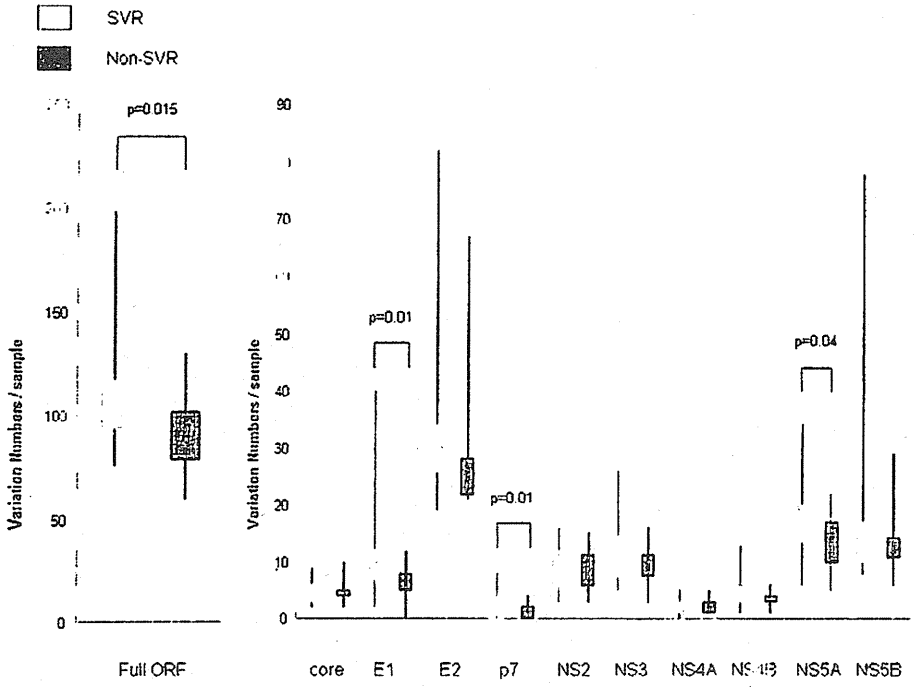


**Figure 2. Number of amino acid substitutions per sample in the sustained virological responders (SVR) and the non-sustained virological responders (non-SVR) group.** The numbers of variations, relative to a population consensus, that were unique to either SVR or non-SVR patients are shown for the complete open reading frame (ORF) (Fig. 1, left) and for each HCV protein (Fig. 1, right).
doi:10.1371/journal.pone.0024514.g002

(PEGINTRON®, Schering-Plough, Tokyo, Japan) plus RBV (REBETOL®, Schering-Plough) between 2005 and 2009 at University of Yamanashi, Tokyo Medical and Dental University, and related institutions were first included in the study. They all fulfilled following criteria: (1) negative for hepatitis B surface antigen, (2) high viral load (≥100 KIU/ml), (3) absence of

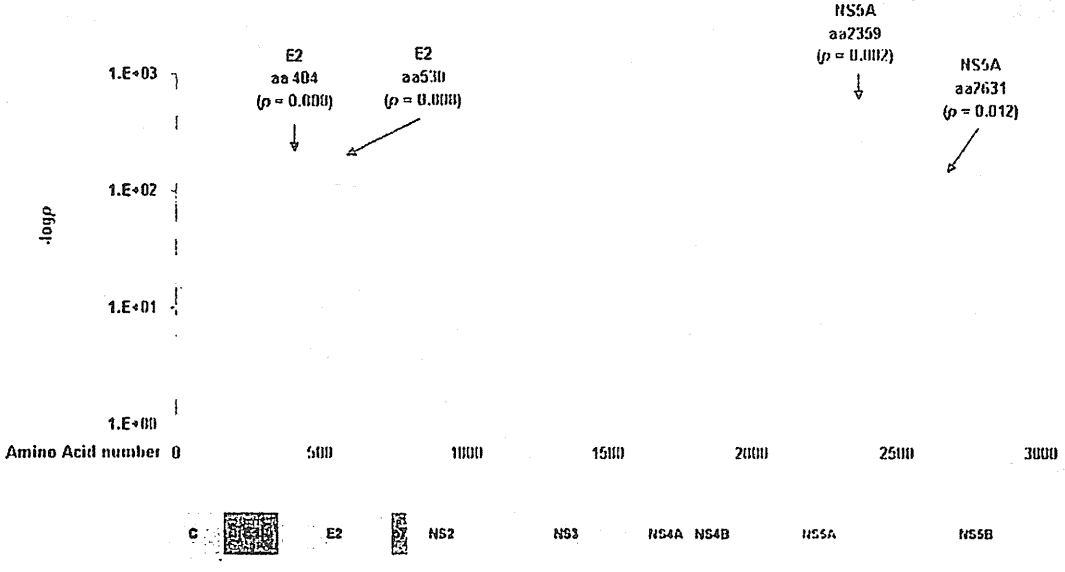


**Figure 3. Different amino acid usage at each viral amino acid position between the sustained virological responders (SVR) and the non-sustained virological responders (non-SVR) patients.** (a) Different amino acid usage at each viral amino acid position between the SVR and the non-SVR patients was analyzed by Fisher's exact probability test. The longitudinal axis shows the −logP value. (b) Sequence alignment in the Core region is demonstrated. Dashes indicate amino acids identical to the consensus sequence and substituted amino acids are shown by standard single letter codes.
doi:10.1371/journal.pone.0024514.g003

**Table 2.** Variation at each Amino Acid Position and SVR rate.

| | E2 aa 404 non T | E2 aa 530 non T | NS5A aa 2359 N | NS5B aa 2631 non P |
|---|---|---|---|---|
| SVR rate | 86.1% (31*/36**, p=0.008) | 87.9% (29/33, p=0.008) | 82% (41/50, p=0.002) | 94.7% (18/19, p=0.012) |

*SVR number in patients fulfilling the criteria.
**Number of patients fulfilling the criteria.
doi:10.1371/journal.pone.0024514.t002

hepatocellular carcinoma, (4) no other form of hepatitis, such as primary biliary cirrhosis, autoimmune liver disease, or alcoholic liver disease, (5) free of co-infection with human immunodeficiency virus. To clearly disclose the non-SVR viral characteristics, we have considered only those patients who achieved total drug administration of 60% or more for both PEG-IFN and RBV, with the completion of the standard treatment duration. Moreover, although we excluded patients with extended therapy to make the studied population uniform, we have included non-SVR patients with extended therapy to clarify the specific characteristics of non-SVR patients, a minor population group. As a result, 17 patients were excluded for the following reasons: 1 patient received insufficient dose, 4 patients were discontinued from the therapy within 12 weeks, and 12 SVR patients received extended therapy. Finally, 60 patients were considered as eligible for the study. During the combination therapy, blood samples were obtained at least once every month before, during and after treatment and were analyzed for blood count, ALT and HCV RNA levels. Liver biopsy specimens were obtained from most of the patients. All patients gave written informed consent to the study. The study was approved by the ethics committees of University of Yamanashi, Tokyo Medical and Dental University, and related institutions. The therapy was performed according to the standard treatment protocol of PEG-IFN/RBV therapy for Japanese patients established by a hepatitis study group of the Ministry of Health, Labour, and Welfare, Japan (PEG-IFNα-2b 1.5 μg/kg body weight, once weekly subcutaneously, and RBV 600–800 mg daily per os for 24 weeks).

## Complete HCV-ORF Sequence Determination by Direct Sequencing from Pretreatment Sera

HCV RNA was extracted from pretreatment serum samples by the AGPC method using Isogen (Wako, Osaka, Japan) according to the following protocol. Briefly, 150 μl of serum were mixed with 700 μl of Isogen, and an aqueous phase was extracted with 150 μl of chloroform. RNA was precipitated with 600 μl of isopropanol and with 2 μl of Glyco Blue (Ambion, Tokyo, Japan) as a carrier. The purified RNA was washed once with ethanol and finally dissolved in 15 μl of distilled water and stored at −70°C until use.

Complementary DNA was synthesized according to the following protocol. 30 μl of the reverse transcription mixture were adjusted to contain 3 μl of the RNA solution, 300 U of Superscript

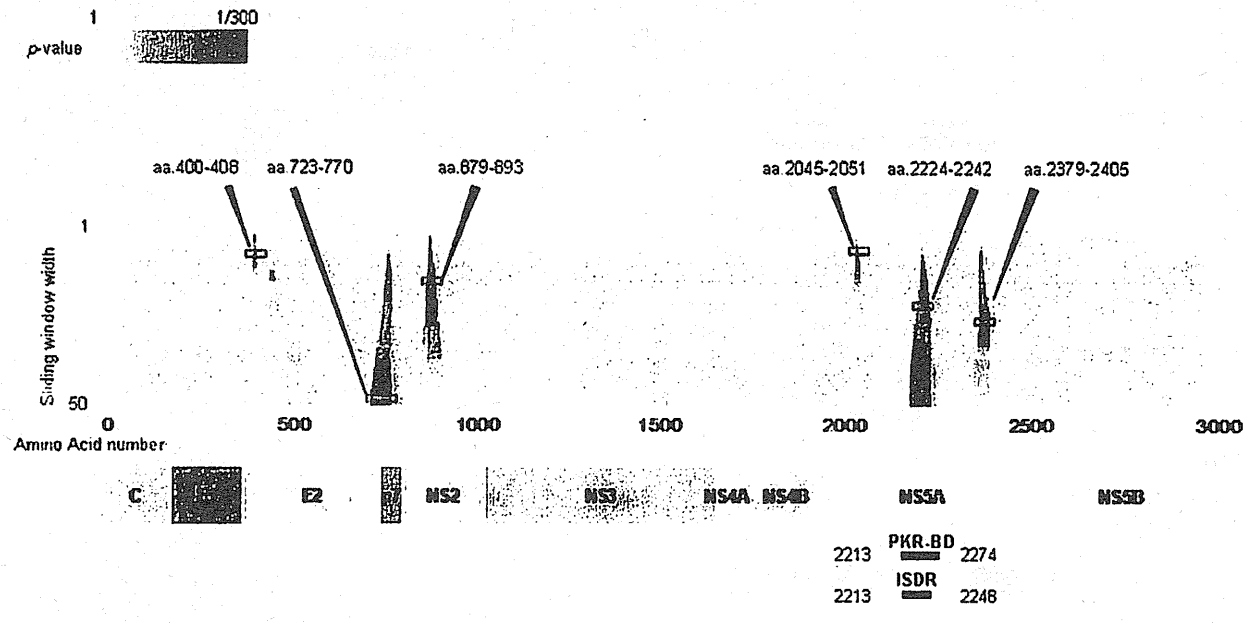


**Figure 4. Sliding window analysis.** (a) Comparison of amino acid variation between the SVR and non-SVR patients across HCV "regions" using sliding window analysis was performed. Viral regions affecting treatment outcome are shown as red areas. There are six hot areas: amino acid 400–408 and 723–770 in the E2 region, amino acid 879–893 in the NS2 region and, amino acid 2045–2051, 2224–2242 and 2379–2405 in the NS5A region. (b) Sequence alignment in the nonstructural (NS)5A around amino acids 2213 to 2274 is demonstrated. Dashes indicate amino acids identical to the consensus sequence and substituted amino acids are shown by standard single letter codes.
doi:10.1371/journal.pone.0024514.g004

**Table 3.** Number of Amino Acid Substitutions in each Region and SVR rate.

| | E2 aa 400–408 mutation ≥4 | E2 aa 723–770 mutation ≥2 | NS2 aa 879–893 mutation ≥2 | NS5A aa 2045–2051 absense of mutation | NS5A ISDR (aa 2213–2248) mutation ≥1 | NS5A aa 2224–2242 mutation ≥1 | NS5A aa 2379–2405 mutation ≥2 |
|---|---|---|---|---|---|---|---|
| SVR rate | 86.5% (32*/37**) p=0.006 | 100% (18/18) p=0.001 | 94.7% (18/19) p=0.01 | 89.7% (35/39) p=0.0002 | 86.1% (31/36) p=0.008 | 90.9% (30/33) p=0.001 | 90.9% (20/22) p=0.03 |

*SVR number in patients fulfilling the criteria.
**Number of patients fulfilling the criteria.
doi:10.1371/journal.pone.0024514.t003

II (Invitrogen, Tokyo, Japan) with an accompanied buffer according to the manufacturer's instructions, 60 units of RNase inhibitor (Promega Corp., Madison, WI), and 300 pg of random primers (Invitrogen). The mixture was incubated at 37°C for 30 min. The HCV genome was amplified with 24 partially overlapping primer (Table S6) sets, designed specifically for this study, to perform two-step nested PCR. As previously reported, a M13 forward primer (5′-TGTAAAACGACGGCCAGT-3′) and a M13 reverse primer (5′-CAGGAAACAGCTATGACC-3′) were attached to the 5′ termini of the sense and antisense second-round PCR primers, respectively, to facilitate direct sequencing. All samples were initially denatured at 95°C for 7 min., followed by 40 cycles with denaturation at 95°C for 15 seconds, annealing at 55°C for 15 seconds, and extension at 72°C for 45 seconds with BD Advantage™ 2 PCR Enzyme System (BD Biosciences Clontech, CA, USA). PCR amplicons were sequenced directly by Big Dye Terminator Version 3.1 (ABI, Tokyo, Japan) with universal M13 forward/M13 reverse primers using an ABI prism 3130 sequencer (ABI). The sequence files generated were assembled using Vector NTI software (Invitrogen) and base-calling errors were corrected following visual inspection of the chromatogram. When several peaks were observed at the same nucleotide position in the chromatogram, the highest chromatogram peak was read as the dominant nucleotide. In sequence analysis, multiple sequence alignment was performed with ClustalW, and the mean genetic distance was calculated using the p-distance algorithm in the MEGA version 4 DNA software. As a result, 60 genotype-2b HCV full open reading frame sequences were determined. In Table S1, obtained GenBank accession numbers for these sequences determined in this study are listed.

**Table 4.** Multivariate Logistic Regression Analysis.

| Factor | odds (95% CI) | p value |
|---|---|---|
| Age | 0.94 (0.85–1.04) | 0.20 |
| E2 aa 530 non T | 4.33 (0.48–39.3) | 0.19 |
| NS5A aa 2359 N | 3.22 (0.18–57.7) | 0.43 |
| NS5B 2631 non P | 5.14 (0.29–91.2) | 0.26 |
| NS2 aa 879–893 mutations ≥2 | 9.77 (0.52–182) | 0.13 |
| NS5A aa 2045–2051 no mutations | 4.46 (0.39–50.6) | 0.23 |
| NS5A aa 2224–2242 mutations ≥1 | 11.0 (1.13–107) | 0.04 |
| NS5A aa 2379–2405 mutations ≥1 | 7.03 (0.62–79.8) | 0.12 |

To evaluate the optimal threshold of amino acid variations for SVR prediction in each viral region extracted, a receiver operating characteristic curve was constructed and the most optimal cut off value was determined for each region.
doi:10.1371/journal.pone.0024514.t004

## Sliding Window Analysis

A sliding window analysis was introduced to search through HCV amino acid "regions", rather than single amino acid positions, related to the final outcome of PEG-IFN/RBV therapy. Briefly, the total number of amino acid substitutions compared to the consensus sequence within a given amino acid length were counted at each amino acid position in each HCV sequence. The consensus sequence was generated from these 60 patients. Then the relation of substitution numbers and the final outcome was compared statistically between the SVR and non-SVR groups by Mann-Whitney's U test for each amino acid position. In this study, we changed the window length from 1 to 50 to search for those HCV regions. To visualize the result, significantly lower p-values were colored in red and non-significant p-values were colored in green using Microsoft Excel software to generate a "heat map" appearance. In the present study, p-value of 1/300 or lower was colored in the maximum red.

## Statistical Analysis

Statistical differences in the parameters, including all available patients' demographic, biochemical, hematological, and virological data such as sequence variation factors, were determined between the various groups by Mann-Whitney's U test for numerical variables and Fisher's exact probability test for categorical variables. To evaluate the optimal threshold of variations for SVR prediction, a receiver operating characteristic curve was constructed and the area under the curve as well as the sensitivity and specificity were calculated. Variables that achieved statistical significance ($p<0.05$) in univariate analysis were entered into multiple logistic regression analysis to identify significant independent factors. We also calculated the odds ratios and 95% confidence intervals. All p values of $<0.05$ by the two-tailed test were considered significant.

# Results

## Characteristics of the patients studied

The SVR rate of the patients analyzed was 75.9% (44/58) with the standard therapy (two non-SVR patients received extended therapy). The baseline characteristics of the patients classified according to achievement of SVR are shown in Table 1. Rapid virological response (RVR; undetectable serum HCV RNA within 4 weeks) and early virological response (EVR; undetectable serum HCV RNA within 12 weeks) rates were significantly higher in SVR patients ($p=0.0008$ and $0.004$). In addition, patients with non-SVR were older ($p=0.04$). Pretreatment HCV RNA titer, which is known to affect the treatment outcome in genotype 1 and 2a HCV infection, did not differ significantly between two groups. Achievement of RVR reached 42.4% when all patients were included, and this rate was high compared to achievement of RVR in patients with genotype 1b infection (~10%) observed in

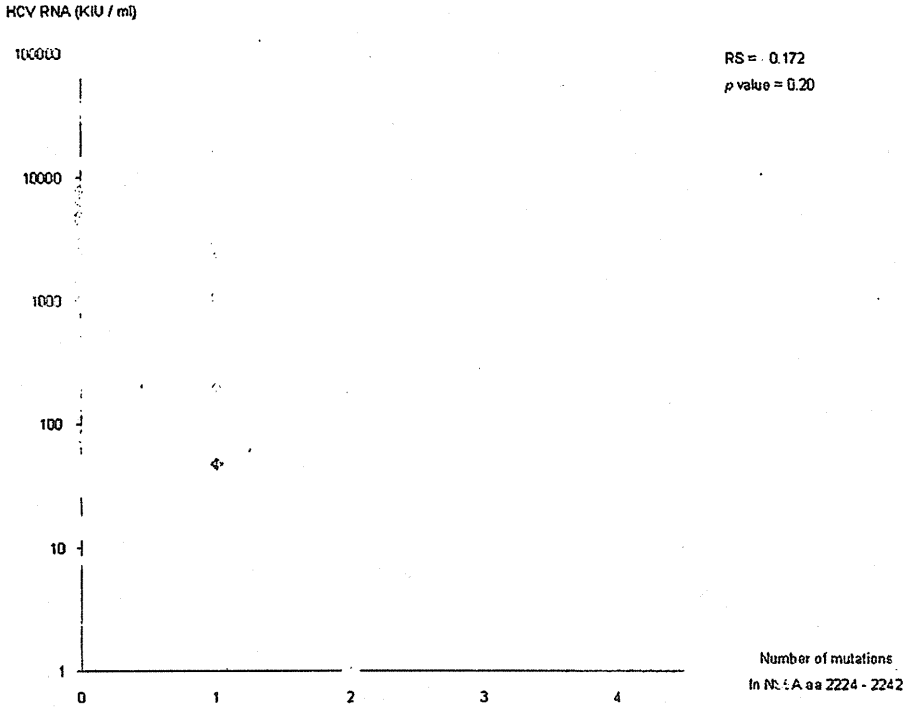


**Figure 5. Correlation between pretreatment HCV RNA levels and the number of substitutions in the NS5A region aa 2224 to 2242.** Spearman's correlation coefficient by rank test is demonstrated.
doi:10.1371/journal.pone.0024514.g005

University of Yamanashi (data not shown). The early virological response (EVR) rate was equally high in the SVR (97.7%) and non-SVR (68.8%) groups. Interestingly, most of the non-SVR patients (14/16, 87.5%) in genotype-2b HCV infection showed end-of-treatment response (ETR; undetectable serum HCV RNA at the end of therapy), demonstrating that the main cause of non-SVR was relapse (reappearance of hepatitis C viremia during the follow-up period after stopping therapy in patients with an ETR, n = 14), and not null response (detectable serum HCV RNA at the end of therapy, n = 2).

## Phylogenetic analysis of SVR and non-SVR patients using the complete HCV amino acid sequence

To determine the viral sequence characteristics in the SVR and non-SVR groups, we first aligned all 60 HCV complete ORF amino acid sequences obtained from the patients' pretreatment sera along

**Table 5.** Baseline Characteristics of patients with NS5A aa 2224–2242 variations none or 1≤.

| Characteristic | Variation 1≤ (n = 33) | No variation (n = 27) | *P* value |
|---|---|---|---|
| Gender (Male/Female) | 17/16 | 18/9 | NS† |
| Age (yrs) | 57 (29–72)* | 57 (22–80) | NS‡ |
| ALT (IU/l) | 72 (19–380) | 47 (17–390) | NS‡ |
| Platelet ($\times 10^4/mm^3$) | 19.3 (7.1–31.8) | 17.5 (10.4–36.7) | NS‡ |
| Fibrosis score (0–2/≥3)§ | 26/5 | 19/3 | NS† |
| HCV RNA (KIU/ml) | 1600 (100–16000) | 2450 (140–13000) | NS‡ |
| IFN dose (≥80%/60–80%) | 26/7 | 23/4 | NS† |
| Ribavirin dose (≥80%/60–80%) | 24/9 | 19/8 | NS† |
| RVR rate (%) | 53.1 | 29.6 | NS† |
| EVR rate (%) | 96.9 | 81.5 | NS† |
| SVR rate (%) | 90.9 | 51.9 | 0.001† |
| Replapse rate (%) | 40.7 | 9.1 | 0.006† |

§: 1≤ : n = 31, 0 : n = 22.
*: median (range).
†: Fisher's exact probability test.
‡: Mann-Whitney's U test.
doi:10.1371/journal.pone.0024514.t005

with reference sequences (2b.HC-J8.D10988, 2.JP.MD2b9-2, and 2a.JP.JFH-1.AB047639 obtained from the Los Alamos HCV Database as representative sequences for genotype 2b and genotype 2a HCV) and constructed a phylogenetic tree (Fig. 1). As demonstrated in the tree, no evident clustering was apparent according to the difference of responses.

## Comparison of amino acid variation between the SVR and non-SVR in the complete HCV polyprotein and each HCV protein

Next, we compared amino acid variations that were unique, relative to a population consensus, to either the SVR or non-SVR patients for the complete HCV polyprotein and each HCV protein. The number of amino acid variations in the sequences from the SVR patients was significantly higher than in those from the non-SVR patients, when the entire HCV polyprotein was analyzed (Fig. 2, left). These differences were especially significant in E1, p7 and NS5A (Fig. 2, right). This result demonstrated that HCV sequences from patients with SVR comprised a heterogeneous population, while HCV sequences from patients with non-SVR comprised a rather homogeneous population, indicating the existence of unique non-responsive HCV sequences in those regions in E1, p7, and NS5A.

## Comparison of HCV sequence variation between the SVR and non-SVR patients at each amino acid position

Each amino acid position in the HCV ORF was compared to detect any differences between the SVR and non-SVR patients. In Fig. 3a, differences in amino acid resides at each position are shown as dots demonstrating $-\log P$ values. As shown in Table 2, four points were extracted: amino acid (aa) 404 in the E2 region ($p = 0.008$), aa 530 in the E2 region ($p = 0.008$), aa 2359 in the NS5A region ($p = 0.002$) and aa 2631 in the NS5B region ($p = 0.012$). Among them, the residue at aa 2359 in the NS5A region differed most frequently between the SVR and non-SVR patients. Amino acids 4 and 110 in the Core region, residues that have been reported to vary according to the virological responses in genotype 2a infection [22,23], did not differ significantly in this genotype 2b HCV study. Meanwhile, amino acids 70 and 91, which have been reported to vary according to virological response to PEG-IFN/RBV therapy in genotype 1b infection, were conserved irrespective of the outcome (Fig. 3b).

## Comparison of amino acid variation between the SVR and non-SVR patients across HCV "regions" using sliding window analysis

Fig. 4a and Table 3 shows the result of sliding window analysis. This approach was used to detect differing HCV amino acid "regions", rather than single amino acid positions, between the SVR and the non-SVR patients. According to the result, six regions were associated with the final outcome (p-values less than 1/20): aa 400–408 in the E2 region ($p = 0.006$), aa 723–770 in the E2 and the N-terminus of p7 region ($p = 0.001$), aa 879–893 in the NS2 region ($p = 0.01$), aa 2045–2051 in the NS5A region ($p = 0.0002$), aa 2224–2242 in the NS5A region ($p = 0.001$) and aa 2379–2405 in the NS5A region ($p = 0.03$). Interestingly, aa 2224–2242 in the NS5A was located in the interferon sensitivity determining region (ISDR). Fig. 4b shows the aligned sequences of amino acids around 2213–2274 of HCV NS5A. Among these 6 regions, aa 723–770, aa 879–893, aa 2224–2242, and aa 2379–2405 were correlated with the final outcome in an incremental manner according to the number of substitutions in those regions (Table S2, S3, S4, S5). The number of substitutions in the ISDR was also correlated to the final outcome in an incremental step-up manner (data not shown).

## Multivariate analysis to detect independent predictive factors contributing to the SVR

Next, multivariate analysis was undertaken to identify pretreatment variables correlated with the final outcome. To evaluate the optimal threshold of amino acid variations for SVR prediction in each viral region extracted, a receiver operating characteristic curve was constructed and the most optimal cut off value was determined for each region. E2 aa404–408 was excluded from the analysis because we considered that the region was unlikely to be truly associated to the outcome as it is located in the hypervariable region, the region of the highest mutation rate in the HCV genome as a result of host's immune attack. E2 aa 723–770 was excluded from the analysis because all the patients above the cut-off value in the region achieved SVR and an odds calculation was not possible. The ISDR was also excluded because NS5A aa2224–2242 was completely contained in the ISDR. In addition, variables of EVR and RVR were excluded because they were post treatment variables. The multivariate analysis revealed that only NS5A aa 2224–2242 (odds ratio 11.0, $p = 0.039$) was finally identified as the independent variable predicting the final outcome (Table 4).

## Biological relevance of variation in NS5A in this study group

Because NS5A aa 2224–2242 is located within the ISDR, for which the amino acid substitution numbers have been reported to be correlated with the HCV RNA titer in genotype 1 and 2a HCV infection [13], we analyzed the relationship between amino acid variations in that region and pretreatment HCV RNA titers. Contrary to our expectation, no evident relationship was found between variations in the NS5A region aa 2224–2242 and HCV RNA titer (Fig. 5). On the other hand, as shown in Table 5, although the initial viral responses (RVR or EVR) did not show evident association with the amino acid variations in the region, treatment relapse was significantly correlated with the amino acid variations in the region. In addition to NS5A aa 2224–2242, there was no evident relationship between HCV RNA level and variations in the other regions found in this study (data not shown).

## Discussion

In this study, we showed that genotype 2b HCV sequences from Japanese patients who achieved SVR were more diverse than the sequences from patients with non-SVR. The result that SVR patients were more diverse in their HCV sequences than non-SVR patients is in accordance with previous studies of genotype 1 HCV infection, although the diverse viral genes varied according to genotype [18,19]. We found that these diversities were primarily found in E1, p7 and NS5A.

In systemic searching for single amino acid positions or consecutive amino acid regions in the HCV ORF associated with the treatment outcome, several regions were extracted in E2, p7, NS2, NS5A and NS5B. Among those identified regions, E2 aa 723–770, NS2 aa 879–893, NS5A aa2224–2242, and NS5A aa2379–2405 were correlated with the final outcome in an incremental manner according to the number of amino acid substitutions. Specifically, the sequences of those regions in non-SVR patients were almost homogeneous, while the sequences of the region in SVR patients were significantly diverse and multiple amino acid substitutions were found compared to the consensus sequence. Interestingly, among those regions, aa 2224–2242 was completely included in the ISDR, in which the number of amino acid substitutions is known to show significant correlation with

the treatment response to IFN-based therapy in genotype 1b, and also in genotype 2 [21,24].

In recent studies of genotype 1b infection, amino acid variation of residues 70 and 91 in the Core were reported to be associated with the treatment response to IFN-based therapy. The correlation of amino acid variation in the Core (residues 4 and 110) with the response to PEG-IFN/RBV therapy was also identified in genotype 2a infection [22,23]. In genotype 2b infection, however, we could not find such associations between amino acid variation in the core region and the response to PEG-IFN/RBV therapy (Fig. 3b). Amino acid residues of aa 70 and 91 were conserved irrespective of differences in the PEG-IFN/RBV responses. On the other hand, although amino acid variations were also sometimes found at residues 4 and 110 in genotype 2b HCV, their frequency was low, and no evident association between the variation and the treatment response was found. Although the reason of the lack of association between the Core and the PEG-IFN/RBV treatment response in genotype-2b HCV infection is unknown, it suggests that a different mechanism affecting the treatment response might exist, depending on genotype-specific viral features.

In genotype 1 HCV, variations within the PKR-binding region of NS5A, including those within the ISDR, were reported to disrupt the NS5A-PKR interaction, possibly rendering HCV sensitive to the antiviral effects of interferon [25]. Clinically, the number of substitutions within the ISDR has been reported to correlate with the serum HCV RNA level in genotype 1 and 2a infections [13]. In addition, a recent study reported that mutations in the ISDR also show the correlation with the relapse in the PEG-IFN/RBV therapy in genotype 1b infection [26]. Because NS5A aa2224–2242, part of ISDR, was extracted as one of those regions related to the treatment response in genotype 2b infection, we undertook further analysis to investigate the correlation between amino acid variation numbers and serum HCV RNA level. Though the reason is unknown, we could not find evidence of a relationship between variation in the NS5A aa 2224–2242 and HCV RNA titer in genotype 2b infection, unlike genotypes 1 and 2a. Of note, a high SVR rate in genotype1 and genotype 2a infection is known to be closely correlated with a low HCV RNA level and multiple substitutions in ISDR. However, in genotype 2b infection in our study, there was no significant difference in the HCV RNA level between SVR and non-SVR patients, as shown in Table 1. Previously, the role of the ISDR in the contribution to SVR in genotype 1 and 2a has been discussed in detail in the context of serum HCV RNA level, and multiple substitutions in the ISDR are related to a low HCV RNA level and high SVR rate. However, it is not known which of these two factors is directly associated with viral clearance. Consideration of this three-sided relationship of ISDR, HCV RNA level and SVR rate in genotype-2b infection leads to the suggestion that amino acid variation in ISDR to be more direct contributor for SVR.

In spite of these findings, there were still limitations in our study. First, because genotype 2b infection only accounts for 10% of all HCV infection in Japan, the number of studied patients was rather small, especially non-SVR patients. In addition, because genotype 2b HCV contains as many as 3033 amino acids, it is possible that incorrect amino acids or regions were judged as significant in the complete HCV ORF comparison study as a result of type I errors. Therefore, if more patients were available for the analysis, the statistical power detecting the meaningful differences would be greater. Secondly, we could not include the IL28B SNP analysis in this study. If we could have combined the information of IL28B SNPs with the full HCV ORF information, a more comprehensive analysis would have been achieved.

In conclusion, we have shown that viral sequences were more diverse in SVR patients infected with genotype 2b HCV. Through systematic comparison between SVR and non-SVR patients, we have also shown that several localized regions were extracted as hot spots whose amino acid substitutions were closely related to the final outcome by affecting the relapse rate in the PEG-IFN/RBV therapy.

## Supporting Information

**Table S1 GenBank Accession Numbers.** Obtained GenBank accession numbers for 60 genotype-2b HCV full open reading frame sequences are listed.
(DOC)

**Table S2 Substitutions in NS5A aa 2224–2242 Amino Acid Regions and SVR rate.** SVR rate increased with the number of substitutions in this region.
(DOC)

**Table S3 Substitutions in NS5A aa 2379–2405 Amino Acid Regions and SVR rate.** SVR rate increased with the number of substitutions in this region.
(DOC)

**Table S4 Substitutions in NS2 aa 879–893 Amino Acid Regions and SVR rate.** SVR rate increased with the number of substitutions in this region.
(DOC)

**Table S5 Substitutions in E2 aa 723–770 Amino Acid Regions and SVR rate.** SVR rate increased with the number of substitutions in this region.
(DOC)

**Table S6 PCR Primer List.** Primers designed to perform two-step nested PCR for this study are listed. Dominant genotype-2b HCV full open reading frame sequences was determined by the 24 partially overlapping amplicons amplified by these primers.
(XLS)

## Acknowledgments

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## Author Contributions

Conceived and designed the experiments: MK SM NE. Performed the experiments: MK. Analyzed the data: MK SM NE. Contributed reagents/materials/analysis tools: RS MM HS KK. Wrote the paper: MK SM NE. Critical revision of the manuscript for important intellectual content: FA TU TI MS MN NS MW.

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ORIGINAL ARTICLE

# Analysis of the complete open reading frame of hepatitis C virus in genotype 2a infection reveals critical sites influencing the response to peginterferon and ribavirin therapy

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## Abstract

*Purpose* A proportion of patients infected with genotype 2a hepatitis C virus (HCV) cannot achieve a sustained virological response (SVR) to pegylated-interferon plus ribavirin therapy (PEG-IFN/RBV) but the reason remains unclear. The present study aimed to clarify the possible correlation between viral sequence variations and final outcome.

*Methods* The pretreatment complete open reading frame (ORF) sequences of genotype 2a HCV were determined by direct sequencing for two independent groups of patients (43 patients as test; group 1 and 35 as validation; group 2), and the correlation with the final outcome was explored.

*Results* Patients with SVR ($n = 58$) and with non-SVR ($n = 20$) differed significantly in pretreatment HCV RNA level ($p = 0.002$), fibrosis score ($p = 0.047$), and cumulative RBV dosage ($p = 0.003$). By comparison of all amino acid positions in the complete HCV ORFs, threonine at amino acid (aa) 110 in the core region was remarkably frequent in SVR ($p = 0.01$ for group 1, $p = 0.004$ for group 2, and $p = 5E{-}05$ for combined). A sliding window analysis revealed that the total number of amino acid variations within the NS5A aa 2258–2306 region were significantly high in SVR compared to non-SVR patients ($p = 0.01$ for group 1, $p = 0.006$ for group 2, and $p = 0.0006$ for combined). Multivariate analyses revealed that core aa 110 ($p = 0.02$), NS5A aa 2258–2306 ($p = 0.03$), and cumulative RBV dosage ($p = 0.02$) were identified as independent variables associated with the final outcome.

*Conclusions* The outcome of PEG-IFN/RBV therapy is significantly influenced by variation in the core and NS5A regions in genotype 2a HCV infection.

**Abbreviations**

| | |
|---|---|
| EVR | Early virological response |
| IFN | Interferon |
| IRRDR | Interferon ribavirin resistance determinant region |
| ISDR | Interferon sensitivity determinant region |
| ORF | Open reading frame |
| PEG-IFN | Pegylated-interferon |
| PePHD | PKR-eIF2 phosphorylation homology domain |
| PKR-BD | Double-stranded RNA-activated protein Kinase binding domain |
| RBV | Ribavirin |
| RVR | Rapid virological response |
| SVR | Sustained virological response |

## Introduction

Worldwide, 180 million of people are estimated to be infected with hepatitis C virus (HCV), and HCV is a major cause of chronic hepatitis, liver cirrhosis, and

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**Table 1** Baseline characteristics of all patients (groups 1 and 2)

| Characteristic | SVR ($n = 58$) | | | Non-SVR ($n = 20$) | | | $p$ value$^{\Delta}$ |
|---|---|---|---|---|---|---|---|
| | Group 1 ($n = 36$) | Group 2 ($n = 22$) | Combined ($n = 58$) | Group 1 ($n = 7$) | Group 2 ($n = 13$) | Combined ($n = 20$) | |
| Gender (male/female) | 20/16 | 9/13 | 29/29 | 4/3 | 5/8 | 9/11 | 0.80$^{\dagger}$ |
| Age (years) | 50.0 ± 12.5* | 57.3 ± 10.0 | 52.4 ± 12.1 | 55.0 ± 9.7 | 59.8 ± 6.4 | 58.1 ± 7.8 | 0.058$^{\ddagger}$ |
| ALT (IU/l) | 86.6 ± 86.6 | 71.2 ± 50.4 | 80.5 ± 74.2 | 52.9 ± 29.3 | 88.1 ± 90.1 | 75.8 ± 75.5 | 0.81$^{\ddagger}$ |
| Platelet ($\times 10^4$/mm$^3$) | 20.8 ± 6.2 | 19.0 ± 5.2 | 20.1 ± 5.8 | 14.7 ± 7.1 | 19.1 ± 4.9 | 17.6 ± 6.0 | 0.11$^{\ddagger}$ |
| Fibrosis score (0–2/≥3)$^{\S}$ | 34/1 | 19/2 | 53/3 | 4/3 | 11/2 | 15/5 | 0.049$^{\dagger}$ |
| HCV RNA (KIU/ml) | 760 (2–3,100)** | 340 (54–3,600) | 550 (12–3,600) | 1,300 (350–30,000) | 1,400 (180–5,000) | 1,300 (180–30,000) | 0.002$^{\parallel}$ |
| IFN dose (≥80%/60–80%)$^{\P}$ | 28/4 | 21/1 | 49/5 | 4/3 | 11/2 | 15/5 | 0.12$^{\dagger}$ |
| Ribavirin dose (≥80%/60–80%)$^{\P}$ | 27/5 | 17/5 | 44/10 | 4/3 | 5/8 | 9/11 | 0.003$^{\dagger}$ |
| RVR rate (%) | 87.5 | 54.5 | 74.1 | 33.3 | 46.1 | 42.1 | 0.022$^{\dagger}$ |
| EVR rate (%) | 100 | 100 | 100 | 66.7 | 100 | 89.4 | 0.07$^{\dagger}$ |

* Mean ± SD; ** median (range); $^{\dagger}$ Fisher's exact probability test; $^{\ddagger}$ Student $t$ test; $^{\parallel}$ Mann–Whitney's $U$ test; $^{\Delta}$ $p$ values between all SVR ($n = 58$) versus all non-SVR ($n = 20$)

Several clinical characteristics listed above were unavailable in some patients. § SVR: $n = 56$ (35 in group 1, 21 in group2), non-SVR: $n = 17$ (7 in group 1, 10 in group 2); ¶ SVR: $n = 54$ (32 in group 1, 22 in group 2)

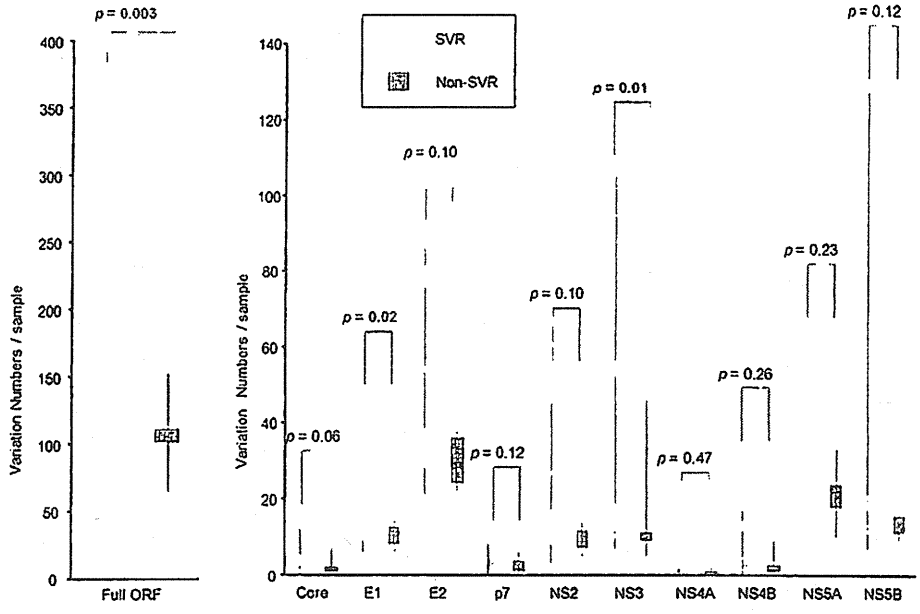


**Fig. 1** Number of amino acid substitutions per sample in the sustained viral responders (SVR) and the non-sustained viral responders (non-SVR) group. The numbers of variations, relative to a population consensus, that were unique to either SVR or non-SVR patients are shown for the full ORF (*left*) and for each HCV protein (*right*)

## Comparison of HCV sequence variation between the SVR and non-SVR patients at each amino acid position

Next, each amino acid position in the HCV ORF was compared to detect any differences between the SVR and non-SVR patients after determination of the consensus sequence from all 78 patients. In Fig. 2a, the final differences of the two independent studies combined are shown as dots demonstrating –log $P$ values. As shown in the figure, amino acid usage at amino acid 110 in the core region differed strikingly between the two groups ($p = 5E{-}05$). The site was detected in group 1 ($p = 0.01$) and was validated in group 2 ($p = 0.004$) (Table 2), and the final $p$ value became remarkably high, making the $p$ value at this site most significantly low. Variations of aa 773 in p7, aa 2099 in the NS5A, and aa 3013 in NS5B were also shown to differ significantly between the SVR and the non-SVR patients when the two studies were combined; however, they were not confirmed by one of the studies (Table 2). Figure 2b shows the aligned sequences of amino acids 1–120 of the core region. Substitutions at aa 110 from

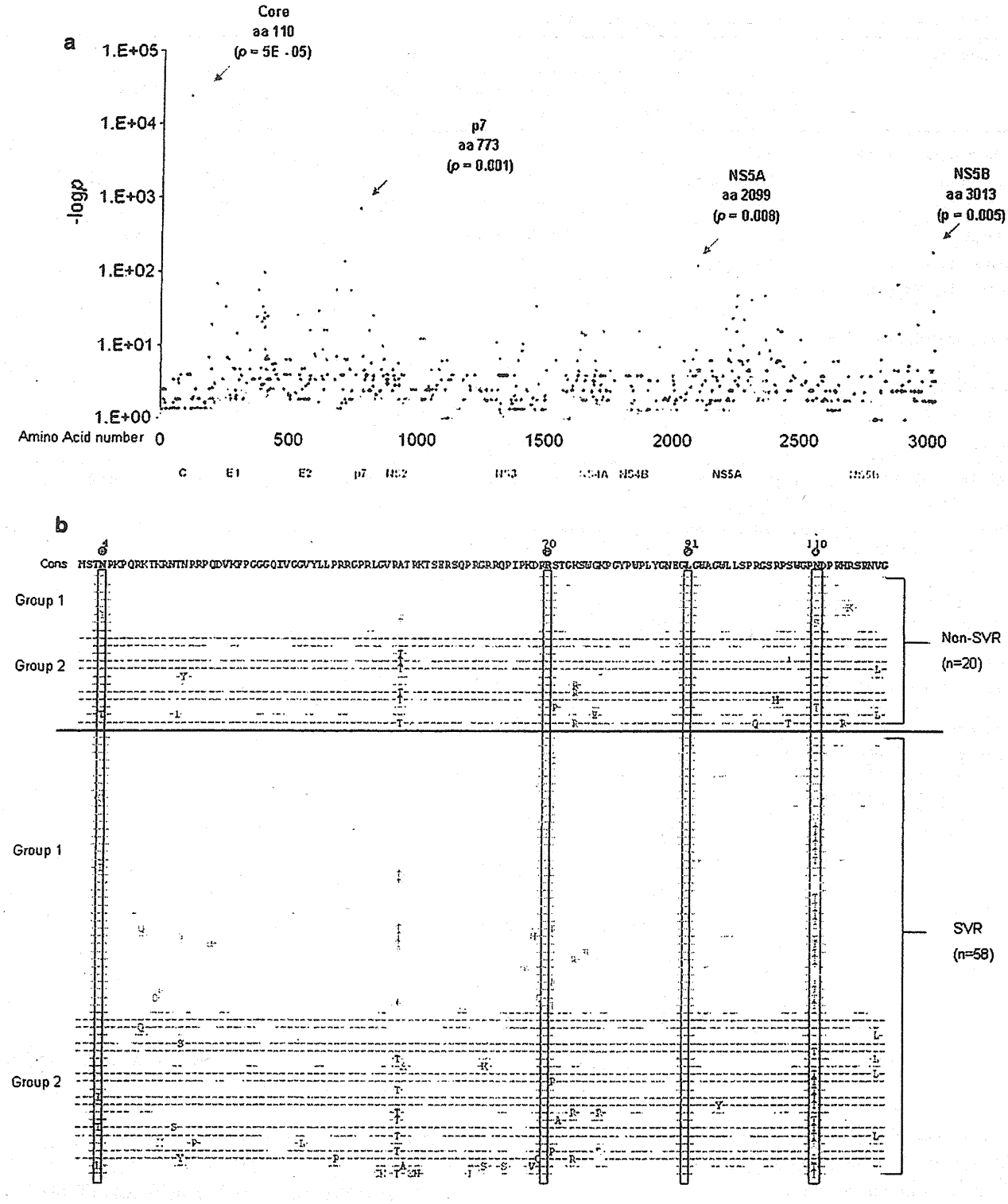


**Fig. 2** a Different amino acid usages at each viral amino acid position between the sustained viral responders (SVR) and the non-sustained viral responders (non-SVR) patients. Amino acid variation was determined between SVR and non-SVR patients by Fisher's exact probability test. The longitudinal axis shows the –log *P* value. b Sequence alignment in the core region. *Dashes* indicate amino acids identical to the consensus sequence and substituted amino acids are shown by standard single letter codes. c Sliding window analysis. Viral regions affecting treatment outcomes are shown in *dark spots*. There are four hot spots: at core amino acid 110, amino acids 400–403 (i.e., the hypervariable region) in Envelope 2 (*E2*) region, amino acids 724–743 in E2, and amino acids 2258–2306 in the nonstructural (*NS*) 5A. d Sequence alignment amino acids in the nonstructural (*NS*) 5A around amino acids 2258–2306. *Dashes* indicate amino acids identical to the consensus sequence and substituted amino acids are shown by standard *single letter* codes

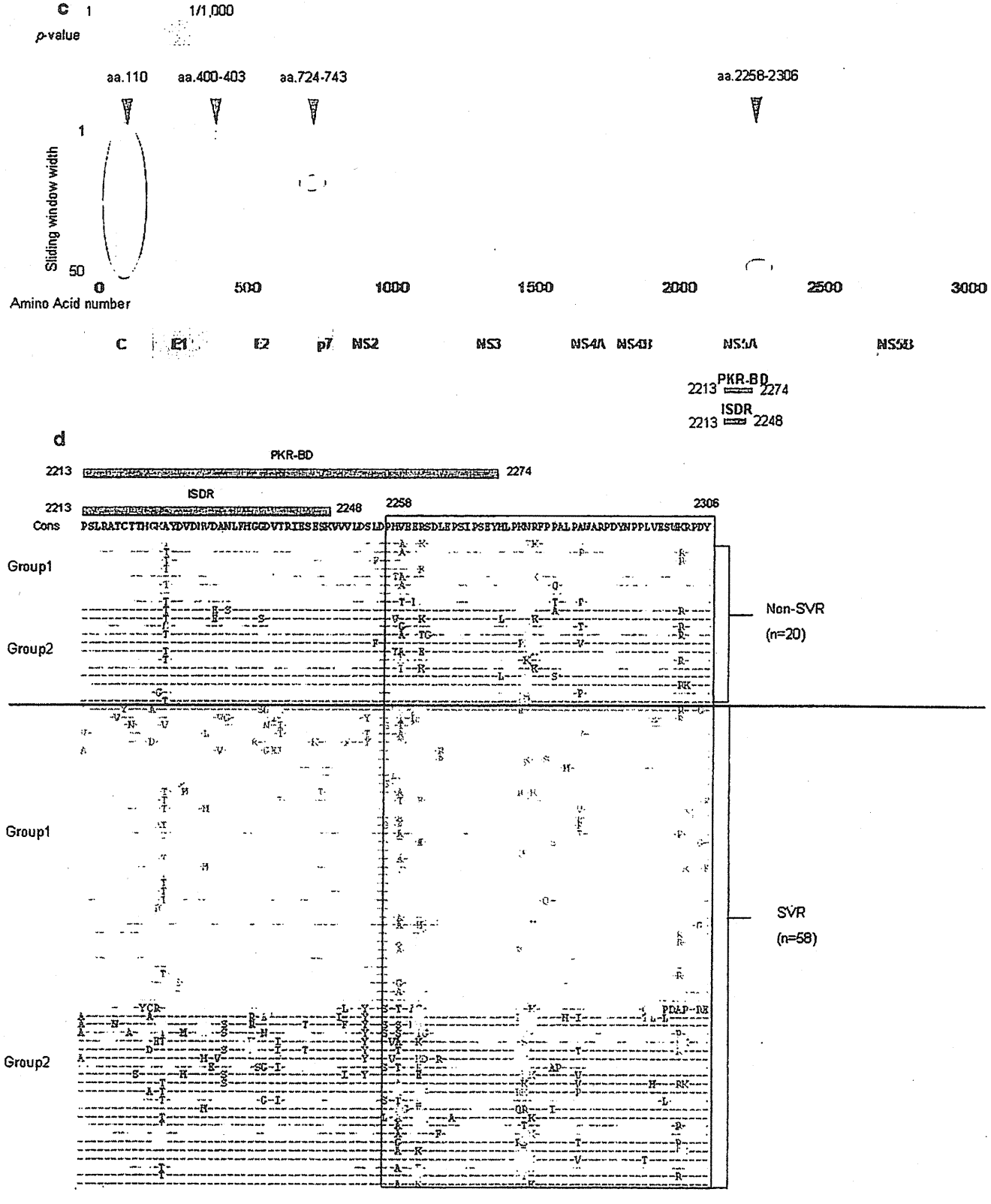


Fig. 2 continued

non-T (N/S) to T were significantly more frequent in SVR (32/58, 55.2%) than in non-SVR (1/20, 3.6%, $p = 5E{-}05$). Amino acid 4, the site reported recently to vary according to the viral response in genotype 2a infection, did not differ significantly in our study. Amino acid 70 and 91, which have been reported to vary according to viral response to

**Table 2** Variations in each amino acid position and SVR rate

| Position | Group 1 ($n = 43$) | $p$ value | Group 2 ($n = 35$) | $p$ value | Combined ($n = 78$) | $p$ value |
|---|---|---|---|---|---|---|
| Core aa 110 | | | | | | |
| T | 100% (19/19) | 0.01 | 92.9% (13/14) | 0.004 | 97% (32/33) | 5E−05 |
| Non T | 70.8% (17/24) | | 42.9% (9/21) | | 57.8% (26/45) | |
| p7 aa 773 | | | | | | |
| V | 77.4% (24/31) | 0.16 | 53.6% (15/28) | 0.03 | 66.1% (39/59) | 0.002 |
| Non V | 100% (12/12) | | 100% (7/7) | | 100% (19/19) | |
| NS5A aa 2099 | | | | | | |
| R | 92.9% (13/14) | 0.40 | 91.7% (11/12) | 0.01 | 92.3% (24/26) | 0.01 |
| Non R | 79.3% (23/29) | | 47.8% (11/23) | | 65.4% (34/52) | |
| NS5B aa 3013 | | | | | | |
| L | 78.9% (26/33) | 0.17 | 47.8% (11/23) | 0.01 | 66.1% (37/56) | 0.008 |
| Non L | 100% (10/10) | | 91.7% (11/12) | | 95.5% (21/22) | |

**Table 3** Number of amino acid substitutions in each region and SVR rate

| Region | Group 1 ($n = 43$) | $p$ value | Group 2 ($n = 35$) | $p$ value | Combined ($n = 78$) | $p$ value |
|---|---|---|---|---|---|---|
| E2 aa 400–403 | | | | | | |
| Mutation ≥ 2 | 89.3% (25/28) | 0.22 | 100% (11/11) | 0.002 | 92.3% (36/39) | 0.0005 |
| Mutation 0–1 | 73.3% (11/15) | | 45.8% (11/24) | | 56.4% (22/39) | |
| E2 aa 724–743 | | | | | | |
| Mutation ≥ 1 | 100% (28/28) | 0.0002 | 72% (18/25) | 0.12 | 86.8% (46/53) | 0.0006 |
| No mutation | 53.3% (8/15) | | 40% (4/10) | | 48% (12/25) | |
| ISDR(aa 2213–2248) | | | | | | |
| Mutation ≥ 2 | 100% (15/15) | 0.08 | 86.7% (13/15) | 0.02 | 93.3% (28/30) | 0.003 |
| Mutation 0–1 | 75% (21/28) | | 45% (9/20) | | 62.5% (30/48) | |
| NS5A aa 2258–2306 | | | | | | |
| Mutation ≥ 5 | 100% (19/19) | 0.01 | 84.2% (16/19) | 0.006 | 92.1% (35/38) | 0.0006 |
| Mutation 0–4 | 70.8% (17/24) | | 37.5% (6/16) | | 57.5% (23/40) | |

PEG-IFN/RBV therapy in genotype 1b infection, were conserved irrespective of the outcome.

## Comparison of amino acid variation between the SVR and non-SVR patients across HCV "regions" using sliding window analysis

Figure 2c shows the combined result of sliding window analysis for study groups 1 and 2. This approach was used to detect differing HCV amino acid "regions", rather than single amino acid positions, between the SVR and the non-SVR patients. According to the result, four regions were notably associated with the final outcome ($p$ values less than 1/1,000). Core aa 110, detected as a single amino acid position discriminating between the SVR and the non-SVR patients, was also identified as one of these regions. Because core aa 110 was already known for its strong correlation with the response as above, the region was excluded from further analysis. Among the other three regions, only NS5A aa 2258–2306 showed significant differences in the two independent study groups (Table 3). Interestingly, the NS5A region overlapped the PKR-binding domain, which includes the IFN sensitivity determining region (ISDR). Figure 2d shows the aligned sequences of amino acids around 2258–2306 of HCV NS5A. As with previous studies, variations in the ISDR were also significantly more frequent in SVR patients.

## Multivariate analysis to detect independent factors contributing to the SVR

Multivariate analysis revealed that variation of core aa 110, the total number of substitutions within NS5A aa 2258–2306, and total RBV dose ≥80% were finally

**Table 4** Multivariate logistic regression analysis

| Factor | Odds (95% CI) | *p* value |
|---|---|---|
| Age | 1.01 (0.91–1.13) | 0.85 |
| HCV RNA | 1.00 (1.00–1.00) | 0.09 |
| Fibrosis score ≥3/0–2 | 2.37 (0.21–26.7) | 0.48 |
| RVR achievement | 3.46 (0.54–22.1) | 0.19 |
| Ribavirin dose ≥80% | 16.0 (1.66–153) | 0.02 |
| Core aa 110 T | 24.7 (1.72–353) | 0.02 |
| NS5A aa 2258–2306 mutations 0–4/≥5 | 11.5 (1.23–108) | 0.03 |

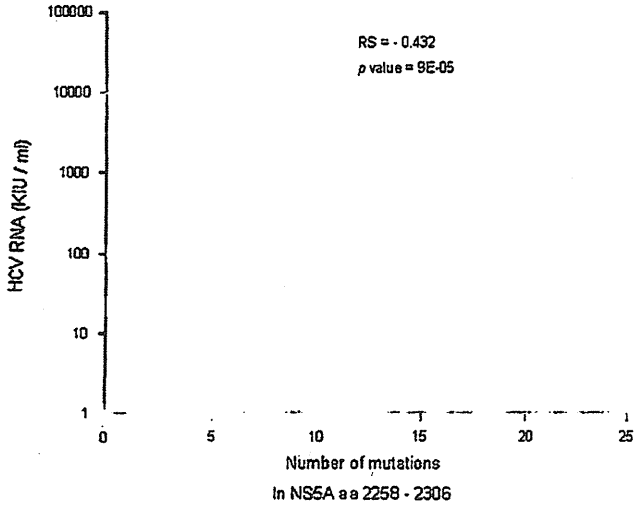


**Fig. 3** Correlation between pretreatment HCV RNA levels and the number of substitutions in the NS5A region aa 2258–2306. Spearman's correlation coefficient by rank test is demonstrated

identified as the independent variables influencing the final outcome (odds ratio 24.7, 11.5, and 16.0; $p = 0.02$, 0.03 and 0.02; Table 4).

### Biological relevance of variation in core and NS5A in this study group

To determine biological relevance of core aa110 and NS5A aa2258–2306, we investigated their relationship with clinical background factors. Multiple variations in the NS5A region aa 2258–2306 were significantly related to pretreatment HCV RNA titer ($p = 9E-05$, Fig. 3; Table 5). Interestingly, variation of the core aa110 was significantly associated with the patients' age ($p = 0.03$, Table 6).

## Discussion

In this study, based on analysis of complete HCV-ORF sequences and comparison of SVR and non-SVR patients in two independent study groups, we have shown that amino acid variations in the core and NS5A correlate most significantly with the final outcome in the treatment for genotype 2a chronic hepatitis C. The study is unique in that the patients studied were all Japanese, excluding any effect of racial differences and providing a clearer analysis of the viral differences.

From the analysis of the characteristics of patients infected with genotype 2a HCV, it was clear that most non-SVR patients responded to the PEG-IFN/RBV therapy at least transiently, given that most of these non-SVR patients (89%) achieved EVR. This result demonstrated that most non-SVR patients were relapsers, but were not null-responders as observed frequently among genotype 1b patients treated with PEG-IFN/RBV therapy. Therefore, we compared the different viral responses according to the final outcome of SVR or non-SVR.

Variation of core aa 110 was identified as the single amino acid residue most significantly related to the final outcome ($p = 5E-05$). In recent studies of treatment of genotype 1b infection with PEG-IFN/RBV, amino acid variation in the core region was reported to be associated with response. It is interesting that the core region was also identified as a HCV gene associated with the response to PEG-IFN/RBV therapy of genotype 2a infection, although the amino acid residues of core in genotype 1b were different, being aa 70 and aa 91. It is also interesting that amino acids aa 70 and aa 91 are conserved as arginine and leucine, respectively, in genotype 2a, as reported to be associated with favorable PEG-IFN/RBV responses in genotype 1b infection, consistent with the association with a high SVR rate in genotype 2a infection. Very recently, a correlation was reported between amino acid variations in the core region and viral responses of genotype 2a HCV infection [20]. Though the result seems discrepant from our study, we suspect the inconsistent results were at least partially attributable to the different groups used in comparison: we compared the difference between non-SVR patients and SVR patients while they compared the difference between non-SVR and RVR patients.

In systemic searching for the viral "regions" associated with the treatment outcome, NS5A aa2258–2306 was identified by two independent studies. Interestingly, the region overlaps the PKR-binding domain (PKR-BD), including the ISDR, in which the number of amino acid substitutions is known to be related to the response to IFN-based therapy in genotype 1b, and also in genotype 2a [17, 18]. Therefore, we also confirmed that total number of substitutions in the ISDR and PKR-BD is significantly associated with the final outcome in this group of patients when the two studies were combined.

Some viral regions other than core and NS5A also showed the potential association with the final outcome. Viral single amino acid substitutions of aa 773 in p7, aa

**Table 5** Baseline characteristics of patients with NS5A aa 2258–2306 mutations 0–4 or ≥5 (groups 1 and 2)

| Characteristic | Mutation 0–4 ($n = 40$) | Mutation ≥ 5 ($n = 38$) | $p$ value |
|---|---|---|---|
| Gender (male/female) | 22/18 | 16/22 | NS† |
| Age (years) | 54.3 ± 11.4* | 53.5 ± 11.5 | NS‡ |
| ALT (IU/l) | 73.8 ± 70.3 | 85.3 ± 78.7 | NS‡ |
| Platelet ($\times 10^4/mm^3$) | 18.0 ± 5.9 | 21.0 ± 5.7 | 0.03‡ |
| Fibrosis score (0–2/≥3)§ | 33/5 | 33/2 | NS† |
| HCV RNA (KIU/ml) | 1,100 (99–30,000)** | 380 (12–5,000) | 0.02‖ |
| IFN dose (≥80%/60–80%)¶ | 31/8 | 33/2 | NS† |
| Ribavirin dose (≥80%/60–80%)¶ | 25/14 | 28/7 | NS† |
| RVR rate (%) | 65.8 | 62.9 | NS† |
| EVR rate (%) | 94.7 | 100 | NS† |
| Relapse rate (%) | 35.9 | 7.9 | 0.002† |
| SVR rate (%) | 57.5 | 92.1 | 0.0006† |

* Mean ± SD; † Fisher's exact probability test; ‡ Student $t$ test; § mutation 0–4 $n = 38$, mutation ≥5: $n = 35$; ** median (range); ‖ Mann–Whitney's $U$ test; ¶ mutation 0–4: $n = 39$, mutation ≥5: $n = 35$

**Table 6** Baseline characteristics of patients with core 110 T or N/S (groups 1 and 2)

| Characteristic | Core 110 T ($n = 33$) | Core 110 N/S ($n = 45$) | $p$ value |
|---|---|---|---|
| Gender (male/female) | 18/15 | 20/25 | NS† |
| Age (years) | 50.4 ± 13.0* | 56.4 ± 9.5 | 0.032‡ |
| ALT (IU/l) | 64.5 ± 48.2 | 88.8 ± 86.2 | NS‡ |
| Platelet ($\times 10^4/mm^3$) | 19.3 ± 4.9 | 19.5 ± 6.6 | NS‡ |
| Fibrosis score (0–2/≥3)§ | 30/1 | 36/6 | NS† |
| HCV RNA (KIU/ml) | 580 (54–3,600)** | 980 (12–30,000) | NS‖ |
| IFN dose (≥80%/60–80%)¶ | 26/3 | 38/7 | NS† |
| Ribavirin dose (≥80%/60–80%)¶ | 23/6 | 30/15 | NS† |
| RVR rate (%) | 72.4 | 59.1 | NS† |
| EVR rate (%) | 100 | 95.5 | NS† |
| Relapse rate (%) | 3.0 | 38.6 | 9E−05† |
| SVR rate (%) | 97.0 | 57.8 | 5E−05† |

* Mean ± SD; † Fisher's exact probability test; ‡ Student $t$ test; § core 110 T: $n = 31$, core 110 N/S: $n = 42$; ** median (range), ‖ Mann–Whitney's $U$ test; ¶ core 110 T: $n = 29$

2099 in the NS5A, and aa 3013 in NS5B, or viral regions in E1 aa 400–403 and in E2 aa 724–744 were more frequent in SVR. However, because these were not extracted as significant in one of the two studies when analyzed separately, additional studies are needed to confirm the association with the final outcome. On the other hand, we could not find an association with the final outcome and the PePHD or IRRDR, including the V3 regions (data not shown) reported 1b HCV infection [21, 22].

It is interesting that the variation of the core region showed clear association with age. Younger patients with core aa 110T showed favorable responses, while older patients with core aa 110 non-T showed unfavorable responses. It is possible that different response rates according to the patients' ages in genotype 2a infection might have been related to the core substitutions, although further study is needed. In NS5A, it was reported that the variations within the PKR-binding region, including those within the ISDR, can disrupt the NS5A-PKR interaction, possibly rendering HCV sensitive to the antiviral effects of IFN [23]. Clinically, the number of substitutions within the region has been reported to correlate with the serum HCV RNA level [12]. We also confirmed that the number of substitutions within the NS5A aa 2258–2306 was significantly associated with the pretreatment HCV RNA titers.

Multivariate analysis of the combined group of patients showed that variation of core aa 110, NS5A aa 2258–2306, and total RBV dose ≥80% were independent variables associated with the final outcome (Table 4). The association of RBV dose and HCV relapse rate was reported previously [24] and that result was confirmed in this study. On the other hand, the total PEG-IFN dosage was not identified when it was administered at greater than 60% of the initially scheduled amount. Indeed, when the drug dosage was excluded, the strongest association was seen in the viral elements of core and NS5A, revealing the

importance of these two regions in the treatment of genotype 2a HCV infection with PEG-IFN/RBV therapy.

On the other hand, our study still has some limitations. In recent studies, IL28B single nucleotide polymorphisms were reported to be correlated significantly with the treatment response in genotype 1b HCV infections [25, 26]. In genotype 2a HCV infection, a correlation was also reported to exist between the IL28B SNP and the treatment response [27]. However, we could not investigate the association of the IL28B single nucleotide polymorphisms in the treatment response in genotype 2a HCV infections. In addition, the number of analyzed patients was rather small, especially in non-SVR patients.

In conclusion, by comprehensive investigation of the complete HCV ORF in patients showing different responses to PEG-IFN/RBV therapy, we have demonstrated that amino acid variation in the core and NS5A are significantly associated with the final outcome of treatment of genotype 2a chronic hepatitis C. Considering this result, determination of those HCV regions before treatment might provide further benefits for the patients infected with genotype 2a HCV.

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# Mutations in the core and NS5A region of hepatitis C virus genotype 1b and correlation with response to pegylated-interferon-alpha 2b and ribavirin combination therapy

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SUMMARY. Mutations in two regions of hepatitis C virus (HCV) have been implicated in influencing response to interferon (IFN) therapy. Substitutions in the NS5A region of HCV have been associated with response to IFN therapy, and this region has been known as the IFN sensitivity-determining region (ISDR). The mutations in the core region of HCV have also been reported to predict IFN response. The aim of this study was to investigate whether amino acid substitutions in the core region and ISDR among patients with HCV genotype 1b affect the response to IFN therapy. A total of 213 patients who completed IFN treatment were randomly selected. All patients received pegylated-IFN-alpha 2b once each week, plus oral ribavirin daily for 48 weeks. Of the 213 patients, 117 (54.9%) showed early virologic response (EVR), with HCV-negativity, at 12 weeks. Factors related to EVR on multivariate analysis were non-Gln70 and Leu91 in the core region, and ISDR mutant-type. One hundred and two (47.9%) showed a sustained virologic response (SVR). SVR occurred more frequently in patients without Gln70 (55.4%) than in those with Gln70 (21.3%) ($P < 0.0001$). SVR was achieved in 43.6% of patients with wild-type ISDR and 62.5% of patients with mutant-type ($P = 0.0227$). Of the 34 patients who simultaneously had non-Gln70 and mutant-type ISDR, 26 (76.5%) achieved SVR. Factors related to SVR on multivariate analysis were non-Gln70 and ISDR mutant-type. In conclusion, amino acid substitutions in the core region and ISDR were useful for predicting the response to IFN in patients with HCV genotype 1b.

*Keywords*: core region, genotype 1b, hepatitis C virus, interferon sensitivity-determining region, interferon therapy, NS5A.

## INTRODUCTION

Hepatitis C virus (HCV) is a member of the Flaviviridae family and causes chronic hepatitis that can develop into potentially fatal cirrhosis and hepatocellular carcinoma [1]. It has been estimated that 170 million people are infected with HCV worldwide. Therefore, HCV infection is a major global health problem. HCV consists of four structural proteins (core, envelope 1, envelope 2 and p7) and six nonstructural proteins (NS2–NS5) [2]. HCV core protein was thought to inhibit the antiviral action of interferon (IFN) through down-regulation of transcription of IFN-induced antiviral genes [3,4]. The NS5A region includes the PKR-binding domain, which is associated with viral replication that is affected by IFN [5]. Thus, the core and NS5A regions of HCV appear to be important factors that may affect the response to IFN therapy, and mutations in the core and NS5A regions of HCV have been reported to affect response to IFN therapy [6–10]. The core region of HCV is well conserved, but substitutions of amino acid (aa) 70 and aa 91 are frequently found. Several studies reported a relation between these substitutions in the core region and IFN responsiveness [8,10]. The substitutions in the NS5A region of HCV have been closely associated with response to IFN therapy, and this region is known as the IFN sensitivity-determining region (ISDR) [6]. However, these

Abbreviations: Aa, amino acid; ALT, alanine aminotransferase; EVR, early virologic response; HCV, hepatitis C virus; IFN, interferon; ISDR, interferon sensitivity-determining region; SVR, sustained virologic response.

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relationships are little known and still controversial [10]. The aim of this study was to investigate whether amino acid substitutions in the core region and ISDR among patients with HCV genotype 1b affect the response to pegylated-IFN-alpha 2b and ribavirin combination therapy.

## MATERIAL AND METHODS

A total of 891 patients with chronic hepatitis C genotype 1b and high viral load who were treated at Nagoya University Hospital and Affiliated Hospitals were enrolled; 213 patients who completed IFN treatment were randomly selected for this study. The patients' clinical characteristics are summarized in Table 1. Patients whose HCV-RNA levels were <100 KIU/mL were excluded. The core region (aa 30–110) and ISDR (aa 2209–2248) were examined by direct sequencing. All patients received subcutaneous injections of pegylated-IFN-alpha 2b (1.5 μg/kg) once each week plus oral ribavirin daily for 48 weeks. HCV-RNA in serum samples was examined at 12 weeks, at the end of IFN therapy and at 6 months after the end of treatment. Serum was stored at −80 °C for virologic examination. Early virologic response (EVR) was defined as HCV-negative at 12 weeks. Patients who were persistently negative for serum HCV-RNA and who had a normal serum alanine aminotransferase (ALT) level at 24 weeks after withdrawal of IFN treatment were considered to have sustained virologic response (SVR). Written informed consent was obtained from each patient, and the study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki.

### *Virologic analysis*

HCV-RNA quantitative viremia load was determined by polymerase chain reaction (PCR). HCV was genotyped by direct sequencing of the 5′-untranslated region and/or E1 regions as described previously [11,12]. Genotypes were classified according to the nomenclature proposed by Simmonds *et al.* [13]. Direct sequencing of the core and NS5A-ISDR region was carried out as reported previously, but with modifications [7,14]. In brief, RNA was extracted from 140 μL serum with a commercial kit (QIAamp Viral RNA Kit; Qiagen, Valencia, CA, USA) and dissolved in 50 μL diethylpyrocarbonate-treated water. RNA (10 ng) was used for reverse transcription with oligo and random hexamer primers with a commercial kit (iScript cDNA Synthesis Kit; Bio-Rad, Hercules, CA, USA). HCV core region and NS5A-ISDR were amplified by nested PCR. In brief, each 50-μL PCR reaction contained 100 nM of each primer, 1 ng template cDNA, 5 μL GeneAmp 10 × PCR buffer, 2 μL dNTPs and 1.25 U AmpliTaq Gold (Applied Biosystems, Foster City, CA, USA). Primers for core region were sense 5′-GGGAGGTCTCGTAGACCGTGCACCATG-3′ and antisense 5′-GAGMGGKATRTACCCCATGAGRTCGGC-3′ and primers for the NS5A-ISDR were sense 5′-TGGATGGAGTGCGGTTGCACAGGTA-3′ and antisense 5′-TCTTTCTCCGTGGAGGTGGTATTG-3′. Amplification conditions consisted of 10 min at 94 °C, followed by 40 cycles of 94 °C for 10 s, 55 °C for 30 s and 72 °C for 30 s in a thermal cycler (GeneAmp PCR System 9700; Applied Biosystems). The second PCR was performed in the same reaction buffer with the first-round PCR product as template, and the following sets of primers: for the core region, sense primer 5′-AGACCGTGCACCATGAGCAC-3′ and antisense 5′-TACGCCGGGGGTCAKTRGGGCCCCA-3′; and for the NS5A-ISDR, sense 5′-CAGGTACGCTCCGGCGTGCA-3′ and antisense 5′-GGGGCCTTGGTAGGTGGCAA-3′. PCR products were separated by electrophoresis on 2% agarose gels, stained with ethidium bromide, and visualized under ultraviolet light. PCR products were then purified and sequenced with the second-round PCR primers with a dye terminator sequencing kit (BigDye Terminator v1.1 Cycle Sequencing Kit; Applied Biosystems) and an ABI 310 DNA Sequencer (Applied Biosystems). A mutation mixture was defined as viral mutants that constituted 50% or more of the total viral population.

Table 1 Clinical characteristics

| Clinical characteristics | *N* =2 13 |
|---|---|
| Age (years) | 55.2 ± 10.6 |
| Sex: male/female | 120/93 |
| AST(IU/L) | 58.5 ± 37.7 |
| ALT(IU/L) | 66.0 ± 53.9 |
| Platelet count ($10^4$/uL) | 17.1 ± 5.1 |
| HCV RNA level (KIU/mL) | 1720 (100–7200) |
| Treatment: naive/retreatment | 117/96 |
| Body weight (kg) | 55.3 ± 19.9 |

Data are expressed as mean ± standard deviation HCV RNA level was shown by median (range). AST, aspartate aminotransferase; ALT, alanine aminotransferase; HCV, hepatitis C virus.

### *Statistical analysis*

Data are expressed as means ± standard deviation (SD). The paired *t*-test, the chi-square and the Fisher's exact tests were used to analyze differences in variables. A *P*-value of <0.05 was considered statistically significant. Multiple logistic regression models were used to identify factors predictive of EVR and SVR. Statview 5.0 software (SAS Institute, Inc., Cary, NC, USA) was used for all analyses.

## RESULTS

### *Genetic heterogeneity in NS5A-ISDR and core regions of the HCV genome*

The mutations in the HCV core region were measured by direct sequencing. The core region of HCV is well conserved,

Table 2 Prevalence of amino acid substitutions at 70, 75, and 91

| | |
|---|---|
| Core 70 | |
| Histidine | $n = 6$ |
| Glutamine | $n = 46$ |
| Glutamine/Histidine | $n = 1$ |
| Arginine | $n = 160$ |
| Core 75 | |
| Alanine | $n = 112$ |
| Alanine/Serine | $n = 1$ |
| Alanine/Threonine | $n = 2$ |
| Glutamine | $n = 1$ |
| Serine | $n = 5$ |
| Threonine | $n = 91$ |
| Valine | $n = 1$ |
| Core 91 | |
| Leucine | $n = 162$ |
| Methionine | $n = 51$ |

but substitutions of aa 70, aa 75 and aa 91 were frequently found, as previously reported. The distribution of mutations in the HCV core region at aa 70, aa 75 and aa 91 is shown in Table 2. The sequence of the HCVJ strain was defined as the consensus sequence, and the approach of counting the number of mutations to the chosen consensus sequence in ISDR was used to analyze the ISDR system. The number of NS5A-ISDR mutations was as follows: none ($n = 102$), 1 ($n = 63$), 2 ($n = 14$), 3 ($n = 8$), 4 ($n = 8$), 5 ($n = 7$), 6 ($n = 2$), 7 ($n = 4$) and 8 ($n = 5$). The relationships between substitutions of amino acids in the HCV core region and NS5A-ISDR are shown in Fig. 1. There were no significant relationships between the two regions. Thus, the HCV core region and the NS5A-ISDR were independent factors.

### *Virological response*

Of 213 patients, 117 (54.9%) showed EVR, with HCV-negativity, at 12 weeks, and 76 became HCV-negative after 12 weeks; overall, 187 patients became HCV-negative at the end of treatment (87.8%). However, 85 patients continued to be HCV-positive after withdrawal of IFN treatment, and 102 of 213 (47.9%) patients were defined as achieving a SVR. Of 117 patients with EVR, 87 (74.4%) achieved SVR. Of 96 patients without EVR, 81 became non-SVR (84.4%). Thus, EVR was strongly associated with SVR.

### *Factors associated with early virologic response*

The results of univariate analysis for factors predictive of EVR are shown in Table 3. The EVR rate according to amino acid substitutions of ISDR are shown in Table 4. The EVR rate of patients with more than two mutations in the ISDR (mutant-type) was 68.9%. Of 166 patients without glutamine (Gln) at aa 70 in the core region, 100 achieved EVR. The EVR rate of patients with Leu91 in the core region was 61.1%. The results of multivariate analysis for factors predictive of EVR are shown in Table 5. Factors related to EVR on multivariate analysis were non-Gln70, Leu91 and ISDR mutant-type.

### *Factors associated with sustained virologic response*

The results of univariate analysis for factors predictive of SVR are shown in Table 6. The SVR rate according to amino acid substitutions of ISDR are shown in Table 4. SVR occurred more frequently in patients without Gln70 (55.4%) than in those with Gln70 (21.3%) (odds ratio, 0.217; 95% confidence interval (CI), 0.101–0.466; $P < 0.0001$). SVR was achieved in 43.6% of patients with wild-type ISDR and 62.5% with mutant-type ISDR (odds ratio, 0.465; 95% CI, 0.240–0.899; $P = 0.0227$). Factors related to SVR on multivariate analysis were non-Gln70 and ISDR mutant-type, as shown in Table 7.

### *The virological response according to amino acid substitutions in the 70 core region and ISDR*

The SVR and EVR rates according to amino acid substitutions in the 70 core region and ISDR are shown in Table 8. The best response for both SVR and EVR was achieved in patients with non-Gln70 and mutant-type ISDR, and the

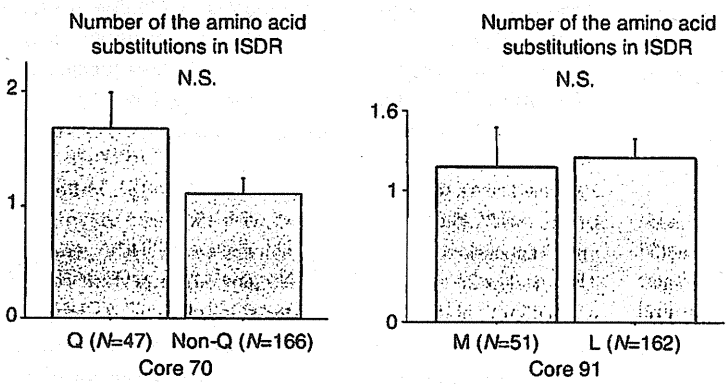


Fig. 1 The association between amino acid substitutions in core region and ISDR. ISDR, interferon sensitivity-determining region; Q, glutamine; L, leucine; M, methionine; NS, not significant.

Table 3 Univariate analysis: Factors predictive of EVR

| Factors | EVR ($n$ = 117) | Non-EVR ($n$ = 96) | $P$-value |
|---|---|---|---|
| Age (years) | 54.7 ± 11.3 | 55.9 ± 9.7 | 0.4511 |
| Gender: male/female | 63/54 | 57/39 | 0.7830 |
| ALT (IU/L) | 69.6 ± 64.8 | 61.5 ± 36.2 | 0.3002 |
| AST (IU/L) | 59.4 ± 40.9 | 57.3 ± 33.5 | 0.7026 |
| PLT ($\times 10^4/mm^3$) | 17.4 ± 5.1 | 16.9 ± 5.18 | 0.4955 |
| HCV RNA level (KIU/mL) | 2051.3 ± 1373.4 | 2006.1 ± 1462.7 | 0.8216 |
| Core 70:non-Q/Q | 100/17 | 66/30 | 0.0046 |
| Core 75: A/non-A | 58/59 | 54/42 | 0.3387 |
| Core 91: L/M | 99/18 | 63/33 | 0.0020 |
| ISDR: wild/mutant | 84/33 | 81/15 | 0.0327 |

EVR, early virologic response; AST, aspartate aminotransferase; ALT, alanine aminotransferase; PLT, platelet count; HCV, hepatitis C virus; Q, glutamine; A, alanine; L, leucine; M, methionine; ISDR, interferon sensitivity-determining region

Table 4 Amino acid substitutions of ISDR and virologic response

| ISDR: number of the amino acid substitutions | 0 $N$ = 102 | 1 $N$ = 63 | 2 $N$ = 14 | 3 $N$ = 8 | 4 $N$ = 8 | 5 $N$ = 7 | 6 $N$ = 2 | 7 $N$ = 4 | 8 $N$ = 5 |
|---|---|---|---|---|---|---|---|---|---|
| EVR rate (%) | 51 (50.0) | 33 (52.4) | 10 (71.4) | 4 (50.0) | 7 (87.5) | 4 (80.0) | 0 (0) | 3 (75.0) | 5 (100) |
| SVR rate (%) | 41 (40.2) | 31 (49.2) | 10 (71.4) | 4 (50.0) | 4 (50.0) | 5 (71.4) | 0 (0) | 3 (75.0) | 4 (80.0) |

EVR, early virologic response; SVR, sustained virologic response.

Table 5 Multivariate analysis: Factors predictive of EVR

| Factors | $P$-value | Risk ratio | 95% CI | |
|---|---|---|---|---|
| Gender: male | 0.3760 | 0.754 | 0.403 | 1.410 |
| Age: <60 years | 0.8247 | 0.915 | 0.416 | 2.012 |
| AST: <60 IU/L | 0.3301 | 1.525 | 0.652 | 3.569 |
| ALT: <60 IU/L | 0.2484 | 0.613 | 0.267 | 1.407 |
| PLT: $<17 \times 10^4/mm^3$ | 0.0666 | 0.530 | 0.269 | 1.044 |
| Core 70: nonQ | 0.0242 | 2.406 | 1.121 | 5.165 |
| Core 91: A | 0.0022 | 3.409 | 1.557 | 7.463 |
| Core 75: M | 0.0683 | 1.863 | 0.954 | 3.635 |
| ISDR: mutant | 0.0085 | 0.338 | 0.151 | 0.759 |

EVR, early virologic response; AST, aspartate aminotransferase; ALT, alanine aminotransferase; PLT, platelet count; HCV, hepatitis C virus; ISDR, Interferon sensitivity-determining region; Q, glutamine; A, alanine; L, leucine; M: methionine.

worst response was achieved in patients with Gln70 and wild type ISDR. The SVR rates according to amino acid substitutions in the 70 core region and ISDR and EVR are shown in Table 9. The positive predictive values for SVR and non-SVR improved to 88.9% and 90.9%, respectively, when EVR was considered with the 70 core region and ISDR.

## DISCUSSION

Peginterferon and ribavirin combination therapy has been standard treatment for patients with chronic hepatitis C. However, the SVR rate was almost 50% for HCV genotype 1b, which is a refractory strain. The standard doses and duration of peginterferon plus ribavirin may be suboptimal for half of the patients; patients need a new approach for eradicating HCV. Peginterferon and ribavirin therapy has been a useful treatment, but cost and adverse events have been problems. To select patients who could attain cure from HCV by current standard treatment, it is necessary to predict the response before therapy. Current guidelines for HCV treatment recommend that the selection of IFN treatment regimen depends on HCV genotypes and viral loads. Several studies have focused on sequence variation of the HCV genome and response to IFN therapy, but prediction of IFN responsiveness has been less well characterized. NS5A-ISDR heterogeneity is an important factor that may affect response to IFN, especially in Asia [6,7,9]. The ISDR interacts with PKR and regulates replication of HCV *in vitro* [5]. Mutations in the ISDR affect the interaction with PKR and may inhibit viral replication. Therefore, ISDR of not only HCV genotype 1b but also 2a and 2b could also play an important role as a predictor of IFN responsiveness in clinical research of standard IFN or Peg-IFN monotherapy [15,16]. The differences in HCV 1b subtype and race affect the utility of ISDR

Table 6 Univariate analysis: factors predictive of SVR

| Factors | SVR ($n$ = 102) | Non-SVR ($n$ = 111) | $P$-value |
|---|---|---|---|
| Age (years) | 53.6 ± 10.8 | 56.7 ± 10.2 | 0.0319 |
| Gender: male/female | 57/45 | 63/48 | 0.7830 |
| ALT (IU/L) | 69.6 ± 66.7 | 62.6 ± 38.5 | 0.3606 |
| AST (IU/L) | 58.8 ± 40.9 | 58.3 ± 34.8 | 0.9469 |
| PLT ($\times 10^4/mm^3$) | 17.7 ± 5.1 | 16.7 ± 5.0 | 0.1563 |
| HCV RNA level (KIU/mL) | 2111.1 ± 1504.9 | 1956.4 ± 1319.8 | 0.4386 |
| Core 70:non-Q/Q | 92/10 | 74/37 | 0.0001 |
| Core 75: A/non-A | 50/52 | 62/49 | 0.3388 |
| Core 91: L/M | 82/20 | 80/31 | 0.1984 |
| ISDR: wild/mutant | 72/30 | 93/18 | 0.0227 |

SVR, sustained virologic response; AST, aspartate aminotransferase; ALT, alanine aminotransferase; PLT, platelet count; HCV, hepatitis C virus; Q, glutamine; A, alanine; L, leucine; M, methionine. ISDR, Interferon sensitivity-determining region.

Table 7 Multivariate analysis: factors predictive of SVR

| Factors | $P$-value | Risk ratio | 95% CI | |
|---|---|---|---|---|
| Age: <60 years | 0.5219 | 0.770 | 0.346 | 1.714 |
| Gender: male | 0.6775 | 1.140 | 0.614 | 2.116 |
| AST: <60 IU/L | 0.1017 | 0.487 | 0.206 | 1.153 |
| ALT: <60 IU/L | 0.1690 | 1.799 | 0.779 | 4.157 |
| PLT: $<17 \times 10^4/mm^3$ | 0.4067 | 1.324 | 0.682 | 2.573 |
| HCV RNA levels: <106 IU/mL | 0.6409 | 0.841 | 0.405 | 1.743 |
| Core70: nonQ | 0.0004 | 0.220 | 0.094 | 0.512 |
| Core91: M | 0.5643 | 0.799 | 0.373 | 1.711 |
| Core75: A | 0.3993 | 0.757 | 0.396 | 1.446 |
| ISDR: mutant | 0.0096 | 2.879 | 1.294 | 6.407 |

SVR, sustained virologic response; AST, aspartate aminotransferase; ALT, alanine aminotransferase; PLT, platelet count, HCV, hepatitis C virus; ISDR, interferon sensitivity-determining region; Q, glutamine; A, alanine; L, leucine; M, methionine.

Table 8 The SVR and EVR rate according to amino acid substitutions in 70 core region and ISDR

| Core70/ISDR | SVR ($n$ = 102) | EVR ($n$ = 117) |
|---|---|---|
| Q/wild ($n$ = 33) | 6 (18.2%) | 11 (33.3%) |
| Q/mutant ($n$ = 14) | 4 (28.6%) | 6(42.9%) |
| Non-Q/wild ($n$ = 132) | 66 (50.0%) | 73 (55.3%) |
| Non-Q/mutant ($n$ = 34) | 26 (76.5%) | 27 (79.4%) |

SVR, sustained virologic response; EVR, early virologic response; SDR, interferon sensitivity-determining region; Q, Glutamine; ISDR, interferon sensitivity-determining region.

sequences for predicting IFN responsiveness [7,17,18]. Thus, ISDR was found to be good for predicting IFN outcome of patients in Asian countries rather than of patients in Western countries. The approach of counting the number of mutations to the HCV-J strain in the ISDR was used in the original report by Enomoto *et al.*, [6] and they classified the mutations into three groups: wild type (no mutation), intermediate (1–3 mutations) and mutant-type (more than four mutations). SVR did not occur in any of the 30 patients with wild type ISDR in the original report using standard IFN monotherapy. In the present study, 41 of 102 patients (40.2%) with the wild type ISDR (no mutation) achieved SVR because of improvement of Peg-IFN plus RBV combination therapy. We examined the association between the number of mutations and SVR with adjustment for current standard treatment. We were unable to identify a significant relation between no mutation and one mutation in ISDR and SVR. Thus, sequences of the HCV-J strain and HCV-J strain with single substitutions were defined as the wild-type, and ISDR sequences with more than two mutations were defined as the mutant-type. SVR was achieved in 43.6% of patients with wild-type ISDR and 62.5% of patients with mutant-type ISDR in this study. ISDR alone was insufficient to predict IFN responsiveness in patients who received peginterferon plus ribavirin combination therapy. We speculated that the other region would explain differences in IFN sensitivity in patients infected with wild type ISDR. HCV core, E2-PePHD and NS5A-V3 regions were reported to be associated with IFN response [8,10,19,20]. The HCV core interacts with several cell factors and modulates numerous gene expressions, including down-regulating transcription of IFN-induced antiviral genes, and it affects the inhibition of the antiviral action of IFN. Several studies indicated that the HCV core region could predict IFN responsiveness [8,10]. Therefore, the utility of substitutions of amino acids in the HCV core region combined with NS5A-ISDR sequences for predicting