[1]. Among these NS3/4A PIs, telaprevir, boceprevir, SCH446211, danoprevir (ITMN-191), nalaprevir (SCH900 518), and TMC435 are now under clinical trials [1, 3–7]. In PROVE1 and PROVE2 studies [3, 4] undertaken in North America and Europe, the SVR rate was favorable (67 and 69%, respectively) in a triple therapy regimen including telaprevir. In addition, some studies have suggested that shortening of treatment duration may be possible for patients who achieve a rapid virologic response (RVR) [8, 9].

However the sole use of STAT-C drugs, such as PIs, promotes production and selection of drug-resistant variants in patients experiencing viral rebound during treatment [3, 10, 11] as well as in HCV replicon experiments [11, 12]. Therefore, these drugs should be used in combination with the PEG-IFN/RBV to prevent the appearance of drug-resistant variants. However, Kuntzen et al. [13] demonstrated the presence of these drug-resistant variants in high frequencies (8.6–16.2%) by population-based sequencing in patients not treated with the drugs [1, 13]. Gaudieri et al. [14] have suggested that regions of NS3 protease and NS5B polymerase are likely to be under HLA immune pressure and therapeutic selection, and that drug-resistant variants may occur naturally to escape the immune system. These observations seem quite astonishing and troubling, since a substantial number of patients may not respond to the new therapies such as STAT-C drugs.

In the present study, to assess the prevalence of NS3 mutations conferring PI resistance in HCV genotype 1b-infected Japanese patients who had not been previously treated with PIs, as well as to assess the influence of those mutations in response to PEG-IFN/RBV therapy, the dominant HCV-NS3 sequences were determined in 261 HCV-1b patients before starting the PEG-IFN/RBV therapy.

Methods

Patients

Serum samples were acquired from 261 HCV genotype 1b-infected adult Japanese patients before combination therapy with PEG-IFN (PEGINTRON®, Schering-Plough, Tokyo, Japan) plus RBV (REBETOL®, Schering-Plough) between 2004 and 2008 at the University of Yamanashi, Musashino Red Cross Hospital and Kanazawa University. The therapy was administered according to the standard PEG-IFN/RBV treatment protocol established for Japanese patients by a hepatitis study group of the Ministry of Health, Labor, and Welfare, Japan. Specifically, the patients were subcutaneously administered PEG-IFNα-2b, 1.5 μg/kg body weight, once weekly and RBV 600–800 mg daily per os for 48 weeks. These patients were not infected with human immunodeficiency virus (HIV). The study was approved by the ethics committees of all participating universities and the hospital, and the protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in a priori approval by the Institutional Review Board at Massachusetts General Hospital. Written informed consent was obtained from each study participant.

Amplification and sequencing of full-length HCV genomes

Viral loads were determined using the Amplicor HCV RNA kit, version 2.0 (Roche Diagnostics, Tokyo, Japan) or the Cobas TaqMan test (Roche Diagnostics). HCV RNA was extracted from pretreatment serum samples by the AGPC method using Isogen (Wako, Osaka, Japan) according to the manufacturer's protocol. Complementary DNA was synthesised using Superscript II (Invitrogen, Tokyo, Japan) and random primers (Invitrogen), and then amplified by two-step nested PCR using the primers listed in Supplementary Table 1. All samples were initially denatured at 95°C for 7 min, followed by 40 cycles of amplification with denaturation at 95°C for 15 s, annealing at 55°C for 15 s, and extension at 72°C for 45 s using the BD Advantage™ 2 PCR Enzyme system (BD Biosciences Clontech, CA, USA). PCR amplicons were directly sequenced using BigDye Terminator version 3.1 (ABI, Tokyo, Japan) and universal M13 forward/reverse primers using an ABI prism 3130 sequencer (ABI).

Sequence alignment and analysis

Sequences were determined in both directions, particularly for the ambiguous stretches, were assembled using the Vector NTI software (Invitrogen), and base-calling errors were corrected following the inspection of chromatograms. If mixed bases were detected as two different chromatogram peaks at the same residue, only the dominant base was called after evaluation of all overlapping fragments. A consensus sequence was generated from the alignment on the basis of the most common amino acid at each site.

Determination of PI resistance mutations

Multiple viral NS3 mutations were observed in amino acid positions reported to confer PI resistance among 261 patients: V36, Q41, F43, T54, V55, Q80, R109, I153, R155, A156, D168, V170, and M175. NS3 amino acid mutations with proven PI resistance in previously published studies (Table 1) were designated as resistance proven mutations (e.g., V36M/A). Mutations in the PI-resistance site not known to confer drug resistance were designated resistance unproven mutations (e.g., V36I). Patients were allocated to two groups according to the presence of PI-resistance

mutations (including resistance unproven mutations), and clinical characteristics including HCV RNA levels and responses to PEG-IFN/RBV therapy were compared. To assess the influence of PEG-IFN/RBV therapy on NS3 mutational status, posttreatment HCV-NS3 sequences in 39 of 58 non-SVR patients were also examined.

Statistical analysis

Statistical differences in the data, including all available patients' demographic, biochemic, hematologic, and virologic data such as sequence variation factors, were determined among the various groups by Student's *t* test or Mann–Whitney *U* test for numerical variables and Fisher's exact probability test for categorical variables.

Results

Prevalence of dominant PI-resistance-associated nonstructural 3 mutations in untreated patients

Figure 1 shows the frequency of substitutions in 261 patients for each of 181 NS3 protease amino acid residues compared to the consensus sequence. A total of 41 resistance proven mutations were detected in 35 (13.4%) patients: T54S (14 patients, 5.4%), Q80K (1 patient, 0.4%), I153V (22 patients, 8.4%), D168E (4 patients, 1.5%), T54S plus I153V double mutation (4 patients, 1.5%), and I153V plus D168E double mutation (2 patients, 0.8%). The mutation number increased to 54 in 47 (18.0%) patients when resistance unproven mutations were included: V36I (2 patients, 0.8%), I153L (11 patients, 4.2%), and I153V plus V36I double mutation (2 patients, 1.5%). Double mutations were found in 7 patients (2.7%) (Table 1). Q80L was observed in 47 (18%) patients but these were excluded from consideration because a previous study demonstrated that this mutation does not confer resistance [15]. All mutations observed in this study would confer low- to moderate-level PI resistance according to previous studies [6, 15–19]. No mutations conferring high-level resistance such as R155 or A156 [11, 17, 19–22] were observed.

Clinical characteristics of patients with PI-resistance mutations

Table 2 presents the characteristics of patients classified according to the presence of PI-resistance mutations

Table 1 Prevalence of PI-resistance-associated NS3 mutations

Drug-resistance mutations described in the literature				Detected resistance mutations
NS3 residue	Resistance mutations	Drugs	References	Genotype 1b ($N = 261$), (%)
V36	A, M, L, G, C	Telaprevir, Boceprevir	[1, 3, 4, 10, 11, 19, 31, 37]	I × 2 (0.8)
Q41	R	ITMN-191, Boceprevir	[19]	
F43	S, C	ITMN-191, Boceprevir, Telaprevir, TMC435	[15, 19]	
T54	A, S	Telaprevir, Boceprevir, SCH900518	[1, 3, 10, 11, 19, 20, 31, 38]	**S × 14 (5.4)**
V55	A	Boceprevir	[1]	
Q80	R, K	TMC435	[6, 15]	**K × 1 (0.4)**
R109	K	SCH446211	[17]	
I153	V	SCH446211	[17]	**V × 22 (8.4)**, L ×11 (4.2)
R155	K, T, I, M, G, L, S, Q	Telaprevir, Boceprevir, ITMN-191, BILN2061, TMC435	[1, 3, 4, 6, 10, 11, 15, 19, 20]	
A156	S, T, V, I, G	Telaprevir, Boceprevir, ITMN-191, BILN2061, SCH446211, TMC435, SCH900518	[1, 3, 4, 10, 11, 15, 17, 19, 20, 38]	
D168	A, V, E, N, T, H	BILN2061, ITMN-191, TMC435	[6, 15, 20]	**E × 4 (1.5)**
V170	A	Telaprevir, Boceprevir	[1, 19, 20]	
M175	L	Boceprevir	[39]	
Total number (%) of patients with resistance proven mutations				35 (13.4)
Total number (%) of patients with resistance proven and unproven mutations				47 (18.0)

Amino acid mutations conferring PI resistance in the literatures and those observed in PI-treatment-naive patients in this study are indicated. Bold indicates resistance proven mutations, and the others indicate resistance unproven mutations

Double mutations found were as follows: V36I and I153V × 1, T54S and I153V × 4, I153V and D168E × 2

(including resistance unproven mutations). Age, sex ratio, body mass index, alanine aminotransferase (ALT) levels, serum albumin, platelet count, and fibrosis stage did not differ between the NS3 mutation and wild-type groups. No significant difference was observed between the two groups in the parameters of PEG-IFN/RBV treatment response, HCV sequence variations in interferon sensitivity determining region (ISDR), Core 70, interferon plus ribavirin resistance-determining region (IRRDR), or interleukin 28B (IL28B) single nucleotide polymorphism (SNP) (rs8099917; T/G and G/G vs. T/T) [23–30]. These clinical variables were also compared between the mutation group defined as resistance proven mutations and the wild-type group, but no notable differences were observed.

Unimpaired in vivo fitness of viral strains with resistance mutations

Because most PI-resistance mutations described till date have been associated with reduced replicative capacity of varying degrees [1, 10, 11, 13, 17, 20–22, 31, 32], we examined viral replication levels in patients with drug-resistance mutations (Fig. 2). The estimated P value indicated no significant difference between the mutation (median 1,500 KIU/ml) and wild-type (median 1,800 KIU/ml) groups ($P = 0.69$). The results indicate that drug-resistant HCVs were not necessarily impaired in their ability to replicate in vivo. However, patients with double mutations ($N = 7$) tended to have low viral loads (median 1,200 KIU/ml) ($P = 0.09$).

Resistance mutations and virologic response to PEG-IFN/RBV therapy

To determine the difference in virologic response to PEG-IFN/RBV therapy according to the PI mutation, frequency of HCV RNA levels below detection at 4 weeks (rapid viral response, RVR) and 12 weeks (complete early viral response, cEVR), and SVR rate (%) were investigated in each group. The frequency of HCV RNA levels below detection at 4 and 12 weeks was 14 and 50%, respectively, in the mutation group, and was 11 and 46%, respectively, in the wild-type group. The SVR rate was 48 and 40% in the mutation and wild-type groups, respectively ($P = 0.38$). No significant difference was observed between the two groups in any of the indexes investigated (Table 2). The time-dependent viral clearance rate during PEG-IFN/RBV therapy was estimated in 133 patients including 25 patients (19%) with PI-resistance mutations available for the analysis. Kaplan–Meier analysis demonstrated that HCV clearance did not differ between the two groups with and without resistance mutations (log-rank test, $P = 0.30$) (Fig. 3).

Changes in nonstructural 3 amino acid sequence diversity during PEG-IFN/RBV therapy

Full-length NS3 protease sequences were determined in 39 non-SVR patients after PEG-IFN/RBV therapy. A single amino acid change at resistance-associated sites in two patients was observed. In one patient, isoleucine (Ile) at position 153 changed to valine (Val), and glutamic acid (Glu) changed to aspartic acid (Asp) at position 168 in the second (Fig. 4). At the nucleotide level, ATC (Ile) changed to GTC (Val) in I153V, and GAA (Glu) changed to GAC (Asp) in E168D. Both mutations were caused by one nucleotide exchange. No other changes were observed in the other 37 patients.

Discussion

Here we report that in 18% (47/261) HCV genotype 1b-infected patients who had not been previously treated with NS3 PIs, the viral genome contained dominant amino acid mutations within the NS3 PI-resistance sites. Even after confining the data to established PI-resistance mutations, the mutation rate was still significant in 13.4% (35/261). No clinical differences were observed between patients

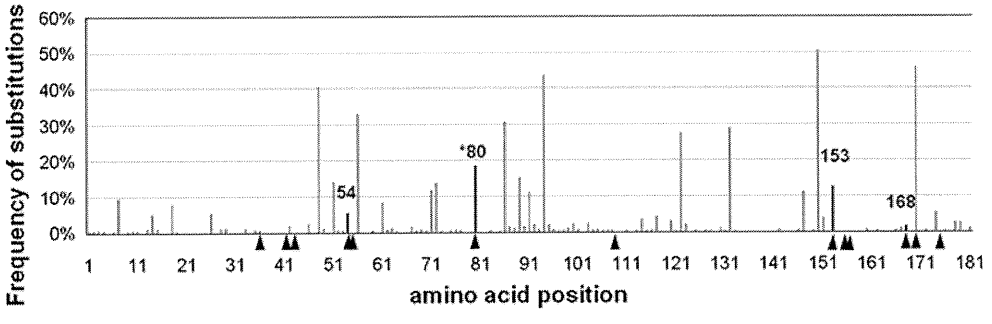

Fig. 1 Frequency of polymorphic mutations for each of the 181 NS3 protease amino acid residues in 261 patients. *Arrowheads* indicate the sites reported to confer PI resistance. *Dark bars* denote the amino acid variations at the resistant sites in this study. **80*, we detected one resistant mutation (Q80K) and 47 (18%) non-resistant variations (Q80L) at the 80th residue

Table 2 Characteristics of patients with or without HCV genomes harboring drug-resistance mutations

Characteristics	Mutation type ($N = 47$)	Wild-type ($N = 214$)	P value
Patients' characteristics			
Age, median (range)	59 (46–72)	57 (19–77)	0.17
Male, no. (%)	26 (55)	112 (52)	0.70
BMI, median (range)	23.2 (15.5–31.9)	22.8 (16.1–31.9)	0.41
ALT IU/ml	81.3 ± 72.6[a]	74.8 ± 51.9	0.93
Serum albumin g/dl	4.00 ± 0.37	4.01 ± 0.36	0.81
Platelet count × 10^4/μl	15.8 ± 4.3	14.5 ± 4.8	0.18
HCV RNA KIU/ml, median (range)	1,500 (58–6,310)	1800 (28–15,849)	0.69
Fibrosis, no. (%)			0.97
F0	0 (0)	7 (3)	
F1	23 (50)	89 (42)	
F2	9 (20)	52 (24)	
F3	9 (20)	40 (19)	
F4	5 (11)	26 (12)	
IFN pre-treatment no. (%)	15/40 (38)[b]	66/172 (38)	1.00
IL28B (rs8099917) T/G or G/G no. (%)	6/20 (30)	19/67 (28)	1.00
Response to PEG-IFN/RBV therapy			
SVR total cases no. (%)	22/46 (48)	83/210 (40)	0.38
RVR in total cases no. (%)	6/44 (14)	22/195 (11)	0.83
cEVR in total cases no.(%)	22/44 (50)	92/200 (46)	0.75
SVR 48w treatment no. (%)	16/29 (55)	55/130 (42)	0.29
End of treatment response no. (%)	26/41 (63)	123/202 (61)	0.91
HCV genome sequence variation			
ISDR mutation ≤1 no. (%)	32/46 (70)	167/210 (80)	0.21
Core70 R no. (%)	26/44 (59)	136/210 (65)	0.56
IRRDR mutation >3 no. (%)	25/38 (66)	107/190 (56)	0.34

[a] Mean ± SD

[b] Number/total number (%)

harboring viruses with and without these mutations. Moreover, no differences were observed in the responses of either group to PEG-IFN/RBV therapy.

Recent studies reported that significant number of patients who were never treated with PI possess viral sequences with PI-resistance-associated NS3 mutations. In these studies, the prevalence of PI-resistance mutations was determined to be 8.6–16.2% [13, 14], in HCV genotype 1- and 3-infected patients in European–American populations. These patients were often coinfected with HIV. Analysis of the public HCV databases (EuHCVdb and Los Alamos) also reported the presence of naturally occurring PI-resistance-associated NS3 mutations in worldwide isolates [33]. However, in vivo and in vitro studies demonstrated that most of the mutations observed conferred only low- to moderate-level PI resistance [7, 13, 14, 34, 35]. Regarding viral fitness, PI-resistant HCVs show lower fitness at varying degrees as revealed by in vitro studies [1, 10, 11, 17, 20–22, 31, 32], but HCV RNA levels in a clinical study did not differ significantly. The response to PEG-IFN/RBV therapy was almost comparable to that in HCV-infected patients without PI-resistance mutations either in HCV replicon experiments or in a clinical study of small number of treated patients [34].

The prevalence of 13.4% for PI-resistance-proven patients observed in the present study was almost comparable to the results of previous studies. Although HIV is known to increase HCV replication in coinfection with HCV [36], and HIV patients are often treated with the HIV-specific PIs, the HIV infection might not affect the natural occurrence of HCV-specific PI-resistance mutations since our studied patients were all proven to be free from coinfection with HIV infection. As shown in Table 1 and Fig. 1, I153 V (22/261, 8.4%), T54S (14/261, 5.4%), and D168E (4/261, 1.5%) were among the most prevalent PI-resistance-proven mutations in the present study. The most frequent mutation detected in our study I153V was reported to appear secondarily to the occurrence of R109K mutations in a HCV replicon system [17]. Although the role of this mutation is not understood, the I153V mutation on its own conferred SCH446211 resistance to the HCV replicon to a lesser degree [17]. Interestingly, I153V was often found in double mutations in our study, as shown in Fig. 2. This suggests analogy between in vitro and in vivo data. T54S and D168E, the other frequent mutations, have been also reported to occur as single dominant mutations in previous in vitro or in vivo studies in HCV genotype 1

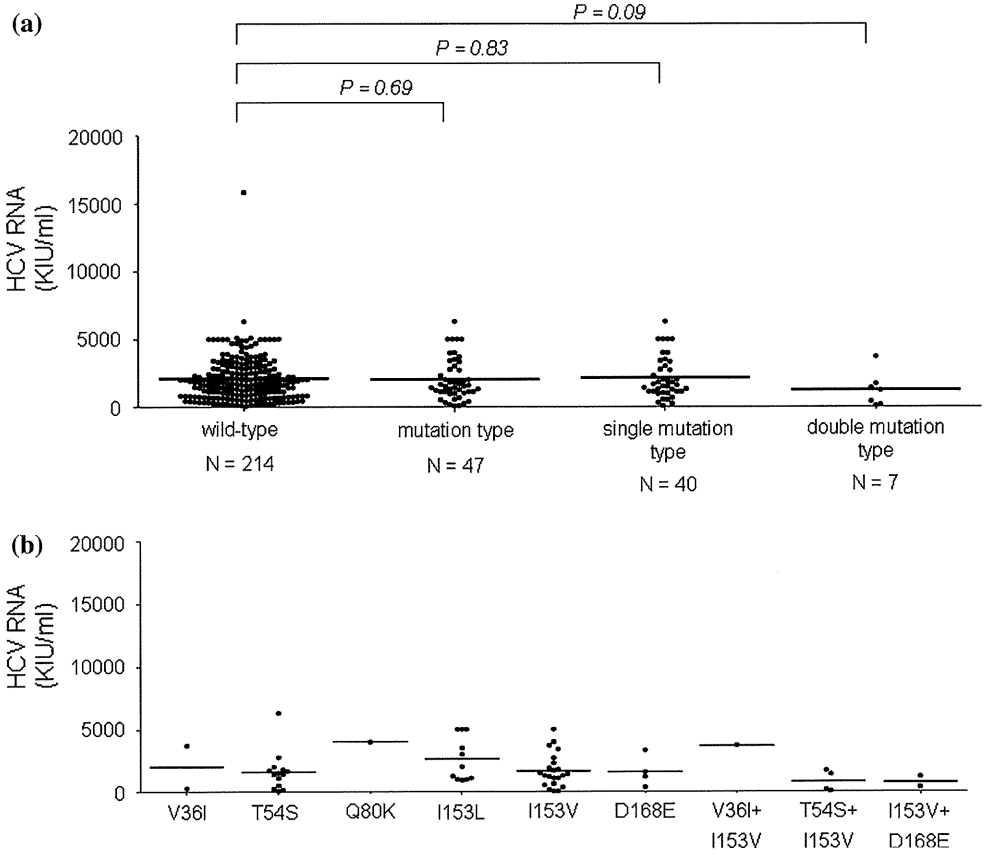

Fig. 2 In vivo fitness of HCV with PI-resistance-associated NS3 mutations. HCV RNA levels were compared between patients with and without NS3 PI-resistance-associated mutations (**a**) and between patients with each resistance mutation (**b**). The estimated *P* value (Mann–Whitney *U* test) indicates no significant difference between the wild-type and other groups (wild-type vs. mutation type, wild-type vs. single mutation type, and wild-type vs. double mutation type). (Wild-type, $N = 214$; mutation type, $N = 47$; single mutation type, $N = 40$; double mutation type, $N = 7$; V36I, $N = 2$; T54S, $N = 14$; Q80K, $N = 1$; I153L, $N = 11$; I153V, $N = 22$; D168E, $N = 4$; E176A, $N = 1$; V36I + I153V, $N = 1$; T54S + I153V, $N = 4$, and I153V + D168E, $N = 2$)

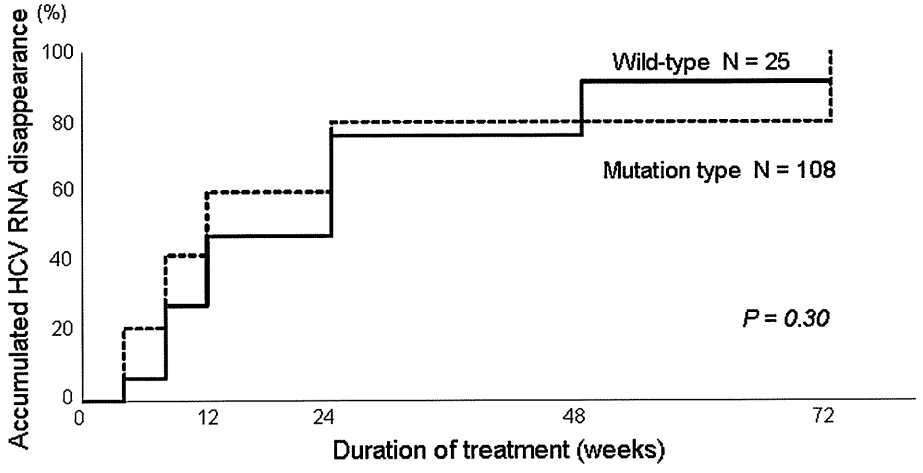

Fig. 3 Comparison of virologic response to PEG-IFN/RBV therapy between HCV-infected patients with and without PI-resistance-associated NS3 mutations. Time-dependent HCV clearance rate analysis was based on serum HCV RNA positivity during PEG-IFN/RBV therapy for HCV isolates with resistance mutations or wild-type sequences. A total of 133 patients for whom the limit of viral genome detection could be determined were analyzed. Among this group, NS3 mutations were detected in 25 patients (19%). The estimated *P* value (log-rank test) shows no significant difference between the two groups ($P = 0.30$)

infections showing moderate degrees of resistance [16, 18, 19].

Most PI-resistance mutations described to date have been associated with varying degrees of reduced replicative capacity [10, 11, 17, 20–22, 31, 32]. In the present study, HCV RNA levels of those patients with low- to moderate-level resistance mutations were similar to those in patients in the wild-type groups, suggesting that in vitro viral fitness

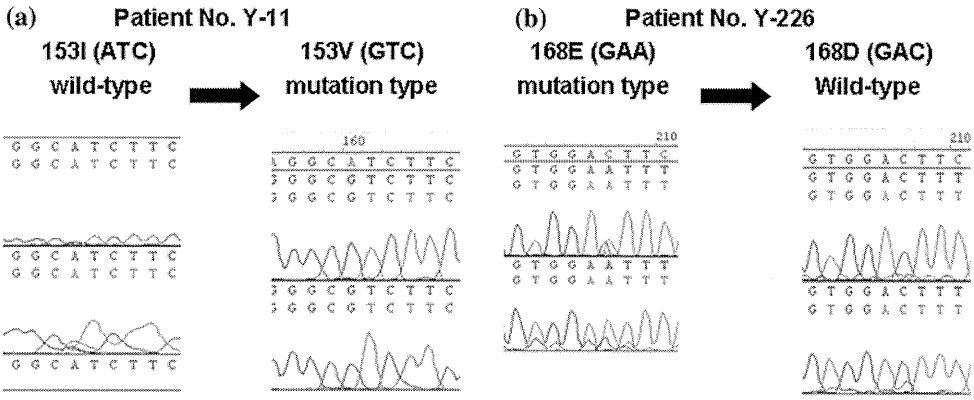

Fig. 4 Appearance of PI-resistance-associated NS3 mutations during the PEG-IFN/RBV therapy. Chromatograms show part of the HCV NS3 sequence demonstrating PI-resistance mutations in two patients receiving therapy. **a** Site 153 isoleucine (Ile) (ATC) changed to valine (Val) (GTC), **b** Site 168 glutamic acid (Glu) (GAA) changed to aspartic acid (Asp) (GAC)

does not necessarily reflect in vivo viral fitness. This, however, does not rule out the possibility that some unknown compensatory viral mutations might have resulted in upregulation of reduced viral fitness. Interestingly, although the replicative capacity conferred by a single mutation seemed to be the same, the HCV RNA levels of double mutations were frequently low, suggesting that double mutations might weaken viral fitness.

In previous studies, clinical characteristics representing the state of liver disease other than HCV RNA levels were not studied in patients with PI-resistance mutations. In this study, we show that those clinical characteristics did not differ according to the presence of viral NS3 mutations. As shown in Table 2, age, sex ratio, fibrosis stage, ALT levels, serum albumin, platelet count, and past history of IFN pretreatment did not differ according to the presence of NS3 mutations. These results suggest that NS3 mutations occur independently of disease progression. Moreover, no evident differences were observed between viral and host factors known to affect IFN-based treatment responses. However, viral amino acid variations in the core and NS5A or the allelic frequency of IL28B SNPs, which were recently reported for the close relationship of responses to PEG-IFN/RBV therapy, did not differ between the two groups.

A significant outcome of the present study is the demonstration that PI-resistance mutations might not affect responses to PEG-IFN/RBV therapy. Previous in vitro studies demonstrated that HCV replicons harboring PI-resistance mutations were also sensitive to IFN treatment [31]. In addition, recent clinical studies also indicated that PI-resistance mutations were sensitive to the PEG-IFN/RBV [10, 34]. However, our analysis was more comprehensive because viral and host factors that contribute to treatment responses were simultaneously analyzed. A unique aspect of the present study is that we investigated the influence of the PEG-IFN/RBV treatment on the occurrence of new PI mutations by direct nucleotide sequencing, and were able to show that the PEG-IFN/RBV might not induce amino acid mutations.

Will the pre-existence of naturally occurring PI-resistance mutations have an influence on future treatment of HCV infections? Since new PIs are on the verge of clinical use, all clinicians should bear in mind the substantial numbers of HCV-infected patients with PI-resistance mutations. Although the degree of resistance is considered to be low or moderate in untreated patients, weak resistance might progress to more potent resistance with additional mutations, when PIs become widely used. Therefore, all clinicians need to be sufficiently prepared for the possibility of later onset of PI-resistance mutations that confer greater drug resistance and concomitant poorer responses to therapy. In SPRINT-1 study, the lead-in therapy was associated with a modestly lower rate of breakthrough than with no lead in [7]. Considering that PEG-IFN/RBV was equally effective for PI-resistant viruses, sufficient "lead-in" therapy before the administration of PIs could be an option in the forthcoming triple therapy modality.

In conclusion, we demonstrate here that PI-resistance-associated NS3 mutations exist in a substantial proportion of untreated HCV-1b-infected patients. Although the degree of resistance might not be strong, clinicians will need to consider this upon the introduction of triple therapy.

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Analysis of the Complete Open Reading Frame of Genotype 2b Hepatitis C Virus in Association with the Response to Peginterferon and Ribavirin Therapy

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Abstract

Background and Aims: Patients infected with genotype 2b hepatitis C virus (HCV) generally can achieve favorable responses to pegylated-interferon plus ribavirin therapy (PEG-IFN/RBV). However, a proportion of patients show poorer responses and the correlation between viral sequence variation and treatment outcome remains unclear.

Methods: The pretreatment complete open reading frame (ORF) sequences of genotype 2b HCV determined by direct sequencing were investigated for correlation with the final outcome in a total of 60 patients.

Results: In this study group, 87.5% (14/16) of non-sustained virological response (non-SVR) patients (n = 16) were relapsers. Compared to sustained virological response (SVR) patients (n = 44), non-SVR patients were older and could not achieve prompt viral clearance after the therapy induction. Comparing each viral protein between the two groups, viral sequences were more diverse in SVR patients and that diversity was found primarily in the E1, p7, and NS5A proteins. In searching for specific viral regions associated with the final outcome, several regions in E2, p7, NS2, NS5A, and NS5B were extracted. Among these regions, part of the interferon sensitivity determining region (ISDR) was included. In these regions, amino acid substitutions were associated with the final outcome in an incremental manner, depending upon the number of substitutions.

Conclusions: Viral sequences are more diverse in SVR patients than non-SVR patients receiving PEG-IFN/RBV therapy for genotype-2b HCV infection. Through systematic comparison of viral sequences, several specific regions, including part of the ISDR, were extracted as having significant correlation with the final outcome.

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Introduction

Worldwide, 180 million people are estimated to be infected with hepatitis C virus (HCV), a major cause of chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma (HCC) [1]. In HCV-infected patients with chronic hepatitis, treatment with interferon (IFN)-based therapy can result in viral clearance as well as biochemical and histological improvements [2]. In this IFN-based therapy, HCV genotype is the most significant factor affecting treatment responses [3,4].

In genotype 2b HCV infection, 80% of patients with high viral titers can achieve a sustained virological response (SVR) to the regimen of pegylated-interferon (PEG-IFN) plus ribavirin (RBV) for 24 weeks [5,6]. This response is high considering that much lower percentages of patients infected with other genotypes can achieve SVR, especially with genotype 1 [1]. However, in other words, 20% of patients infected with genotype 2b HCV still cannot clear the virus and remain at risk of developing HCC. On the other hand, although various studies have been undertaken to clarify the factors contributing to the response to IFN-based therapy in genotype 1 infection, it remains poorly understood which patients with genotype 2b HCV infection will show unfavorable responses. Recently, the significance of IL28B single nucleotide polymorphisms (SNPs) in determining the response to PEG-IFN/RBV therapy was demonstrated in genotype 1 HCV infection [7,8]. However, the significance of IL28B SNPs was rather weak in genotype 2 HCV infection [9].

In terms of the association between HCV sequence variation and treatment responses, previous studies have reported that

Table 1. Baseline Characteristics of Studied Patients.

Characteristic	SVR (n = 44)	non-SVR (n = 16)	*P* value
Gender (Male/Female)	26/18	9/7	NS†
Age (yrs)	56 (22–72)*	59 (30–80)	0.04‡
BMI	23.5 (16.6–30.3)	24.7 (18.5–31.7)	NS‡
ALT (IU/l)	51 (19–380)	41 (17–390)	NS‡
GGTP (IU/l)	36 (11–133)	40 (17–292)	NS‡
T.Chol (mg/dl)	169 (119–225)	178 (145–217)	NS‡
WBC (/μl)	4600 (2620–7200)	5080 (3270–8600)	NS‡
Hb (g/dl)	14.2 (11.5–17.3)	14.6 (11.8–16.4)	NS‡
Platelet ($\times 10^4/mm^3$)	19 (7.1–31.8)	17.8 (8–36.7)	NS‡
Fibrosis score (0–2/≥3)§	38/5	7/3	NS†
HCV RNA (KIU/ml)	2050 (100–16000)	1800 (140–6300)	NS‡
IFN dose (≥80%/60–80%)	36/8	13/3	NS†
Ribavirin dose (≥80%/60–80%)	32/12	10/6	NS†
RVR rate (%)	55.8	6.3	0.0008†
EVR rate (%)	97.7	68.8	0.004†
ETR rate (%)	100	87.5	NS†

§: SVR : n = 43, non-SVR : n = 10.
*: median (range).
†: Fisher's exact probability test.
‡: Mann-Whitney's U test.
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amino acid variation in the NS5A-ISDR [10], NS5A-IRRDR [11], NS5B [12], PKR-eIF2 phosphorylation homology domain (PePHD) of E2 [13], and Core [14] correlate with the clinical outcome of IFN-based therapy, including PEG-IFN/RBV therapy for genotype 1b HCV infection. In the meantime, these viral sequence studies have been controversial regarding their true clinical importance, because the results of different studies were not always coincident [15,16,17]. On this background, recent studies trying to analyze the correlation of complete HCV open reading frame diversity, clinical characteristics, and the response to PEG-IFN/RBV therapy for genotype 1 HCV infection, in the most comprehensive approach yet attempted, have clarified that viral amino acid variation is associated with treatment responses, with consideration of racial background [18,19]. In genotype 2 infection, however, only a few studies have investigated the association of HCV sequence variation and treatment response [20,21] and the clinical significance has been yet established. We reported recently that variation of amino acid (aa) 110 in Core and amino acids (aa) 2258–2308 in NS5A were significantly associated with treatment outcome of the PEG-IFN/RBV therapy for genotype 2a HCV infection, through the analysis of the complete HCV ORFs in Japanese patients [22].

In this study, to assess comprehensively the influence of viral sequence variation on the response to the PEG-IFN/RBV therapy in genotype 2b HCV infection, we determined the complete pretreatment HCV ORFs from Japanese patients and investigated amino acid variation and its correlation with the response to combination therapy with PEG-IFN plus RBV.

Methods

Patients

A total of 77 adult Japanese patients infected with genotype 2b HCV, who received the combination therapy with PEG-IFN

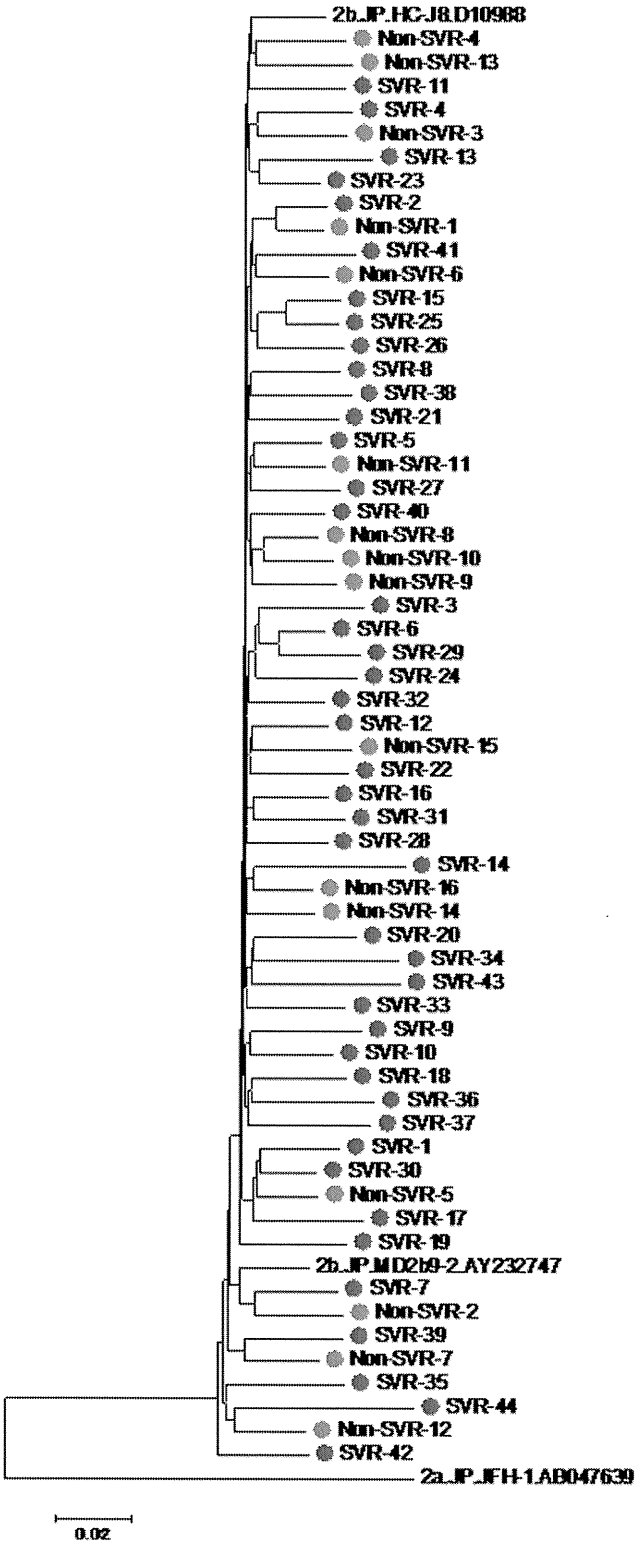

Figure 1. Phylogenetic analysis of the genotype-2b polyprotein sequences. In order to perform the phylogenetic analysis, we first aligned all 60 HCV complete ORF amino acid sequences obtained from the patients along with reference sequences (2b.HC-J8.D10988, 2.JP.MD2b9-2, and 2a.JP.JFH-1.AB047639), using the ClustalW program, and constructed the phylogenetic tree using the Neighbor-Joining method with MEGA version 4 software. Blue circles indicate SVR patients and red circles indicate non-SVR patients.
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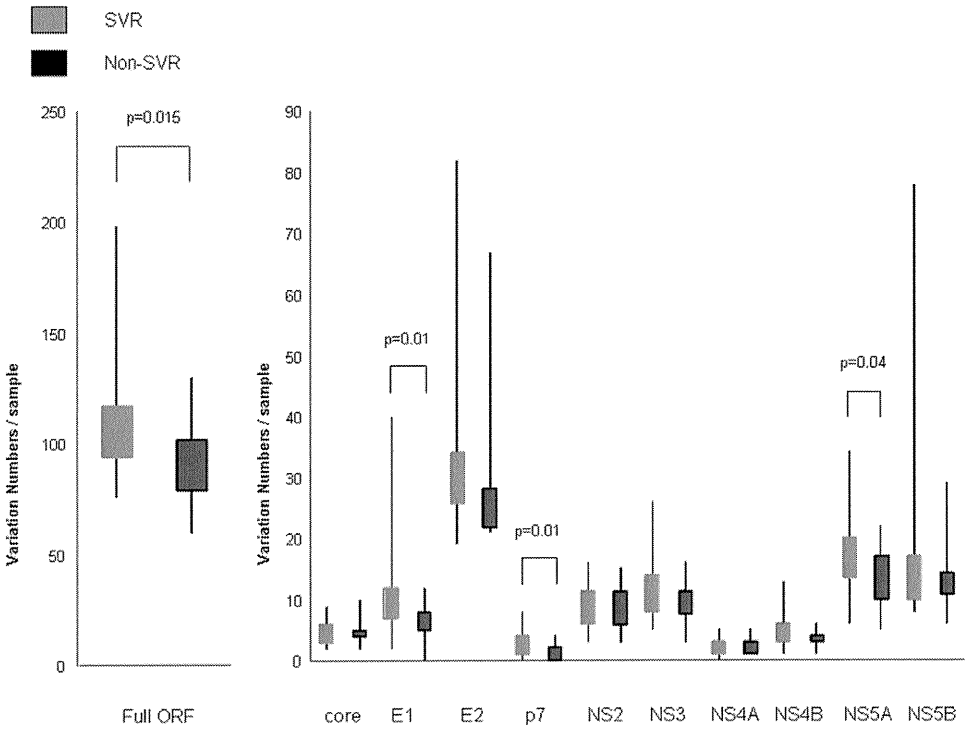

Figure 2. Number of amino acid substitutions per sample in the sustained virological responders (SVR) and the non-sustained virological responders (non-SVR) group. The numbers of variations, relative to a population consensus, that were unique to either SVR or non-SVR patients are shown for the complete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

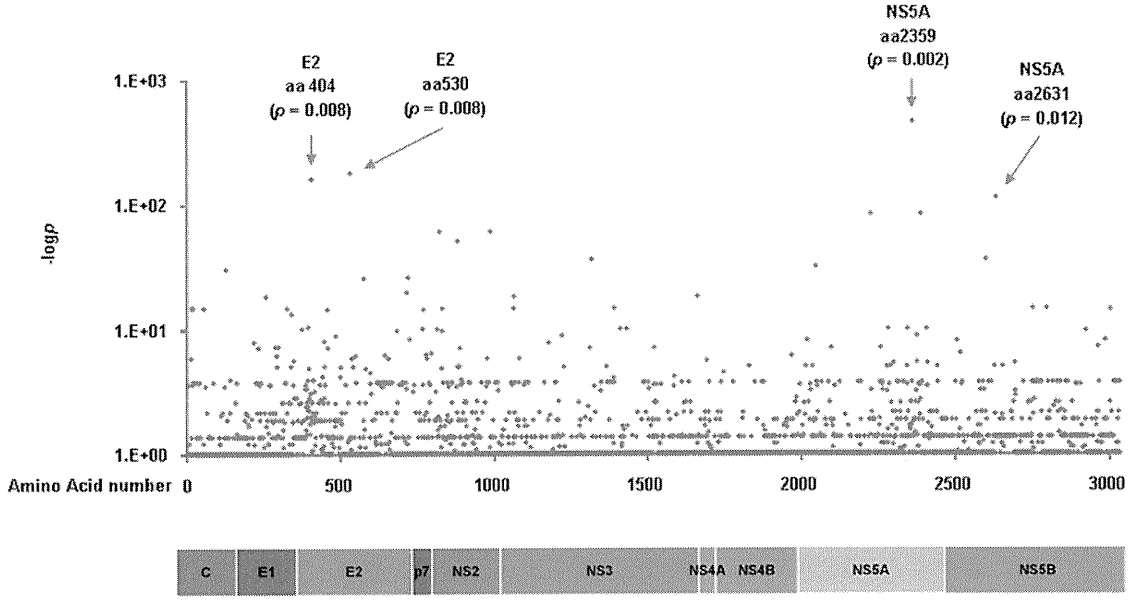

Figure 3. Different amino acid usage at each viral amino acid position between the sustained virological responders (SVR) and the non-sustained virological responders (non-SVR) patients. (a) Different amino acid usage at each viral amino acid position between the SVR and the non-SVR patients was analyzed 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

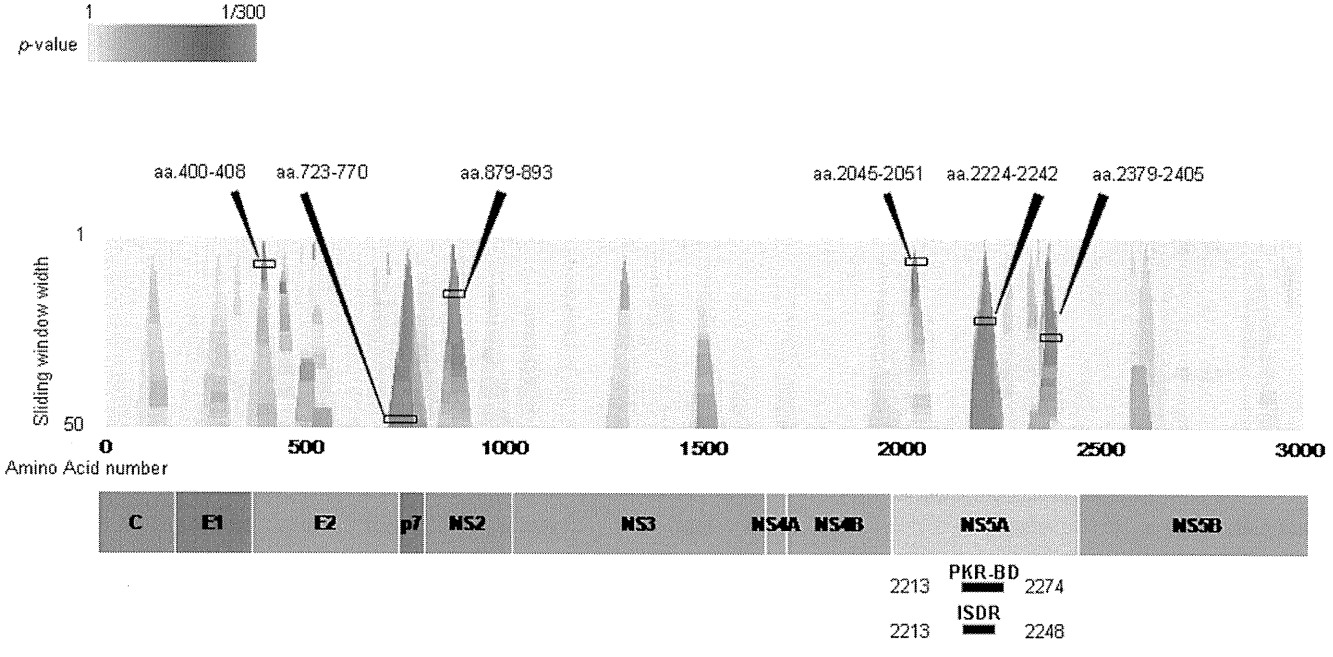

Figure 4. Sliding window analysis. (a) Comparison of amino acid variation between the SVR and non-SVR patients across HCV "regions" using sliding window analysis was performed. Viral regions affecting treatment outcome are shown as red areas. There are six hot areas: amino acid 400–408 and 723–770 in 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.
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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

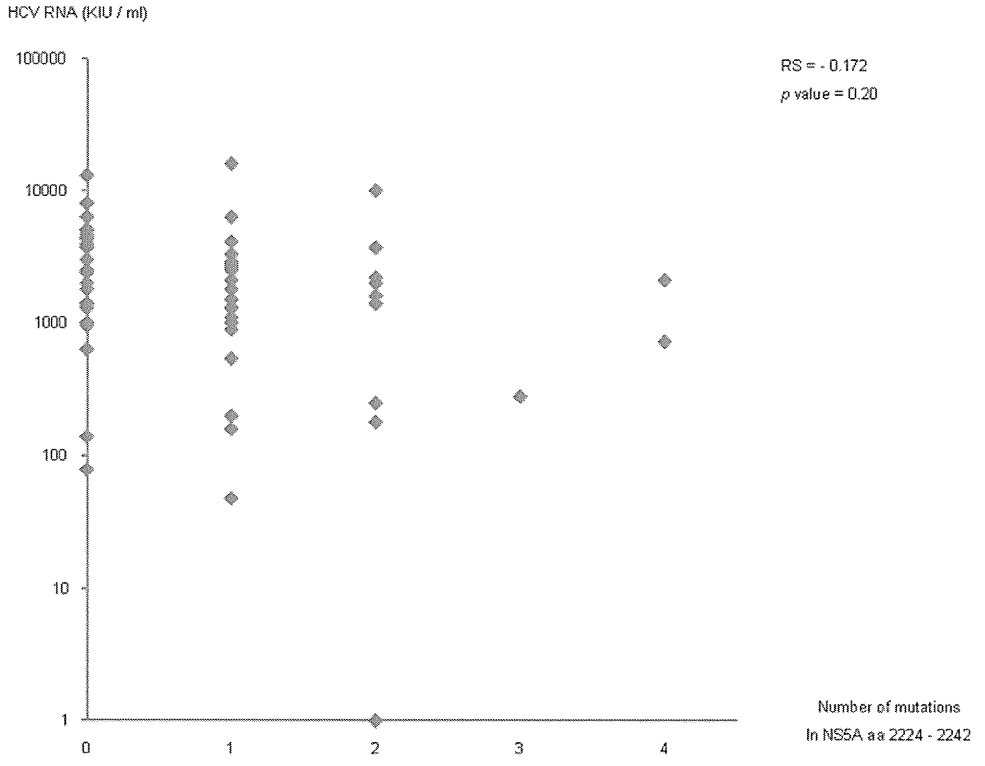

Figure 5. Correlation between pretreatment HCV RNA levels and the number of substitutions in the NS5A region aa 2224 to 2242. Spearman's correlation coefficient by rank test is demonstrated.
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University of Yamanashi (data not shown). The early virological 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.
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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 −logP 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)

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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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hepatocellular carcinoma [1]. In HCV-infected patients with chronic hepatitis, treatment with interferon (IFN) can result in viral clearance and biochemical and histological improvements [2]. The response to the therapy varies according to HCV genotype and pretreatment HCV RNA level [3, 4].

The currently recommended treatment for patients infected genotype 2a HCV with high viral load is pegylated-interferon (PEG-IFN) plus ribavirin (RBV) for 24 weeks [1]. Approximately 80% of patients infected with genotype 2a HCV can achieve a sustained virological response (SVR) with this regimen [5, 6], although much lower percentages of patients infected with other genotypes can achieve SVR, especially with genotype 1 [1]. Because of its high response rate, shorter treatment duration was suggested by some studies, although an agreement has not been reached yet [7, 8]. On the other hand, about 20% of patients infected with this genotype cannot achieve SVR and it remains elusive which patients show poor responses.

Previous studies have reported that amino acid variations in the NS5A-interferon sensitivity determinant region (ISDR) [9], NS5A-interferon ribavirin resistance determinant region (IRRDR) [10], NS5B [11], and PKR-eIF2 phosphorylation homology domain (PePHD) of E2 [12], and core [13, 14] correlate with clinical outcome of IFN-based therapy, including PEG-IFN/RBV therapy in patients infected with genotype 1b HCV. Recent full HCV open reading frame (ORF) analysis for genotype 1 also has reported that core, NS3, and NS5A were associated with early viral response and the outcome in PEG-IFN/RBV therapy [15, 16]. However, in genotype 2a infection, only a few studies have investigated the association between HCV sequence variation and treatment response [17–19], and the role of viral factors has not been established yet, especially in the era of PEG-IFN/RBV therapy. Moreover, these previous studies investigated only several isolated HCV genomic regions, and comprehensive analysis of the full HCV ORF has not been undertaken so far.

In the present study, to assess comprehensively the influence of viral variations on response to the PEG-IFN/RBV therapy in genotype 2a HCV infection, we determined the complete pretreatment HCV ORFs from Japanese patients and investigated viral amino acid variation and their correlation with the response to the combination therapy of PEG-IFN plus RBV.

Patients and methods

Study population

A total of 103 adult Japanese patients infected with genotype 2a HCV, who received the combination therapy with PEG-IFN (PEGINTRON®, Schering-Plough, Tokyo, Japan) plus RBV (REBETOL®, Schering-Plough) between 2005 and 2008 at the University of Yamanashi, Tokyo Medical and Dental University, and related institutions were first included in the study. They all fulfilled the following criteria: (1) negative for hepatitis B surface antigen; (2) high viral load (≥100 KIU/ml); (3) absence of hepatocellular carcinoma; (4) no other form of hepatitis, such as primary biliary cirrhosis, autoimmune liver disease, or alcoholic liver disease; and (5) free of co-infection with human immunodeficiency virus. Informed consent was obtained from each patient. The study was approved by the ethics committees of all the participating universities and hospitals. 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). To clearly disclose the non-SVR viral characteristics, we have considered 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 the 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, 25 patients were excluded for the following reasons: 4 patients received insufficient dose, 8 patients were discontinued from the therapy within 12 weeks, and 13 SVR patients received extended therapy. Finally, 78 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.

The 78 patients belonging to the different institutions were separately analyzed: 43 patients registered in Y-PERS (Yamanashi Pegintron Ribavirin Study Group) were included in group 1 (test group), and the 35 patients from Tokyo Medical and Dental University and related institutions (Ochanomizu Liver Conference Group) were included in group 2 (validation group). We divided the patients into these two groups to exclude false positives (type I errors) which might arise in successive HCV-ORF study. Since genotype-2a HCV contains as many as 3,033 amino acids, it was possible that incorrect amino acids can be judged as significant in full HCV-ORF comparison study as a result of type I errors. Therefore, to guard against false positives, HCV-ORF comparison study was undertaken in group 1, group 2, and combined group.

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 manufacturer's protocol. Complementary deoxyribonucleic acid (DNA) was synthesized with Superscript II (Invitrogen, Tokyo, Japan) using random primers (Invitrogen) and then amplified by two-step nested PCR using the primers newly designed for this study. All samples were initially denatured at 95°C for 7 min, followed by 40 cycles with denaturation at 95°C for 15 s, annealing at 55°C for 15 s, and extension at 72°C for 45 s 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). Generated sequence files were assembled using Vector NTI software (Invitrogen) and base-calling errors were corrected following inspection of the chromatogram.

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 was counted in each amino acid position in each HCV sequence. 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 to generate a "heat map" appearance using Microsoft Excel software. In the present study, p value of 1/1,000 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 Student t test or 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, the receiver operating characteristic curve was constructed. 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 <0.05 by the two-tailed test were considered significant.

Results

Characteristics of the patients studied

Of the patients analyzed, the SVR rate was 78.3% (58/74) with the standard therapy (four non-SVR patients received an extended therapy). The baseline characteristics of the patients (group 1, group2, and combined) classified according to SVR achievement are shown in Table 1. Fibrosis score ($p = 0.047$) and HCV RNA levels ($p = 0.002$) were significantly higher in non-SVR patients, but the cumulative RBV dose ≥80% ($p = 0.003$) and rapid virological response (RVR) rate ($p = 0.011$) were significantly higher in SVR patients. In addition, patients with non-SVR had a tendency to be older ($p = 0.058$). Achievement of RVR reached 61.5% when all patients were included, and this rate was extremely high compared to achievement of RVR in patients with genotype 1b infection (~10%) observed in Yamanashi University Hospital (data not shown). The early virological response (EVR) rate was equally high in the SVR (100%) and non-SVR (89%) groups, showing that relapse to be the characteristic feature of the non-SVR patients with genotype 2a HCV. Actually, 18 patients in non-SVR were relapsers, while two patients were null responders.

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

To determine whether the sequence variations differed between the SVR and non-SVR groups, we first 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. 1, left). These differences were especially significant in E1 and NS3 (Fig. 1, 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.