel (between the fingers and the thumb). The substitutions observed in SVR patients only, NR patients only, and both sets of patients are shown in red, blue, and magenta, respectively.

activity. Other studies have found that ribavirin increases the mutation rate in a full-length HCV cDNA plasmid [20] and in an HCV replicon [21]. However, none of these studies were human studies; no study to date has documented that ribavirin has mutagenic activity in the clinical setting. In the absence of clinical data, the association between the mutagenic activity of ribavirin and the clinical and virological responses to IFN/ribavirin combination therapy remains unknown. Detecting gene mutations induced by ribavirin and analyzing their association with clinical responses to IFN/ribavirin therapy are extremely difficult because HCV with error mutations may be immediately eliminated by the concurrently administered IFN. Although issues regarding the mutagenic effects of ribavirin remain controversial, the inclusion of four weeks of ribavirin monotherapy immediately before

IFN/ribavirin combination therapy in our protocol enabled us to clarify the association between gene mutations induced by ribavirin and the ensuing virological response to the subsequent IFN/ribavirin therapy.

Interestingly, the correlation between mutation rate and virological response to therapy was more evident in the NS5A region, including the ISDR, than in the NS5B region. From our previous analysis of the full-length HCV genome [13,22], NS5A sequences were more variable than NS5B sequences among different clones. Therefore, it seems likely that the stronger relationship observed between mutation rates in the NS5A region and SVR status was due to the relatively greater inherent variability of the NS5A region compared with the NS5B region. Even in the NS5A region, however, serial amino acid mutations rarely occur in the same patients during untreated periods [23]. Hence, our ribavirin monotherapy results suggest that in some patients, ribavirin induces non-synonymous mutations which increase sensitivity to IFN. In turn, this increased susceptibility to IFN could lead to SVR status. Conversely, no functionally important mutations were detected in the NS5B region. Since a ribavirin-induced non-synonymous mutation at a functionally important site in the NS5B region is likely to lead to viral death, even without concurrent IFN administration, the substitutions observed in this critical region are more probably the results of positive or negative selection.

The patients who had gene mutations during the non-treatment observation period were prone to also having mutations during ribavirin monotherapy as well and were more likely to achieve SVR status. These correlations suggest that ribavirin easily induced HCV mutations in such patients. Although mutations could have occurred in the absence of ribavirin, the difference in mutation rates between SVRs and NRs was significantly larger during ribavirin treatment than during the non-treatment observation period (23-fold vs. 5-fold, $P=0.01$). The observation that in more than half of the patients, mutations occurred only during ribavirin monotherapy and were not detectable during the non-treatment observation period suggests that mutagenic effects of ribavirin synergistically potentiating the virological response to IFN may play an important role in achieving SVR status.

Gene mutations in the NS5A and/or NS5B regions during ribavirin monotherapy did not occur in all patients in the present study, suggesting that the intensity of the mutagenic effects of ribavirin differed among individual patients. Additionally, some patients who did not have gene mutations in these regions during the non-treatment observation period or during ribavirin monotherapy nonetheless still achieved SVR, suggesting that the synergistic efficacy of ribavirin may not result solely from the mutagenic activity of this agent. Alternatively, ribavirin may have possibly induced mutations during the period of IFN/ribavirin combination therapy in these patients, but as previously discussed, the concurrent IFN might have eliminated the HCV containing

these mutations before the mutations were able to be detected. Additionally, our data did not address the significance of the impact that mutations in regions other than NS5 may have had on viral response to therapy.

HCV populations *in vivo* consist of a quasispecies nature. Our previous cloning analysis detected small number of minor clones in specimens, which were determined as ISDR-wild type by direct sequencing [24]. Hence, it should be noted that our criteria for mutation could not completely distinguish between *de novo* mutation and selection of a minor clone.

In the present study, we found that ribavirin also expressed antiviral activity by reducing viral load, presumably because we used a highly quantitative assay for HCV-RNA measurement [17]. However, contradictory results have been reported previously [1–3]. Since the present study identified only a small reduction in viral load, further investigation is needed to confirm our result.

In conclusion, our data demonstrate that clinical administration of ribavirin induces mutations in HCV genes and suggest that, in some patients, mutagenesis may be one of the mechanisms responsible for the synergistic efficacy of ribavirin in IFN/ribavirin combination therapy.

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Editorial

Genetic changes in the interferon sensitivity-determining region of hepatitis C virus (HCV) during the natural course of infection: an implication for the gene function in the role of chronic infection

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Nonstructural 5A gene variability of hepatitis C virus (HCV) during a 10-year follow up

FAN W, ZHU W, WEI L, et al.

The interferon sensitivity-determining region (ISDR; aa2209–2248 of HCV-J) in the nonstructural 5A region (NS5A) of hepatitis C virus (HCV) was originally identified as the genomic functional element wherein missense mutations were closely related to the clinical efficacy of interferon treatment, as well as to serum viral loads, in genotype-1b HCV infection.^{1,2} After the first reports of the ISDR, controversy arose as to its predictive value for the outcome of interferon therapy, because clinical studies in Europe and North America did not always support the relevance of ISDR,³ although most studies in Japan, Spain, and Italy supported it.^{4,5} However, recent meta-analyses have clearly supported the universal correlation between ISDR sequence and interferon resistance.⁶ It is speculated that the initial discrepancy of the results might have been caused by differences in interferon regimens and patient sources.

After identification of ISDR as the key genomic element for interferon efficacy and viral replication, the molecular function of NS5A protein and its relevance to ISDR structures has been vigorously and intensively studied using NS5A protein expression in vitro or in transgenic mice. A variety of putative NS5A functions were postulated, such as binding to cellular protein kinase R (PKR),⁷ TRADD,⁸ Grb-2,⁹ p21,¹⁰ hVAP-33,¹¹ or other proteins that may influence the pathogenesis of hepatitis C by antiviral effects, apoptosis, signal transduction, cell cycle regulation, or formation of viral replication complex. Much attention has been paid to PKR, because NS5A protein was found to block the antiviral effect of PKR in an ISDR sequence-dependent manner by directly binding to PKR through the so-called PKR-binding domain, which includes the ISDR plus an additional 26 aa stretch located at the C-terminal portion (aa2209–2274). The recently developed HCV

subgenomic replicon system also disclosed the importance of NS5A proteins in intracellular viral replication, because specific mutations called “cell culture-adaptive mutations” needed in its genome for efficient replication in cultured cells clustered in the central region of NS5A, particularly in the serine cluster region immediately upstream to the ISDR or the ISDR itself.^{12,13} Because these mutations possibly affect phosphorylation of NS5A proteins, the role of phosphorylated NS5A protein in viral replication and interferon sensitivity has become the recent target of molecular research.

How does the HCV-ISDR structure change in a host during the natural course of disease? The answer to this question should give us important clinical information about when to start interferon therapy, whether earlier or later in HCV infection. In the current issue of the *Journal of Gastroenterology*, Fan et al.¹⁴ report the genetic evolution of the NS5A gene during a 10-year follow-up of natural HCV infection in 7 patients, focusing on ISDR, PKR-binding domain, serine cluster region, and other functional domains in NS5A gene. To investigate changes of the genetic variability during the natural course, they performed subcloning analysis. As a result, serine residues at positions 2194, 2197, 2201, and 2204 in the serine cluster region, suggested to be important for hyperphosphorylation of NS5A protein, were highly conserved in all patients and within the quasispecies of each patient, suggesting that phosphorylation plays the crucial role in NS5A protein function. Meanwhile, subcloning analysis of the ISDR quasispecies disclosed that the wild-type ISDR (no aa substitution relative to HCV-J), or the intermediate-type ISDR (one to three substitutions) was dominant and stable throughout the observation periods in all patients. However, the ISDR quasispecies decreased over time, and the quasispecies had a tendency gradually to converge to the wild-type ISDR.

Reprint requests to: N. Enomoto

Before the report by Fan et al., several studies already had been conducted for analyzing the natural genetic change of the ISDR.¹⁵⁻¹⁹ Although these previous studies also demonstrated that the wild-type ISDR or the intermediate-type ISDR was generally stable, the observation periods were rather short, and the quasispecies complexity was not investigated in most of the studies. Importantly, Fan et al. demonstrate that the ISDR quasispecies finally tended to converge to the wild-type ISDR with a decrease of the quasispecies complexity, indicating that sequence motif of the wild-type ISDR had a crucial role functionally in establishing chronic HCV infection. Although the mutant-type ISDR (four or more aa substitutions) was not included in the study, the mutant-type ISDR was reported to be rather unstable, because nonsynonymous mutations (63%) were higher than synonymous mutations (37%), indicating that strong selective pressure of the host was exerted on the mutant-type ISDR.¹⁶ This finding coincides with the results by Fan et al. that the HCV of wild-type ISDR ultimately survives in the course of chronic infection.

If the quasispecies complexity of the ISDR subtypes finally converges to the wild-type ISDR, it might be better to start interferon therapy early for chronic HCV infection. It is not clear why the mutant-type ISDR is unstable in a host. Part of the mechanism, however, might be explained by different interaction with cellular proteins, such as PKR induced by endogenous interferon. On the other hand, if the mutant-type ISDR was unstable and easily defeated by the wild-type ISDR in chronic infection, this weak subtype might have disappeared in the world of HCV infection. However, although the distribution is rather small, the mutant-type ISDR is still frequently found in clinical samples. Is it on the way to disappearing? Or does it have an advantage over the other types in a certain phase of infection other than the chronic phase? Recent advances in understanding of the innate immune system have disclosed that mammalian cells have two distinct innate immune pathways protecting cells from the virus: the interferon regulatory factor (IRF) system, and the interferon (IFN)—signal transduction and activator of transcription (STAT) system. In the phase of chronic infection, the IFN-STAT system might have a dominant role in viral suppression, and the wild-type ISDR is supposed to inhibit this pathway, giving a survival advantage to HCV with the wild-type ISDR. In contrast, the IRF system might be dominant in the phase of acute infection. Does the mutant-type ISDR inhibit this IRF pathway, and give a survival advantage to HCV with the mutant-type ISDR? The answer to this question requires further study, but these analyses might go far toward helping understand the mysterious function of the ISDR.

Shinya Maekawa, M.D., Ph.D., and
Nobuyuki Enomoto, M.D., Ph.D.

First Department of Internal Medicine, Yamanashi University, 1110 Shimokato, Tamaho, Nakakoma, Yamanashi 409-3898, Japan

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Viral load change and sequential evolution of entire hepatitis C virus genome in Irish recipients of single source-contaminated anti-D immunoglobulin*

J. Itakura,¹ K. Nagayama^{1,2}, N. Enomoto,¹ K. Hamano,¹ N. Sakamoto,¹ L. J. Fanning,³ E. Kenny-Walsh,³ F. Shanahan³ and M. Watanabe¹ ¹Department of Gastroenterology and Hepatology, Tokyo Medical and Dental University, Tokyo, Japan; ²Departments of Internal Medicine, Tsuchiura Kyodo General Hospital, Tsuchiura City, Ibaraki, Japan; ³Hepatitis C Unit, Department of Medicine, Clinical Sciences Building, National University of Ireland, University College Cork, Cork, Ireland

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SUMMARY. In hepatitis C virus (HCV) infection, serum viral load is important in the prediction of therapeutic efficacy. However, factors that affect the viral load remain poorly understood. To identify viral genomic elements responsible for the viral load, we investigated samples from a population of Irish women who were iatrogenically infected from a single HCV source by administration of HCV 1b-contaminated anti-D immune globulin between 1977 and 1978 (Kenny-Walsh, *N Engl J Med* 1999; 340: 1228). About 15 patients were divided into two groups, viral load increasing group (11 patients) and decreasing group (4 patients). Pairs of sera were collected from each patient at interval between 1.1 and 5.8 years. Full-length sequences of HCV genome were determined, and analyzed for chan-

ges in each patient. Sliding window analysis showed that the decreasing group had significantly higher mutation rates in a short segment of NS5B region that may affect the activity of RNA-dependent RNA polymerase. By comparing each coding regions, significantly higher mutation numbers were accumulated in NS5A region in the increasing group than the decreasing group (0.92 vs 0.16 nucleotides/site/year, $P = 0.021$). The mutation in certain positions of the HCV genome may be determinant factors of the viral load in a relatively homogeneous patient population.

Keywords: anti-D immunoglobulin, full genome sequence, hepatitis C virus.

INTRODUCTION

Hepatitis C virus (HCV) is globally distributed virus that causes chronic inflammation in liver, and may leads to liver cirrhosis and hepatocellular carcinoma over the course of 20–30 years [1–3]. Efficacy of anti-viral therapy based on

*Informed consent was obtained from each patient, and the study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki. There is no conflict of interests for present study.

Abbreviations: HCV, hepatitis C virus; CH, chronic hepatitis; LC, liver cirrhosis; HCC hepatocellular carcinoma; ALT, alanine aminotransferase; E, envelope; NS, nonstructural; PKR, protein kinase R; HLA, human histocompatibility leukocyte antigen; RT, reverse transcription; ISDR, interferon sensitivity determining region; PCR, polymerase chain reaction.

Correspondence: Nobuyuki Enomoto, M.D., Department of Gastroenterology and Hepatology, Tokyo Medical and Dental University, 1-5-45 Yushima, Bunkyo, Tokyo 113-8519, Japan.
E-mail: nenomoto.gast@tmd.ac.jp

pegylated interferon and ribavirin for chronic hepatitis C ranges between 40 and 80% [4–6]. Viral load is an important predictive factor for therapeutic outcome. We have previously demonstrated a close relationship between HCV genomic structures with patients' viral load [7], hepatitis activities [8,9], and ALT levels [10]. However, all of these observations were on the basis of heterogeneous hosts infected with different viral strains, making the role of the viral genetic structures ambiguous. A study using homogeneous host populations infected with a homogeneous viral strain would be ideal to clarify the role of viral gene in determination to the viral load.

There were two outbreaks of HCV infection in the world through usage of the virus-contaminated blood products from a single donor. One was in Ireland and one in Germany [11,12]. In Ireland, from May 1977 to November 1978, 704 women were iatrogenically infected with HCV through administration of a virus-contaminated anti-D immune globulin to prevent rhesus isoimmunization [12,13]. All recipients were female, had same ethnic origin, similar

duration of disease, infected with HCV genotype 1b from a single donor. In this homogeneous clinical setting, the amount of serum HCV has been shown to fluctuate [14]. HLA DR locus and DQ locus were shown to be associated with viral load in this patients group [15]. The HCV genomic determinants of the viral load set point are currently unknown.

The purpose of this study is to clarify the relationships between viral genetic structures and the serum viral load in this Irish patient population. We analyzed the full viral genomic sequences in each patient within the study population, and compared those patients whose had increased viral load with those who had decreased viral load within the time frame under retrospective investigation of approximately 20 years.

PATIENTS AND METHODS

Patients

We determined the HCV sequence of donor's preserved serum (deposited with the DDBJ/Genbank/ENBL data libraries under accession number AF313916). In total, 15 Irish female patients infected with HCV-contaminated anti-D immunoglobulin were used in this study, all of them have never been treated before enrolled into this study. About 25 patients were randomly chosen from the anti-D patients, DNA sequence analysis was attempted on all specimens, and 15 pairs of sera yielded the full length viral genomic sequence information. Each patient in the study population had two sera samples retrospectively analyzed from a bio-bank of specimens prospectively collected as a part of the routine clinical management and viral load quantification in this patient population at Cork University Hospital. The median temporal separation between samples was 39.2 months (13–72 months). All patients were infected by HCV genotype 1b from virus-contaminated anti-D immunoglobulin injections during the period from May 1977 to November 1978. All patients attended the hepatitis C clinic at Cork University Hospital, Cork, Ireland. Serum HCV-RNA levels were determined by a polymerase chain reaction assay (PCR assay; Roche HCV Monitor kit, F. Hoffmann-La Roche Ltd., Basel, Switzerland). The study design is shown in Fig. 1. Standard deviations have previously been reported [16], the 95% confidence interval of the viral load was ± 0.032 viral copies/mL. A viral load increase or decrease over the range $0.062 \log_{10}$ viral copies/mL, was used as the criteria for change in viral load. The patients were classified into two groups. The characteristics of group one were as follows: the viral load was increased over time, $n = 11$, hence, increasing group (group-I). The second group of patients had a decreased or remained stable in viral load over the time investigated, decreasing group (group-D, $n = 4$). All patients were seronegative for HBV markers, had no autoimmune liver disease or competing

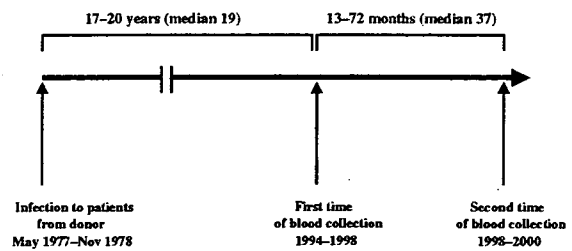


Fig. 1. Study design. A total of 30 sera were acquired from 15 patients. Each patient's sera were collected as part of the routine clinical management of chronic hepatitis C at Cork University Hospital, Ireland. Initial viral load assessment was approximately 19 years post-infection with HCV 1b contaminated anti-D immunoglobulin. The second samples selected for analysis in this study were dated to a further 6 years post-infection. Sequences were analysed by direct sequence from PCR generated amplicons.

risk factors such as excessive alcohol intake or hepatotoxic drugs. All patients had liver biopsy performed as part of their routine clinical assessment. Informed consent was obtained from each patient for liver biopsy, and the study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki. Sera were stored at -80°C storage facility.

RNA extraction and RT-PCR analysis

RNA extraction, cDNA synthesis and PCR analysis were performed as reported by us previously [17]. Briefly, serum RNA was extracted using ISOGEN (Wako Pure Chemical Industries, Osaka, Japan). The extracted RNA was reverse-transcribed by moloney murine leukemia virus reverse transcriptase (MMLV-RT, GIBCO BRL) using random hexamers (Takara, Kyoto, Japan). The full-length HCV genome was amplified by the nested PCR with 21 partially overlapping sets of primers using Advantage cDNA Polymerase Mix (Clontech, California, USA) according to the manufacturer's instructions. 3'UTR and X tail were not uniformly amplified probably because of the condition of storage. Therefore, these regions were not analyzed in this study.

Sequence determination

Each PCR product was purified and residual primers were removed with the column (Suprec O2, Takara, Kyoto, Japan). Thereafter, both strands of the PCR products were cycle sequenced with the Big Dye Terminator Cycle Sequencing Ready Reaction Kits (Applied Biosystems, Tokyo, Japan) using forward and reverse sequencing primers, respectively. The products were purified by the column (Quickspin column, Boehringer Mannheim, Indiana, USA) and sequenced using an automated DNA sequencer (model 373S; Applied Biosystems, Tokyo, Japan). Each sequence

Table 1 Patients' basic characteristics

No.	Duration from infection (year)		Interval time (month)	Viral load (log ₁₀ kcopy/mL)		Rate change of viral load (log ₁₀ kcopy/mL/year)
	1st	2nd		1st	2nd	
1	18	22	29	5.62	7.41	0.741
2	19	22	33	5.48	7.28	0.655
3	17	22	31	4.64	6.27	0.633
4	20	23	37	6.18	7.59	0.459
5	17	20	45	4.38	5.94	0.416
6	17	23	56	5.23	6.88	0.354
7	20	22	69	5.46	7.15	0.293
8	17	23	37	4.71	5.15	0.203
9	20	22	39	4.82	5.38	0.173
10	17	19	72	5.38	5.91	0.089
11	19	22	26	4.96	5.04	0.052
12	19	23	18	5.36	5.36	0.000
13	21	22	24	5.00	4.89	-0.054
14	19	22	13	5.86	5.75	-0.099
15	19	22	59	5.46	4.55	-0.184
Group-I*	18.7 ± 0.3	22.2 ± 0.2	41.0 ± 4.8	5.19 ± 0.15	6.28 ± 0.27	0.37 ± 0.07
Group-D*	19.0 ± 1.1	21.0 ± 1.0	32.0 ± 13.9	5.44 ± 0.25	5.07 ± 0.36	-0.08 ± 0.04

*no. 1-11: group-I, no. 12-15: group-D.

Table 2 Patients' basic characteristics (continued)

No.	ALT value (microkat/L)		HLA DQB1 locus	HLA DRB1 locus	DR 51-53
	1st	2nd			
1	0.50	0.44	0201, -02/0501	01/03	53
2	0.98	0.91	0201, -02/0602, -11	03/15	51/52
3	0.65	0.70	Not typed	Not typed	Not typed
4	1.43	1.56	Not typed	Not typed	Not typed
5	0.72	0.51	0201, -02/0602, -11	0701/15	51
6	0.63	0.59	05031/05031	13/14	52
7	1.26	2.29	0201, -02/0602, -11	0701/15	51/53
8	0.72	0.75	0201, -02/03032, -06	03/0701	53
9	0.56	0.57	0201, -02/03032, -06	0701/0701	52
10	1.70	0.98	0201, -02/03011	03/04	Not typed
11	0.54	0.98	0301/0501	01/13	52
12	0.71	0.59	Not typed	Not typed	Not typed
13	0.32	0.43	Not typed	Not typed	Not typed
14	0.79	0.67	0301/0301	0701/11	52/53
15	0.69	0.64	0402/0604	08/13	52
Group-I†	0.91 ± 0.12	0.97 ± 0.16			
Group-D†	0.63 ± 0.11	0.58 ± 0.06			

Normal range of ALT value was 0.12-0.60 microkat/L.

†no. 1-11: group-I, no. 12-15: group-D.

was confirmed twice with direct sequencing method for sense and anti-sense strands'. Subcloning study was not done, therefore HVR were not mentioned in the present

study. HLA profile was available for 11 individuals study population and was performed as outlined previously reported [16].

Statistical analyses and phylogenetic analysis

For phylogenetic analysis, the nucleotide and deduced amino acid sequences of the patients were compared with a sequence of HCV-J strain [18] as a reference. The nucleotide and amino acid sequences were compared longitudinally in each patient. Amino acid changes were picked up between the paired samples from each patient. Serum viral load changes were defined as increasing status when they were above zero, and defined as decreasing status when equal to or under than zero. Calculation of amino acid mutation rates and the phylogenetic analyses were performed by the Mega software version 2.1 with neighbour-joining method (NJ) [19], and PHYLIP version 3.62 with maximum likelihood method (ML) [20]. NJ tree and NG distances were evaluated

using 1000 bootstrap samples, *P* values for the branches of the ML tree were also calculated. Nucleotide mutation rates were calculated by the Mega ver.2.1. Statistical analyses of the two groups were done by Mann-Whitney's *U* test using the program Stat View 5.0 (SAS institute inc.). All tests of significance were two-tailed, with *P* values of <0.05 considered to indicate statistical significance.

RESULTS

Characteristics of patients

The clinical characteristics were compared between the two groups, 11 patients with increasing viral load status (group-I) and 4 patients with decreasing status (group-D), as shown

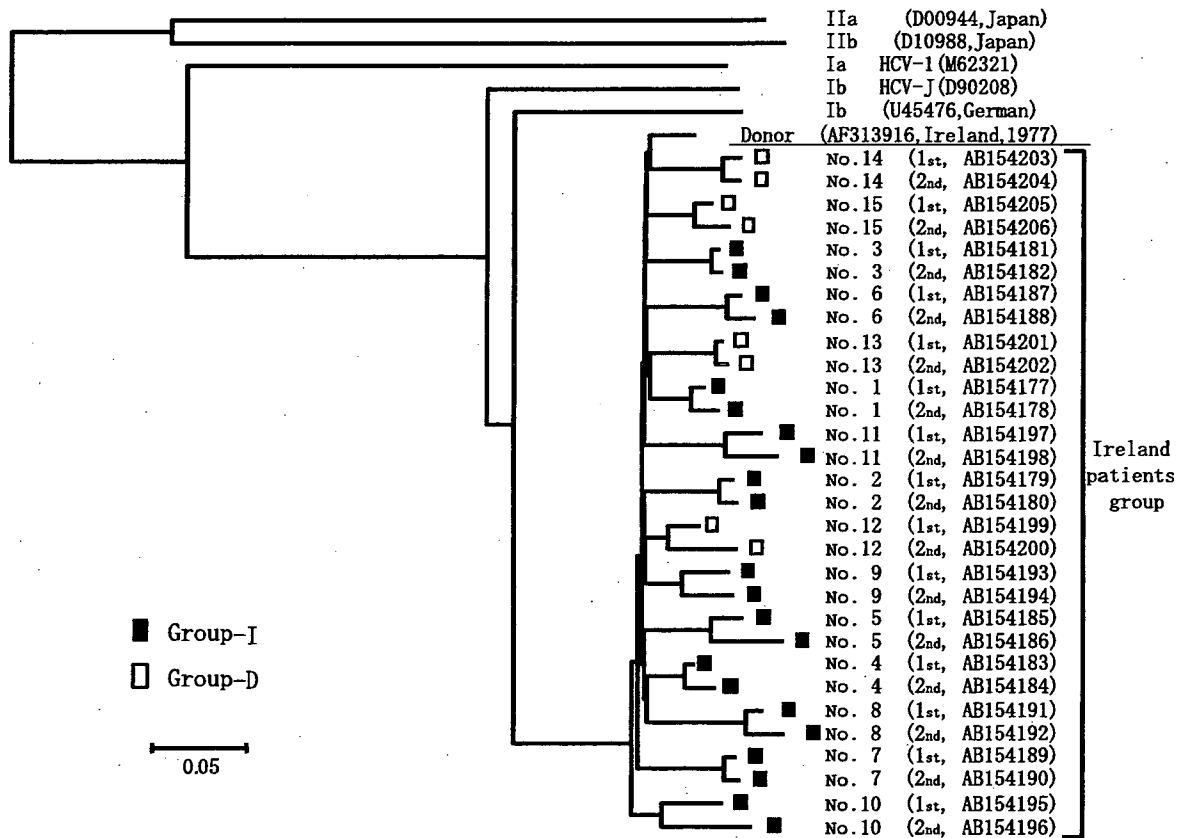


Fig. 2 Phylogenetic analysis with entire amino acid sequences of anti-D recipients, the donor, and representative sequences of genotype 1b, 2a and 2b. A phylogenetic tree was constructed by the neighbour-joining tree method with 10 000 bootstraps supports. The donor-sequence is shown in the 6th from the top. Closed squares show the recipients sequences belonging to the viral load increasing group (group-I), and open squares show them belonging to the decreasing group (group-D), and the bar at the left below is the reference for the distance which length is 0.05. Patients' numbers are matched with Table 1, and ordinal numbers after patients' number indicates the order of collection of serum from each patient. The mutations cluster in one group involving donor's sequence, but are individual to each patient. The distances from donor's sequence to patients' sequences did not segregate with change in viral load. The average distance from donor's sequence are as follows: to 1st acquired serum, 0.058 ± 0.0028 in group-I and 0.051 ± 0.0034 in group-D, and to 2nd, 0.065 ± 0.0030 in group-I and 0.060 ± 0.0021 in group-D, and within each pairs, 0.028 ± 0.0060 in group-I and 0.024 ± 0.0080 in group-D.

in Table 1. No significant difference could be found in age, time intervals between the two-blood sampling of each patient, and the intervals between the time on infection and the first blood sampling. The amounts of serum HCV-RNA were not significantly different between the two groups at first time of blood collection ($5.17 \pm 0.16 \log_{10}$ kcopy/mL in group-I, $5.42 \pm 0.18 \log_{10}$ kcopy/mL in group-D, respectively). The viral load was significantly higher in the group-I at the second serum samples ($6.37 \pm 0.29 \log_{10}$ kcopy/mL in group-I, $5.14 \pm 0.26 \log_{10}$ kcopy/mL in group-D, $P = 0.027$). The mean rate of change was significantly different between two groups ($0.37 \pm 0.07 \log_{10}$ kcopy/mL/year to group-I, $-0.08 \pm 0.04 \log_{10}$ kcopy/mL/year to group-D, $P = 0.004$). Average ALT values were not different between both the groups (Table 2).

Phylogenetic analysis

To clarify the tendency of genomic changes of HCV in each patient (deposited with the DDBJ/Genbank/ENBL data libraries under accession number AF313916 for donor sequence, AB154177 to AB154206 for recipients), phylogenetic analyses were done based on the entire amino acid sequence by the neighbour-joining tree method (Fig. 2). The phylogenetic tree analysis showed that all sequences studied in the present study belonged to genotype 1b cluster (100% bootstrap support, $P < 0.01$ in the maximum likelihood tree), and they were more closely related to the donor's sequence than any other known 1b sequences (100% bootstrap support, $P < 0.01$). The two sets of sequence data derived from the each patient segregated together on the phylogenetic tree (100% bootstrap support, $P < 0.01$). The genetic distance between each specimen of each pair was, as anticipated, less than the distance from donor's sequence (100% bootstrap support, $P < 0.01$). There was no relationship between the genetic distance calculated with amino acid sequence and time intervals of recipient's serum samplings or viral load fluctuations. This suggests that the genetic evolution speed was different among patients independent of viral loads.

Mutations from donor to recipients

We initially analyzed the average rate of HCV amino acid sequence mutation between the donor and the pair of samples from each recipient (Fig. 3). Comparison of the number of mutations between group-I and group-D in the open reading frame revealed no significant differences. Further analyses restricted to small functional regions including PKR eukaryotic initiation factor-2 α phosphorylation homology domain, ALT response related element, PKR-binding domain, interferon sensitivity determining region (ISDR), nuclear localization signal and variable region 3' region showed no difference in mutations number in both groups (raw sample data not shown).

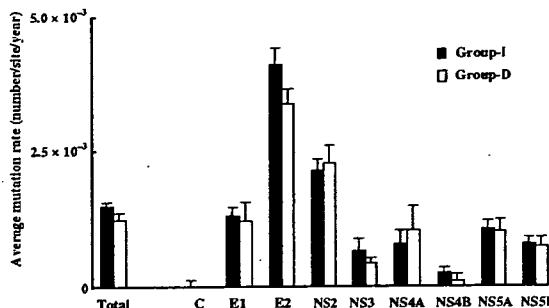


Fig. 3 Average rates of amino acid mutations from donor's sequence to recipients'. The mutation rates are those calculated from the first acquired sera from each patient in the cohort. The mutation rate was calculated for each coding region. The analysis of the number of mutations in both groups indicated that there are differences between regions, but the analysis did not achieve significance.

Genomic analysis of paired sera

The average rates of mutations in amino acids for the entire HCV genome in group-I were similar to that in group-D ($3.4 \times 10^{-3} \pm 0.6 \times 10^{-3}$ numbers/site/year in group-I and $3.3 \times 10^{-3} \pm 1.2 \times 10^{-3}$ numbers/site/year in group-D). A comparison of each coding region revealed that the average number of mutations was significantly higher in group-I than group-D in NS5A ($2.07 \times 10^{-3} \pm 0.41 \times 10^{-3}$ numbers/site/year vs $0.36 \times 10^{-3} \pm 0.41 \times 10^{-3}$ numbers/site/year in average, $P = 0.02$) (Fig. 4). Individual points of mutations in each patient were aligned as outlined in Fig. 5. The majority of the mutations clustered at E2, NS5A and NS5B in each patient. However, no specific position was found to be unique to group-I or group-D.

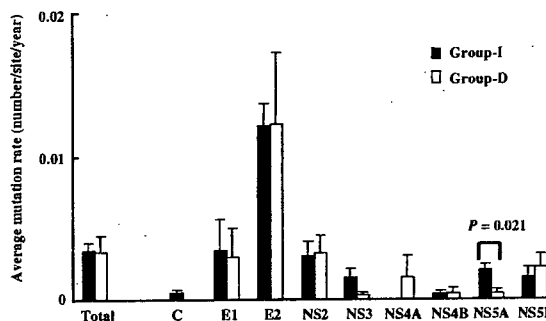


Fig. 4 Average rates of viral sequential mutations within each recipients belonging to two groups. The mutation rates are those calculated from the first to second acquired sera from each patient in the cohort. The mutation rate was calculated for each coding region. The analysis of the number of mutations indicated that there are defined differences in the observed mutation frequency between the group-I and group-D for NS5A. This difference was significantly different ($P = 0.021$).

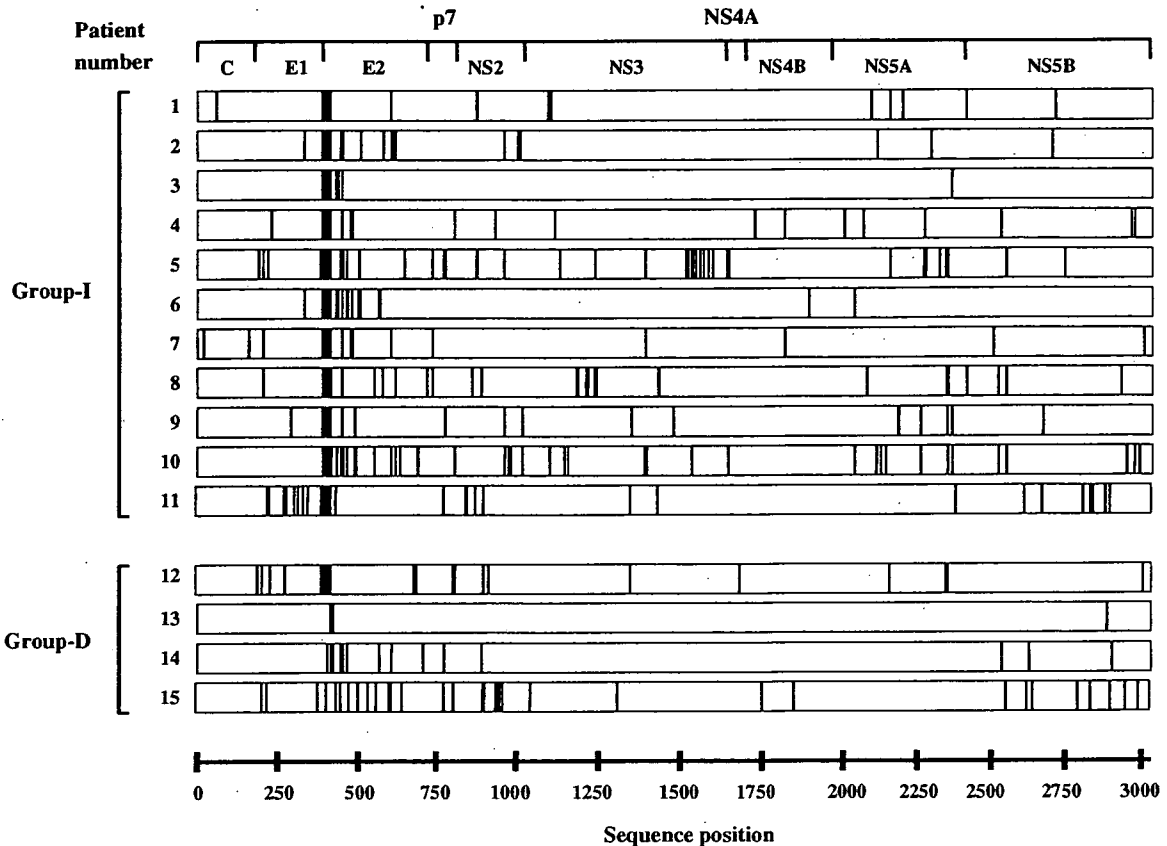


Fig. 5 Plots of mutations in full sequence in each recipients. Every mutation of amino acids has plotted on the bar for each patient. No specific point mutation was detected that was associated with change in viral load.

Sliding-window analysis

We analyzed the sequence data using a method that we previously reported [21], to find any accumulation of mutations related to viral load change across the entire sequences. We summated the total number of mutation recognized in each window constructed by 10 amino acids among all the sequence-pairs after corrected by the observation period (Fig. 6a), and compared by Mann-Whitney's *U* test (Fig. 6b). A correlation between viral load change and NSSB revealed, by collapsing the window to 2 amino acids, at position 2508–2509. These results are in agreement with those published by Qin *et al.* [22]. Qin suggested that mutations within this region have been associated with reduced NS5B activity [22].

Synonymous and nonsynonymous mutations and the molecular clock analysis

To analyze the profile of the nucleotide mutations underlying the amino acid changes, we examined the synonymous and the nonsynonymous mutations of each sequence generated from this population with Nei-Gojobori model and Jukes-

Cantor method. Ratios of the genetic distance of the non-synonymous changes (*dN*) per distance of the synonymous changes (*dS*) were calculated for the respective regions as well as the entire HCV genome (Fig. 7a). High *dN/dS* ratio reflects the immune selective pressure, and the low *dN/dS* reflects the speed of genomic evolution which is dependent on the fidelity of viral genomic replication [23]. The comparison of the *dN/dS* ratio showed that there was no overall difference between group-I and group-D.

In addition, we investigated the viral mutational rate in each patient. Averaged mutation rates of each genome were calculated by dividing distances of synonymous changes (*dS*) by the time interval between paired specimens. The viral mutational rates of both groups were almost same across the HCV genome. The rate of mutation between the donor sequence and (1) the first sera of each pair were $2.81 \times 10^{-3} \pm 0.51 \times 10^{-3}$ nucleotides/site/year in the group-I, $2.75 \times 10^{-3} \pm 0.34 \times 10^{-3}$ nucleotides/site/year in the group-D, and (2) the second sera of each pair were $2.77 \times 10^{-3} \pm 0.49 \times 10^{-3}$ in the group-I, $2.72 \times 10^{-3} \pm 0.36 \times 10^{-3}$ nucleotides/site/year in the group-D. The average mutational rate for the entire HCV genome during

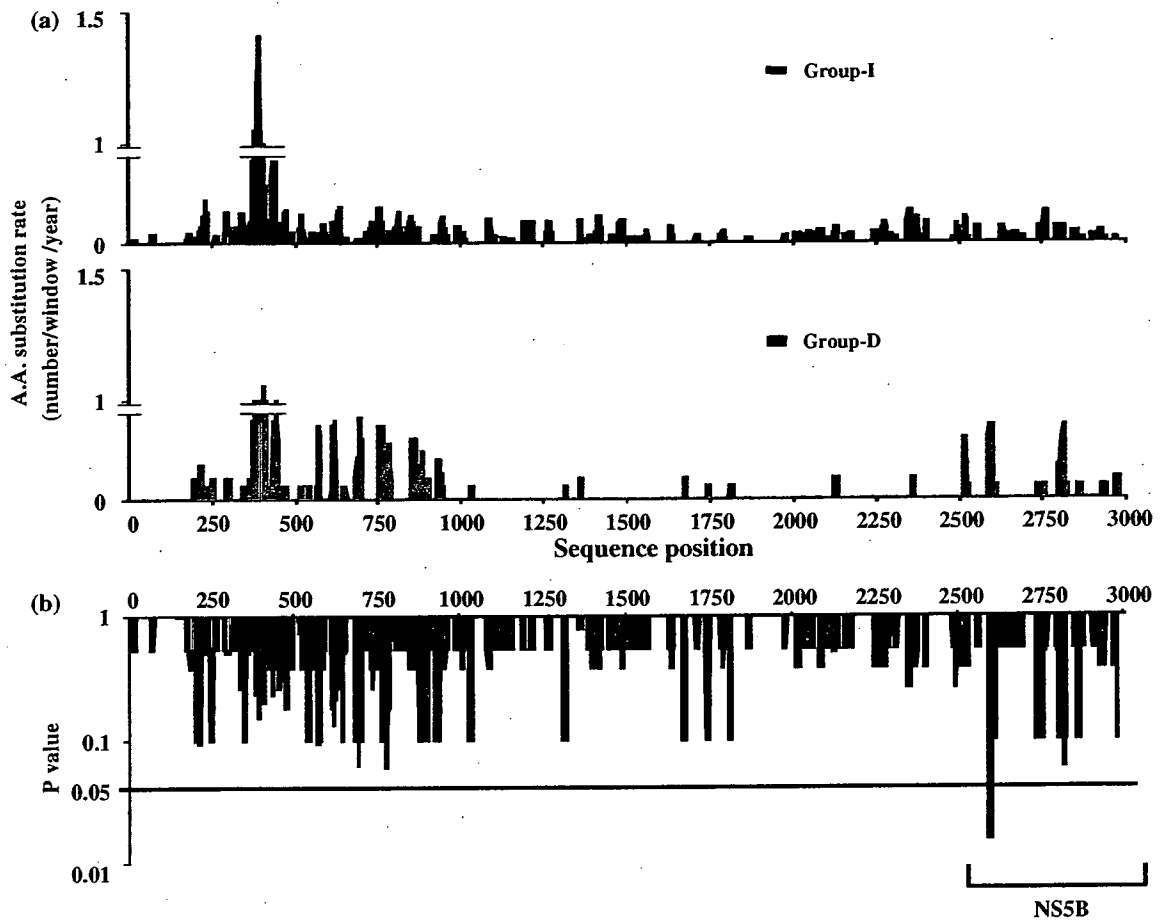


Fig. 6 Sliding-window analysis in full sequences. (a) Average mutation rates in every window are plotted according to the groups. Each window constructed with 10 amino acids. In NS5B region, calculated annual mutation numbers were high in group-D than group-I. (b) The results of statistical analysis are plotted. Longitudinal axis is appeared in logarithmic scale, areas under the bar of 5×10^{-2} indicate statistical significance. Mutation rates of two groups in E2 and NS5B region, showed difference in (a), have significance when tested by Mann-Whitney's *U* test ($P = 0.015$).

the two sampling points was as follows: $8.20 \times 10^{-3} \pm 2.0 \times 10^{-3}$ nucleotides/site/year for group-I and $10.0 \times 10^{-3} \pm 3.4 \times 10^{-3}$ nucleotides/site/year for group-D, respectively (Fig. 7b). The mutational rate of the viral genome did not differ between the two patient groups examined.

DISCUSSION

In the present study, we aimed to clarify the relationship between amino acid substitutions of HCV and serum viral load in Irish women, who were infected by an HCV-contaminated serum from a single donor during a period between 1977 and 1978. We analyzed full sequences of HCV derived from the patients whose viral load set point have increased (group-I), and those with patients whose viral load

set point has decreased (group-D). Serum ALT values and nucleotide mutation rates were not significantly different between the two groups. These results suggested that the mutation pressure is almost equal in across the two groups. Specific points of substitution directly related to the viral load were not found. However, significantly more substitution changes of amino acid were observed in NS5A in group-I when analyzed at the level of the polyprotein. Analysis using a sliding windows method revealed that the numbers of mutations in a short segment in the NS5B region was significantly higher in group-D than in group-I. These regions of NS5B are closely related to the position that is reported to be important to RNA-dependent RNA polymerase (RdRP) activity [22]. These results suggest that the determinants of hepatitis C viral load include, at least in part, virological factors.

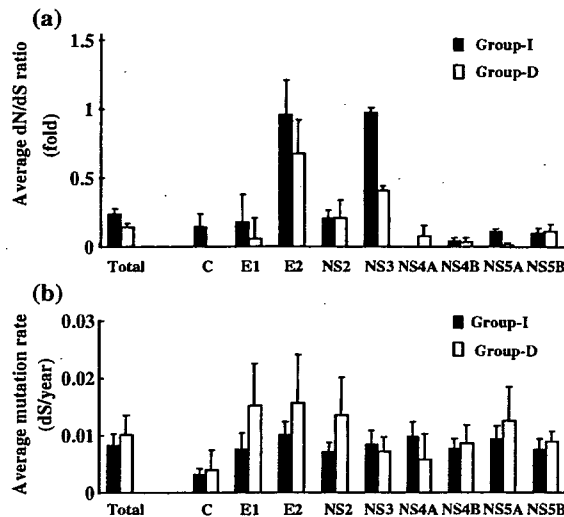


Fig. 7 The analysis of selection bias and mutation rates in RNA level. (a) Distance of nonsynonymous changes (dN) per distance of synonymous changes (dS) ratio of each coding region in comparison with two groups. Only in NS5A is the ratio significantly different between the two groups. (b) Mutation rate calculated by dS/interval time. There were no significant rate differences between two groups.

Clusters of mutations within the NS5B region of the genome were evident in this study group. The RdRP coded by NS5B contains conserved amino acid sequence motifs which essential for RdRP activity [24–26]. The mutation profiles correlated to viral load in the present study were not within these conserved motifs. However, Qin *et al.* and Labonte *et al.* reported that point mutations outside of this latter NS5B motif caused a change in the RdRP activity [22,27]. Qin *et al.* showed that the mutation of amino acid no. 191 of NS5B, co-incident with the region identified in the study reported here, lowered the RdRP activity from 100 to 3%. Furthermore, all mutations identified in our present study altered the polarity of amino acids, and the mutations in this site were only found in group-D. The mutations in the region may affect the replication capacity of the virus resulting in changes in the serum viral load. Further study will be needed which assess the relationship between the RdRP activity of the wild type HCV and those HCV with mutations observed in this study.

We found a significantly different pattern of the mutations in NS5A regions between group-I and group-D. NS5A possess transcriptional activator properties [28,29], and also considered to affect to virus–host interaction [30]. NS5A protein has been reported to bind to cellular RNA-dependent protein kinase R (PKR), a protein activated by double stranded viral RNA. The NS5A inhibition PKR prevents the down regulation of protein translation [31,32], mutations in ISDR, which within PKR-binding domain, have been shown to effect the efficacy of interferon based anti-viral therapy

[17,33]. Several researchers have previously identified a correlation between ISDR mutation and viral load [7,17,34–36]. However sequence of ISDR were highly conserved in the present study. In addition, no such relationship was found to exist in a longitudinal study of patients which were followed 2 years [37,38], or in patients with normal ALT levels [39]. These three studies suggest that ISDR is not mutation-prone region. The role of transcriptional activator properties or affection to interaction may explain the different mutation trends found between two groups in this study.

A unique feature of the anti-D patients analyzed in this study is that they are infected with a single strain genotype, and that they are young and may have an immune system that possess different potency to influence the interaction between host and virus than other less homogeneous cohorts. Seven hundred and four individuals were identified as having being iatrogenically infected through HCV 1b contaminated anti-D immunoglobulin. Three hundred and ninety of these women were viremic [12]. Three hundred and seventy six patients had been evaluated for the clinical outcome in 1997; 55% of them had evidence of elevated alanine aminotransferase. The disease progression in this group is slower than the dogma relating to the natural progression of chronic hepatitis C. Only 7 patients had evidence of cirrhosis on the liver 17 years post-infection. Fanning *et al.* have previously reported an association between HCV RNA titers and the degree of inflammation, and between the degree of inflammation and serum ALT levels [40], but Creedon *et al.* reported no association between viral load and the progression of disease [41]. HLA class II data previously generated on this group was analyzed in an attempt to identify any association between host HLA and modulations in viral load [16,42]. HLA class II, has previously been reported to be associated with changes in viral load. The number of individuals examined does not give this study the power to fully address this question, however all of the patients with the HLA DRB1 15/DQB1 0602 phenotype belonged to the increasing group.

Two previously published studies which investigated this cohort of Irish women infected by contaminated anti-D immune globulin, estimated the mutational rate to be 2×10^{-3} synonymous substitutions per site per year by using Core E1/E2 and NS5 region sequences [43,44]. Other researcher reported that the molecular clock of HCV in Japan and USA are ranged in $1-2 \times 10^{-3}$ synonymous substitutions per site per year [45]. In the previous Japanese investigations of the entire HCV genome in all but one individual, the mutational rates were calculated about 2×10^{-3} base substitutions per site per year in human and chimpanzee over a 10 year period [46,47]. The mutation rates calculated with the first and the second samples in the present study are likely to be over estimated in comparison with those described in the previous investigations. The mutational rates between the donor sequence and the patients' samples used in this study, approximate that of these other studies [43–47]. In fact,

Allain *et al.* reported the evolutionary rate of HCV genome between blood donor and recipient, the range were 3.4×10^{-4} to 4.51×10^{-3} nucleotide substitutions per site per year, which were close to the data presented here [48]. The temporal difference between the first and second samples is too small to determine the mutation rate of the viral genome accurately.

In conclusion, we have shown that the hepatitis C viral genomic mutation patterns are associated with changes in viral load in this patient group infected from a single source. Interestingly, specific small regions within NS5B were identified as associated with changes in serum viral load. Mutations in NS5A regions were correlated with the viral load, when analyzed from the viewpoint of each polyprotein as a unit. These results suggest that the viral genome composition is a determinant of the set point of viral load for the hepatitis C virus.

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Human VAP-B Is Involved in Hepatitis C Virus Replication through Interaction with NS5A and NS5B

Itsuki Hamamoto,¹ Yorihiro Nishimura,^{1,2} Toru Okamoto,¹ Hideki Aizaki,^{2,3} Minyi Liu,³ Yoshio Mori,¹ Takayuki Abe,¹ Tetsuro Suzuki,² Michael M. C. Lai,³ Tatsuo Miyamura,² Kohji Moriishi,¹ and Yoshiharu Matsuura^{1*}

Department of Molecular Virology, Research Institute for Microbial Diseases, Osaka University, Osaka,¹ and Department of Virology II, National Institute of Infectious Diseases, Tokyo,² Japan, and Department of Molecular Microbiology and Immunology, Keck School of Medicine, University of Southern California, Los Angeles, California³

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The hepatitis C virus (HCV) nonstructural protein (NS) 5A is a phosphoprotein that associates with various cellular proteins and participates in the replication of the HCV genome. Human vesicle-associated membrane protein-associated protein (VAP) subtype A (VAP-A) is known to be a host factor essential for HCV replication by binding to both NS5A and NS5B. To obtain more information on the NS5A protein in HCV replication, we screened human brain and liver libraries by a yeast two-hybrid system using NS5A as bait and identified VAP-B as an NS5A-binding protein. Immunoprecipitation and mutation analyses revealed that VAP-B binds to both NS5A and NS5B in mammalian cells and forms homo- and heterodimers with VAP-A. VAP-A interacts with VAP-B through the transmembrane domain. NS5A interacts with the coiled-coil domain of VAP-B via 70 residues in the N-terminal and 341 to 344 amino acids in the C-terminal polyproline cluster region. NS5A was colocalized with VAP-B in the endoplasmic reticulum and Golgi apparatus. The specific antibody to VAP-B suppressed HCV RNA replication in a cell-free assay. Overexpression of VAP-B, but not of a mutant lacking its transmembrane domain, enhanced the expression of NS5A and NS5B and the replication of HCV RNA in Huh-7 cells harboring a subgenomic replicon. In the HCV replicon cells, the knockdown of endogenous VAP-B by small interfering RNA decreased expression of NS5B, but not of NS5A. These results suggest that VAP-B, in addition to VAP-A, plays an important role in the replication of the HCV genome.

Hepatitis C virus (HCV) infects 170 million people worldwide and frequently leads to cirrhosis or hepatocellular carcinoma (6, 29). HCV is classified in the family *Flaviviridae* and possesses a single-stranded positive-sense RNA with a length of 9.6 kb. The HCV genome encodes a single large precursor polyprotein composed of about 3,000 amino acids (aa) that is processed by cellular and viral proteases, resulting in at least 10 structural and nonstructural (NS) proteins (29). Details of HCV's replication cycle are unknown because of the low viral load in the sera of HCV-infected individuals and the lack of a reliable and robust cell culture system to support HCV infection and replication. The development of HCV RNA replicons in which a synthetic HCV genomic or subgenomic RNA replicates efficiently in the human hepatocarcinoma cell line Huh-7 has enabled the study of viral RNA replication in cell culture (4, 20, 24). The HCV RNA replication complex, composed of the viral NS proteins and host cellular proteins, replicates the viral RNA genome at the intracellular membrane. Thus far, the HCV replicon system has greatly contributed to the understanding of HCV replication and pathogenesis associated with the expression of viral NS proteins. Replication of positive-strand RNA viruses generally involves certain intracellular membrane structures, including the endoplasmic reticulum (ER), Golgi apparatus, endosome, and lysosome (39).

Recently, several groups have succeeded in demonstrating cell-free replication activities of replication complexes in crude membrane fractions of HCV subgenomic replicon cells (2, 3, 14, 53). These cell-free systems provide semi-intact polymerase assays for biochemical dissection of HCV RNA replication and are a useful source for the isolation of HCV replication complexes. Replication complexes were detected in detergent-resistant membrane structures, most likely lipid raft structures (2, 14). Although HCV NS proteins presumably form a membrane-associated RNA replication complex with host proteins, the precise components and mechanisms for replication are poorly understood.

HCV NS5A is a phosphoprotein that appears to possess multiple and diverse functions in viral replication, interferon resistance, and pathogenesis (26, 35). Cell culture-adaptive mutations have been shown to cluster in the central portion of NS5A in subgenomic HCV replicons, indicating that NS5A is involved in the viral replication process either directly or by interacting with host cellular proteins (4, 55). This observation, together with the modulation of NS5A hyperphosphorylation by NS3, NS4A, and NS4B and physical interaction with other viral NS proteins, strongly supports the notion that NS5A is an essential component of the HCV replication complex (21, 30, 36). NS5A has been shown to be associated with a range of cellular proteins involved in cellular signaling pathways, such as interferon-induced kinase PKR (11), growth factor receptor-binding protein 2 (Grb2) (45), p53 (27, 37), phosphoinositide-3-kinase p85 subunit (15), and proteins in protein trafficking and membrane morphology, such as karyopherin β 3 (8),

* Corresponding author. Mailing address: Department of Molecular Virology, Research Institute for Microbial Diseases, Osaka University, 3-1, Yamadaoka, Suita, Osaka 565-0871, Japan. Phone: 81-6-6879-8340. Fax: 81-6-6879-8269. E-mail: matsuura@biken.osaka-u.ac.jp.