Fig. 2 Core and NS5A sequences in additional patients were studied. **a** Using sera from 230 additional patients at the time of diagnosis, amino acid usage was compared between HCC and non-HCC patients for the part of core and the NS5A region, and this difference is shown as the *bar height* expressed as reciprocal *p* values. **b** In 230 patients, the association between polymorphisms of core a.a. 70 and the state of liver disease (chronic hepatitis, LC, or HCC) is shown

to R progressively increased in patients in the three major groups of disease activity: chronic hepatitis, cirrhosis, and HCC (Fig. 2b). The association between the disease progression and core a.a. 70 polymorphism also was observed irrespective of IFN-based therapy (data not shown).

Changes in core a.a. 70 over time in patients with and without HCC

We then examined changes in core a.a. 70 over time in association with disease progression (Fig. 3). For this analysis, 55 patients from Group 2, for whom sera from two distant time points were available, were added to the 43 patients in Group 1 and a total of 98 patients were enrolled. When they were classified into two groups according to later HCC onset, the mean observation period was comparable between the groups, 10.4 years for the HCC group and 12 years for the non-HCC group. The occurrence of core a.a. 70Q was 61% (22/36) at the time of
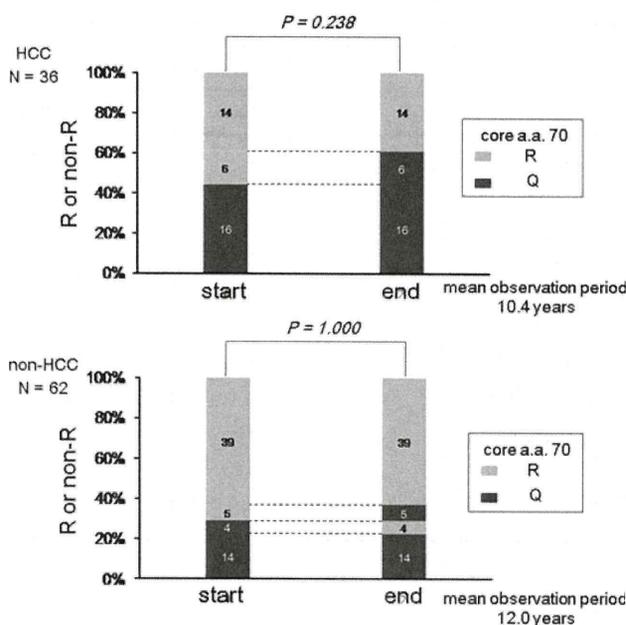

Fig. 3 Changes in core a.a. 70 over time were studied. In 98 patients available for the analysis of 2, sufficiently distant time points, amino acid changes of core a.a. 70 were investigated over time in each patient group. Patients with later HCC development (*upper panel*). Non-HCC patients (*lower panel*)

HCC onset in the HCC group (Fig. 3, upper panel), but 31% (19/62) for the non-HCC group (Fig. 3, lower panel). In contrast, it was 44% (16/36) at the start of observation in the HCC group (Fig. 3, upper panel) and 29% (18/62) for the non-HCC group (Fig. 3, lower panel). Regarding the core a.a. 70 changes over time, R was dominant throughout the observation period in the non-HCC group (71% at the start and 69% at the end), while the dominant amino acid changed from R (56%) to Q (61%) in the HCC group, so that the core a.a. 70 residues of 17% of the HCC patients changed from R to Q during the course of HCC development. In other words, 6 of 22 patients with 70Q at the onset of HCC had 70R originally (6/22, 27%), while 0 of 14 (0%) with 70R at the most recent observation time had 70Q at the beginning. There were no patients with core a.a. 70H throughout the observation period in this population. The result demonstrates that the relationship between 70Q and HCC development is significant at the time of HCC development. At the start of observation, there was also a tendency that the patients with 70Q compared with 70R develop HCC. However, this difference did not reach statistical significance as shown in Supplementary Fig. 1.

Core a.a. 70 changes over time and their association with disease progression

These 98 patients were classified into four groups according to the pattern of core a.a. 70 change (Table 3) and their

Table 3 Progression of liver disease in 98 patients categorized by core a.a. 70 changes over time

	R → R (N = 53)			Q → R (N = 4)		
	Start	End	p value	Start	End	p value
HCC rate (HCC/non-HCC)	–	26.4% (14/39)	–	–	0% (0/4)	–
Sex (male/female)	–	25/28	–	–	4/0	–
Observation period (years)	–	11.1 ± 3.4	–	–	12.9 ± 3.5	–
Age (years)	51.3 ± 11.7	62.4 ± 12.1	<0.001	48.0 ± 11.6	61.0 ± 9.1	0.128
AST (IU/L)	68 ± 73	48 ± 26	0.066	56 ± 32	83 ± 61	0.456
ALT (IU/L)	80 ± 71	48 ± 35	0.003	114 ± 71	96 ± 42	0.678
Platelets (10^{-4}/mm^3)	17.0 ± 5.8	15.0 ± 6.7	0.104	21.3 ± 3.9	17.2 ± 5.2	0.251
Albumin (g/dL)	4.1 ± 0.4	3.9 ± 0.6	0.225	4.4 ± 0.4	4.3 ± 0.4	0.647
γGTP (IU/L)	56 ± 51	38 ± 40	0.052	95 ± 51	61 ± 46	0.371
T.Chol (mg/dL)	172 ± 36	158 ± 33	0.032	152 ± 14	175 ± 32	0.222
Alpha-fetoprotein (ng/mL)	8.3 ± 9.5	12.5 ± 22.1	0.202	6.0 ± 6.0	5.2 ± 2.2	0.816
HCV RNA concentration (kIU/mL)	4,634 ± 8,509	7,070 ± 14,159	0.291	5,798 ± 7,970	13,676 ± 1,881	0.162

	R → Q (N = 11)			Q → Q (N = 30)		
	Start	End	p value	Start	End	p value
HCC rate (HCC/non-HCC)		54.5% (6/5)			53.3% (16/14)	
Sex (male/female)		6/5			13/17	
Observation period (years)		13.7 ± 1.65			10.8 ± 3.5	
Age (years)	56.4 ± 7.5	69.3 ± 9.3	0.002	54.6 ± 8.5	64.9 ± 9.9	<0.001
AST (IU/L)	62 ± 47	46 ± 12	0.285	79 ± 51	60 ± 31	0.087
ALT (IU/L)	100 ± 69	37 ± 15	0.008	95 ± 58	59 ± 36	0.006
Platelets (10^{-4}/mm^3)	17.7 ± 3.9	11.8 ± 4.8	0.005	16.3 ± 6.5	11.9 ± 5.6	0.007
Albumin (g/dL)	4.2 ± 0.2	3.8 ± 0.4	0.005	4.1 ± 0.3	3.8 ± 0.5	0.009
γGTP (IU/L)	73 ± 53	33 ± 16	0.025	101 ± 55	71 ± 65	0.065
T.Chol (mg/dL)	157 ± 21	144 ± 27	0.245	163 ± 28	150 ± 32	0.100
Alpha-fetoprotein (ng/mL)	7.1 ± 4.3	97.8 ± 63.6	0.267	20.8 ± 50.0	35.1 ± 54.7	0.295
HCV RNA concentration (kIU/mL)	2,415 ± 3,163	2,349 ± 1,851	0.957	2,869 ± 3,984	3,229 ± 4,026	0.731

clinical characteristics were investigated. Significant decreases of platelets ($p = 0.007$) and albumin ($p = 0.009$) were observed in the Q unchanged group during the observation period, but not in the R unchanged group ($p = 0.104$ and 0.225, respectively). Because platelets and albumin are markers of liver disease progression, it was considered that the Q unchanged group progressed rapidly with frequent HCC occurrence (53%, 16/30) while the R unchanged group showed stable disease with less frequent HCC occurrence (26%, 14/53). In contrast, the R to Q group showed progressive disease ($p = 0.005$ and 0.005, respectively) similar to the Q unchanged group, while the Q to R group showed stable disease similar to the R unchanged group ($p = 0.251$ and 0.647, respectively), demonstrating that amino acid changes of core a.a. 70 were significantly associated with disease progression.

The IL28B SNP and its association with core a.a. 70 and disease progression

Next, the association between the state of liver disease and IL28B SNP was analyzed for a total of 228 patients through the analysis of the rs8099917 locus. Among them, 162 patients (71%) had the major homozygous TT alleles, while 66 patients (29%) had the minor homozygous or heterozygous alleles (GG/TG). Although some patients had a history of IFN therapy, all patients were positive for HCV RNA at the time of study. The clinical characteristics related to disease progression were compared, as shown in Table 4. Each group consisted of patients with similar distributions of age and sex. Though most clinical factors showed no evident differences in these groups, γGTP was high ($p = 0.020$) and HCV RNA concentration was apt to be low ($p = 0.085$) in TG/GG group. Moreover, the ratio

Table 4 Patient characteristics classified by IL28B SNPs at the time of diagnosis

	TT ($N = 162$)	TG/GG ($N = 66$)	p value
Sex (male/female)	81/81	32/34	0.951
Age (years)	63.3 ± 10.7	63.8 ± 11.9	0.735
Platelets ($10^{-4}/mm^3$)	13.8 ± 5.8	14.3 ± 6.6	0.632
Albumin (g/dL)	4.2 ± 3.0	4.0 ± 0.5	0.528
γGTP (IU/L)	41 ± 39	55 ± 49	0.020
T.Chol (mg/dl)	162 ± 31	157 ± 31	0.237
HCV RNA concentration (kIU/ml)	7,576 ± 10,292	5,069 ± 6,701	0.085
Alpha-fetoprotein (ng/ml)	39.0 ± 152.2	27.3 ± 48.3	0.555
AST (IU/L)	49.6 ± 29.6	51.9 ± 30.5	0.607
ALT (IU/lL)	54.5 ± 53.9	51.6 ± 37.7	0.689
Core a.a. 70R/(Q/H)	106/56	17/49	<0.001
Core a.a. 91L/(M/C)	108/54	38/28	0.253
ISDR	1.2 ± 2.0	0.9 ± 1.5	0.164
IRRDR	5.1 ± 2.4	4.7 ± 2.2	0.207
HCC −/+	115/47	45/21	0.788
IFN −/+	95/67	34/32	0.402

Fig. 4 a Association between the state of liver disease and IL28B SNP. **b** Time-dependent core a.a. 70 changes and its relation to IL28B SNP was investigated in 78 patients
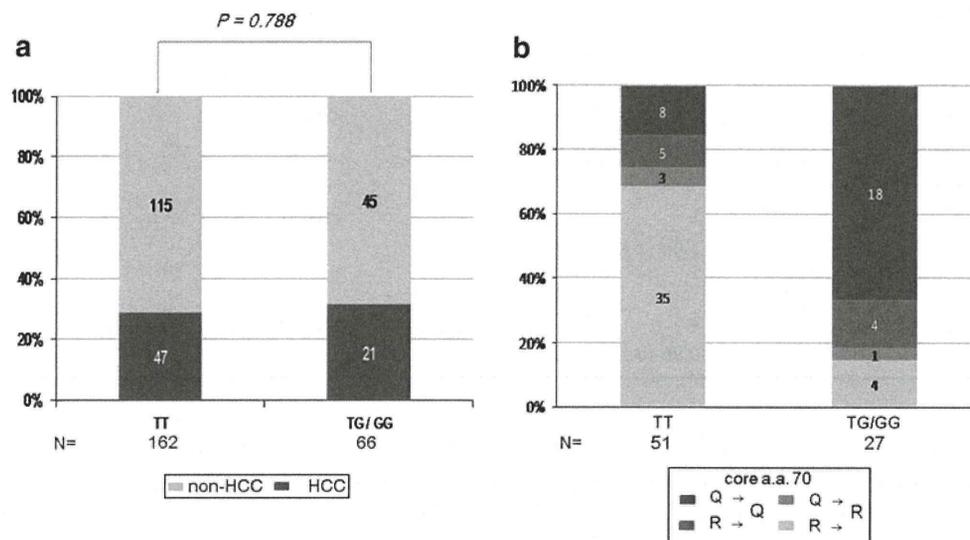
of R/(Q/H) at core a.a. 70 was significantly higher in those with the TT alleles than in those with TG/GG ($p < 0.001$). In association of IL28B SNP with HCC development, there was no evident relationship as demonstrated in Fig. 4.

IL28B SNP and time-dependent core a.a. 70 changes

In Fig. 4b, it is demonstrated that the direction of time-dependent core a.a. 70 change was influenced by IL28B SNPs. In IL28B TG/GG patients, 4 (50%) out of 8 patients with the initial core a.a. 70R changed into 70Q, while only 1 (5%) out of 19 patients with the initial core a.a. 70Q changed into 70R, demonstrating that core a.a. 70 tended to

change into Q over time in IL28B TG/GG patients ($p = 0.034$). On the other hand, there was no evident changing direction in IL28B TT patients; 5 (13%) out of 40 patients with the initial core a.a. 70R changed into 70Q, while 3 (27%) out of 11 patients with the initial core a.a. 70Q changed into 70R ($p = 0.45$).

Multivariate analysis for independent factors influencing core a.a. 70

To investigate further the relationship between core a.a. 70, the IL28B SNP, and HCC development, we divided the patients according to the specification of core a.a. 70 and

Table 5 Factors related to polymorphism of core a.a. 70

Variables	Univariate analysis ($N = 228$)		Multivariate analysis ($N = 228$)	
	Odds ratio (95% CI)	p value	Odds ratio (95% CI)	p value
Sex				
Female	1	0.415	1	0.812
Male	1.23 (0.74–2.01)		1.08 (0.58–1.99)	
Age (years)				
<65	1	0.216	1	0.855
≥65	1.39 (0.82–2.35)		1.06 (0.57–1.96)	
Platelets ($10^{-4}/mm^3$)				
>12	1	0.004	1	0.844
≤12	1.76 (1.03–2.99)		1.07 (0.53–2.16)	
Albumin (g/dL)				
>4	1	0.002	1	0.300
≤4	2.28 (1.33–3.91)		1.46 (0.71–3.00)	
γGTP (IU/L)				
<41	1	0.003	1	0.299
≥41	2.32 (1.32–4.09)		1.42 (0.73–2.79)	
ALT (IU/L)				
<41	1	0.040	1	0.573
≥41	1.74 (1.03–2.94)		1.22 (0.62–2.39)	
IL28B				
TT	1	<0.001	1	<0.001
TG or GG	5.46 (2.88–10.30)		5.74 (2.91–11.31)	
HCC				
–	1	<0.001	1	0.046
+	2.98 (1.65–5.37)		2.21 (1.01–4.83)	
Previous IFN therapy				
–	1	0.874	1	0.644
+	0.96 (0.57–1.62)		0.87 (0.47–1.59)	

those factors, as well as clinical factors, were compared in univariate and multivariate analyses. In Table 5, it may be seen that platelets, albumin, γGTP, ALT, the IL28B SNP, and number of patients with HCC development differed significantly between the two groups in univariate analysis. In contrast, successive multivariate analysis demonstrated that the number of patients with HCC development ($p = 0.046$) and the IL28B SNP ($p < 0.001$) were extracted as independent variables correlated with the core a.a. 70 residue (Table 5).

Multivariate analysis for independent factors influencing HCC development

To disclose factors influencing HCC development, multivariate analysis was performed. As shown in Table 6, age, albumin, and core a.a. 70 residue were extracted as independent factors. On the other hand, IL28B SNP was not extracted as one of those factors.

Discussion

In this study, we have documented several important findings. Through the investigation of HCV sequences, including complete HCV ORFs analysis, we have shown that the core a.a. 70 residue and its changes over time are associated with the disease progression as well as HCC development in genotype-1b HCV infection. Specifically, core a.a. 70Q/H was associated with HCC development and disease progression; core a.a. 70 often changed with time and R70Q substitutions were associated with progressive disease, while Q70R substitutions were associated with the stable disease. Moreover, we have shown that the IL28B SNP and core a.a. 70 showed significant linkage. In contrast, we have also shown that HCC development and disease progression were not apparently correlated with the IL28B SNP.

Recently, core amino acids have been reported in several studies to be associated with HCC [12, 21–25]. In

Table 6 Factors related to influencing HCC development

Variables	Univariate analysis ($N = 228$)		Multivariate analysis ($N = 228$)	
	Odds ratio (95% CI)	p value	Odds ratio (95% CI)	p value
Sex				
Female	1	0.161	1	0.190
Male	1.50 (0.85–2.67)		1.69 (0.77–3.71)	
Age (years)				
<65	1	0.006	1	0.004
≥65	2.30 (1.28–4.16)		3.26 (1.46–7.25)	
Platelets ($10^{-4}/mm^3$)				
>12	1	<0.001	1	0.021
≤12	5.82 (3.11–10.88)		2.59 (1.16–5.82)	
Albumin (g/dL)				
>4	1	<0.001	1	<0.001
≤4	13.75 (6.69–28.24)		7.73 (3.53–16.94)	
γGTP (IU/L)				
<41	1	<0.001	1	0.122
≥41	3.09 (1.70–5.62)		1.87 (0.85–4.13)	
ALT (IU/L)				
<41	1	<0.001	1	0.109
≥41	3.88 (2.06–7.31)		1.98 (0.86–4.56)	
IL28B				
TT	1	0.626	1	0.290
TG or GG	1.17 (0.13–2.17)		0.63 (0.27–1.49)	
Core a.a. 70				
R	1	<0.001	1	0.029
Q/H	2.91 (1.61–5.26)		2.44 (1.09–5.44)	
Previous IFN therapy				
−	1	0.949	1	0.331
+	0.98 (0.55–1.74)		1.46 (0.68–3.16)	

these studies, patients with core a.a. 70Q/H frequently developed HCC with exacerbation of liver damage. In this analysis, we confirmed the previous findings. However, because this association might be a reflection of the core-dependent IFN sensitivity differences often reported in recent studies [12, 22, 25], we restricted the analysis to patients, who were unable to clear HCV RNA previously through IFN-based therapy. Moreover, we also confirmed the relationship of the core sequences and disease development among the populations without a previous history of IFN therapy (data not shown). These findings strongly confirmed the role of core a.a. 70 in disease progression, independent of any IFN response.

It is a focus of interest how the core sequence evolves with time or with the course of disease. If the core sequences were fixed throughout the course of disease, HCV with core 70Q might be an "oncogenic" virus, while HCV with core 70R might be "non-oncogenic", and the initial viral sequence might forecast future liver disease. In

this study, we have demonstrated that core sequences changed in 15% (15/98) of patients during the observation period of around 10 years. Among these changes, R70Q ($N = 11$) was more common than Q70R ($N = 4$). Interestingly, changes in this region were significantly associated with disease activity or HCC development, although patients with R70Q substitutions were significantly more likely to have exacerbation of the disease and Q70R substitutions were associated with the stable disease. These results demonstrate that the core a.a. 70 residue is not fixed, but often changes with time during the course of disease in close association with disease progression and HCC development. Although the molecular mechanism of their interaction needs further exploration, this result highlights the important clinical and basic implications for the association between host and virus.

The importance of the IL28B SNP has been demonstrated recently in HCV infection in terms of a correlation with treatment outcome of pegylated-IFN plus ribavirin

therapy [16–19]. The contribution of the IL28B SNP to the outcome of therapy was confirmed in successive studies, although the mechanism remains under investigation. On this basis, we sought to investigate the impact of the IL28B SNP on disease progression and HCC development, separate from the IFN-based treatment response. As shown in Table 4, we compared the clinical features between the two groups (IL28B GG/TG vs. IL28B TT). Importantly, this comparison disclosed a significant correlation between the core a.a. 70 polymorphisms and the IL28B SNP ($p < 0.001$) and confirmed the existence of a complex interaction between the host and the virus in chronic HCV infection. According to the result, patients with IL28B TG/GG were more likely to be infected with HCV with core a.a. 70Q/H than with core a.a. 70R and vice versa. Although the molecular mechanisms of their relationship remain unknown, it could be speculated that the IL28B SNP has an influence on the viral core sequences, because the host IL28B SNP remains fixed and cannot be influenced by the viral core sequence.

On the other hand, we observed no evident association between the IL28B SNP and HCC development. This was rather unexpected because it is considered that the IL28B SNP has a significant influence on the core a.a. 70 residue. Therefore, to clarify the correlation among core a.a. 70, IL28B, and HCC development, we undertook multivariate analysis to extract the independent variables affecting the core 70 residue. As demonstrated in Table 5, the IL28B SNP and the development of HCC were extracted as variables independently correlated with the core a.a. 70 residue. The result indicates that the core a.a. 70 residue was not only influenced by the IL28B SNP, but also by factors strongly related to HCC development, independent of the IL28B SNP. When considering the result, it is not strange if there is no direct relationship between IL28B SNP and HCC development. In contrast, multivariate analysis undertaken for disclosing factors influencing HCC development revealed that core a.a. 70 residue was a variable independently associated with HCC development other than age, albumin, or platelets even though the IL28B SNP was not extracted (Table 6). However, further comprehensive studies are warranted to disclose the molecular mechanisms for the complicated relationships among core a.a. 70, IL28B, and HCC development.

In conclusion, we have shown that core a.a. 70 was closely associated with disease progression and, often, changes of that residue were accompanied by temporal changes in liver damage, in close relationship with the IL28B SNP.

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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

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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

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 pegy-lated-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 varia-tions in the NS5A-interferon sensitivity determinant region (ISDR) [9], NS5A-interferon ribavirin resistance determi-nant 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 deter-mined the complete pretreatment HCV ORFs from Japa-nese 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 geno-type 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 fol-lowing criteria: (1) negative for hepatitis B surface antigen; (2) high viral load (≥100 KIU/ml); (3) absence of hepa-tocellular 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 subcutane-ously, 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 exten-ded 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 con-sidered 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 institu-tions (Ochanomizu Liver Conference Group) were inclu-ded 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 $\geq 80\%$ ($p = 0.003$) 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 ($\sim 10\%$) 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.

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

Characteristic	SVR ($n = 58$)			Non-SVR ($n = 20$)			p value[△]
	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[†]
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[‡]
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[‡]
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[‡]
Fibrosis score (0–2/≥3)[§]	34/1	19/2	53/3	4/3	11/2	15/5	0.049[†]
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[‖]
IFN dose (≥80%/60–80%)[¶]	28/4	21/1	49/5	4/3	11/2	15/5	0.12[†]
Ribavirin dose (≥80%/60–80%)[¶]	27/5	17/5	44/10	4/3	5/8	9/11	0.003[†]
RVR rate (%)	87.5	54.5	74.1	33.3	46.1	42.1	0.022[†]
EVR rate (%)	100	100	100	66.7	100	89.4	0.07[†]

* Mean ± SD; ** median (range); [†] Fisher's exact probability test; [‡] Student t test; [‖] Mann–Whitney's U test; [△] 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*)
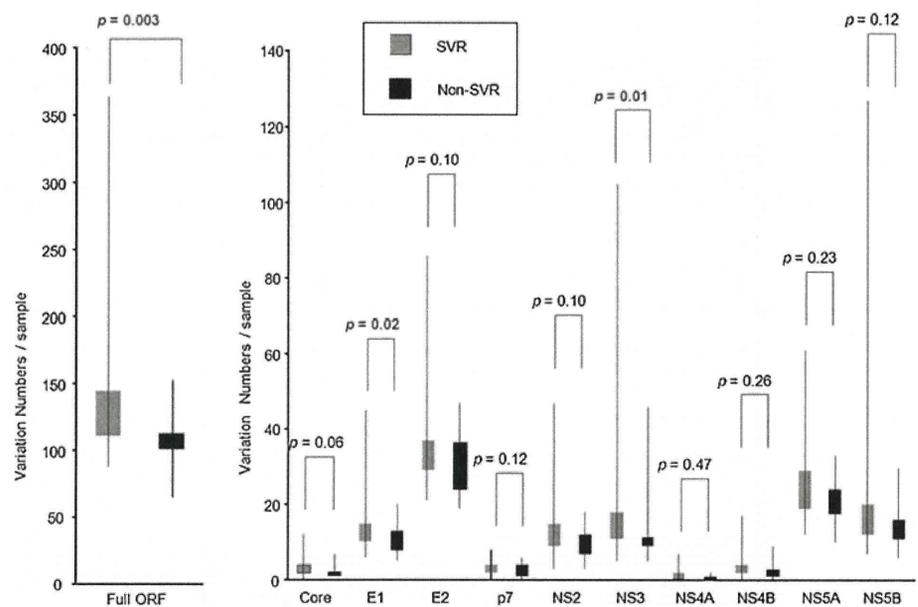
Comparison of HCV sequence variation between the SVR and non-SVR patients at each amino acid position

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
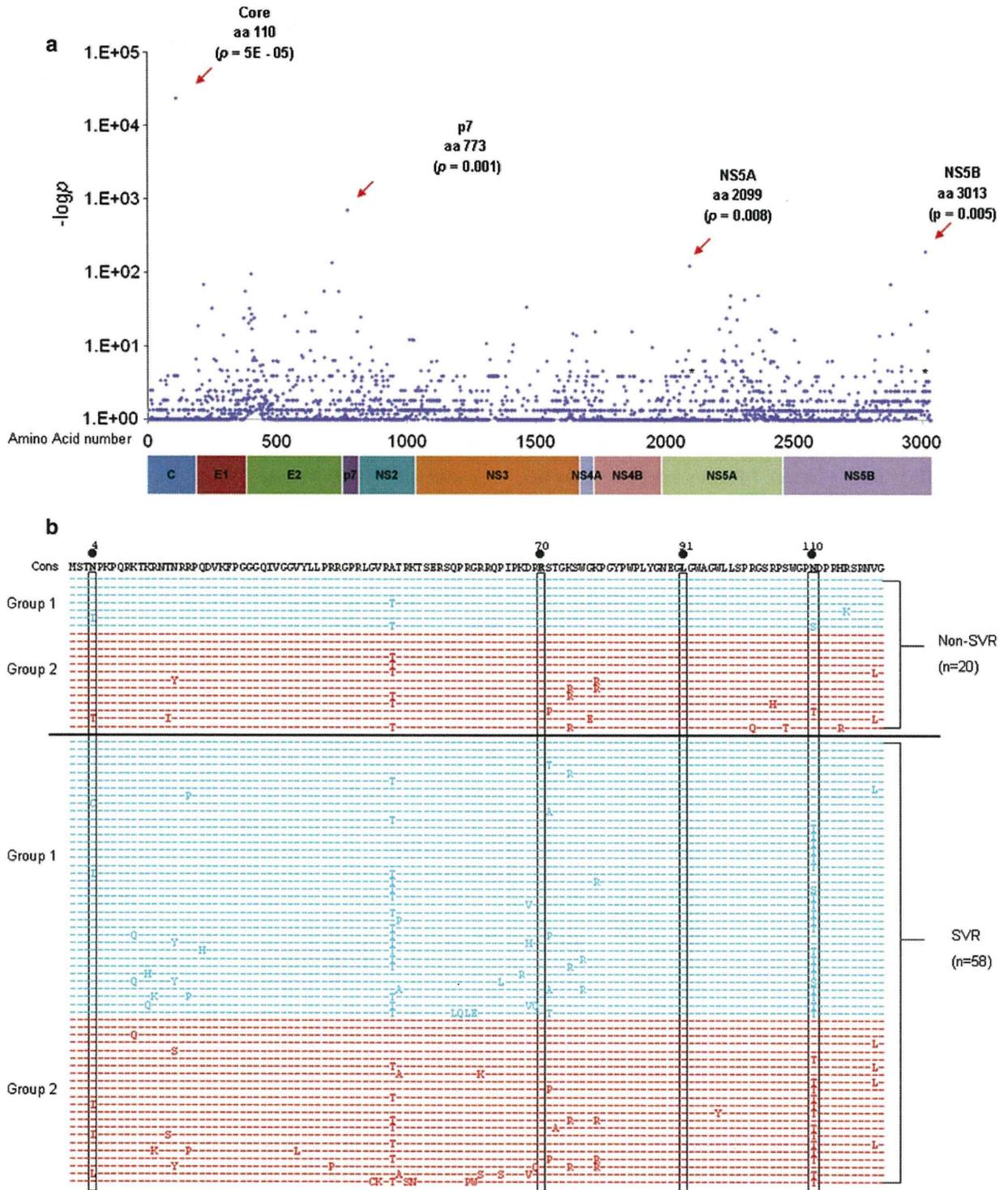
Fig. 2 a Different amino acid usages at each viral amino acid position between the sustained viral responders (SVR) and the non-sustained viral responders (non-SVR) patients. Amino acid variation was determined between SVR and non-SVR patients by Fisher's exact probability test. The longitudinal axis 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
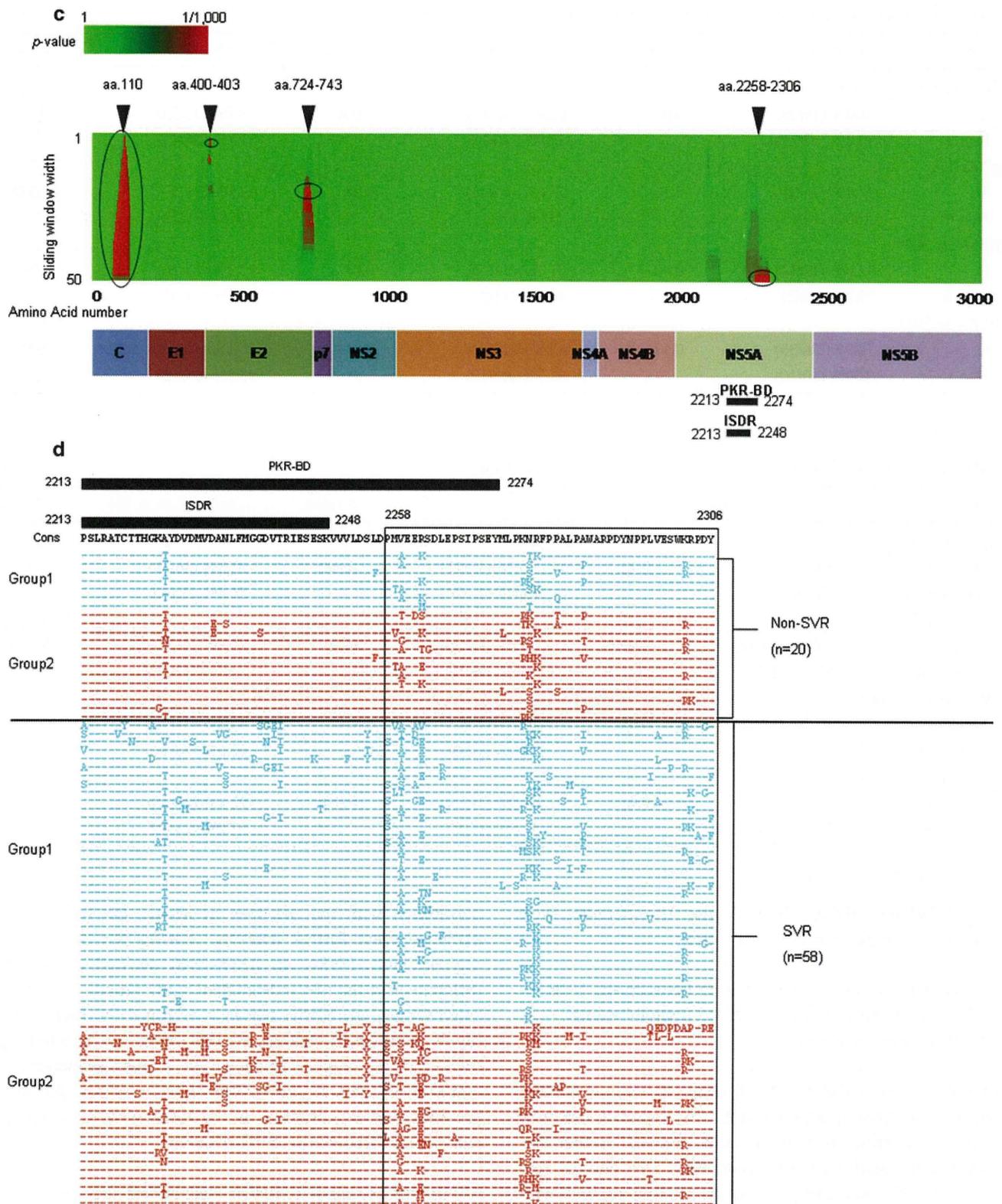
Fig. 2 continued

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 $\geq 80\%$ 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
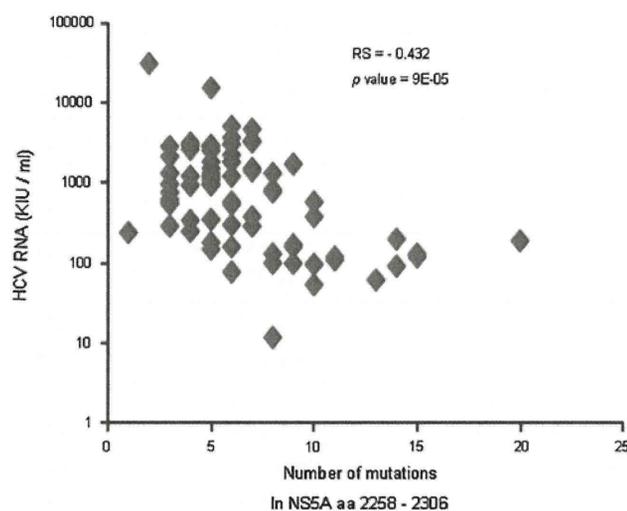
Fig. 3 Correlation between pretreatment HCV RNA levels and the number of substitutions in the NS5A region aa 2258–2306. Spearman's correlation coefficient by rank test is demonstrated

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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Characterization of naturally occurring protease inhibitor-resistance mutations in genotype 1b hepatitis C virus patients

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Abstract

Background and aims Protease inhibitor (PI)-resistant hepatitis C virus (HCV) variants may be present in substantial numbers in PI-untreated patients according to recent reports. However, influence of these viruses in the clinical course of chronic hepatitis C has not been well characterized.

Methods The dominant HCV nonstructural 3 (NS3) amino acid sequences were determined in 261 HCV genotype 1b-infected Japanese patients before pegylated interferon plus ribavirin (PEG-IFN/RBV) therapy, and investigated the patients' clinical characteristics as well as treatment responses including sustained virological response (SVR) rate. HCV-NS3 sequences were also determined in 39 non-SVR patients after completion of the therapy.

Results Four single mutations (T54S, Q80K, I153V, and D168E) known to confer PI resistance were found in 35 of 261 patients (13.4%), and double mutations (I153V plus T54S/D168E) were found in 6 patients (2.3%). Responses to PEG-IFN/RBV therapy did not differ between patients with and without PI-resistance mutations (mutation group, SVR 48%; wild-type group, SVR 40%; $P = 0.38$). On the other hand, two mutations appeared in two non-SVR patients after PEG-IFN/RBV therapy (I153V and E168D, 5.1%).

Conclusions PI-resistance-associated NS3 mutations exist in a substantial proportion of untreated HCV-1b-infected patients. The impact of these mutations in the treatment of PIs is unclear, but clinicians should pay attention to avoid further development of PI resistance.

Introduction

Hepatitis C virus (HCV) infects more than 170 million persons worldwide and thus represents a global health problem. At least 130 million infected individuals are chronic carriers of HCV and are at significant risk of developing liver cirrhosis and hepatocellular carcinoma [1]. The current standard treatment with pegylated interferon plus ribavirin (PEG-IFN/RBV) is complicated by frequent adverse reactions, and a sustained virologic response (SVR) can be achieved only in 50% of patients infected with the most prevalent genotype 1 [2]. In Japan, since 70% of patients are infected with intractable genotype 1b HCV, more effective treatments are urgently required.

A promising approach is the development of specifically targeted antiviral therapies for hepatitis C (STAT-C). HCV-specific protease inhibitors (PIs) target an essential step in HCV replication by blocking the nonstructural 3/4A (NS3/4A) protease-dependent cleavage of the HCV polyprotein

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[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