Table 4. Correlation between NS5A sequence heterogeneity and SVR or RVR in HCV-2a and HCV-2b infections.

Factor	SVR	Non-SVR	*P* value	RVR	Non-RVR	*P* value
IRRDR[2a]≥4	42/49* (86%)	2/9 (22%)	**0.0003**	42/46 (93%)	5/15 (33%)	<0.0001
IRRDR[2a]≤3	7/49 (14%)	7/9 (78%)		4/46 (7%)	10/15 (67%)	
ISDR/+C[2a]≥1	35/49 (71%)	2/9 (22%)	**0.008**	32/46 (70%)	7/15 (47%)	0.1
ISDR/+C[2a] = 0	14/49 (29%)	7/9 (78%)		14/46 (30%)	8/15 (53%)	
IRRDR/N[2b]≥2	17/34 (50%)	6/13 (46%)	1.0	22/34 (65%)	3/17 (18%)	0.0025
IRRDR/N[2b]≤1	17/34 (50%)	7/13 (54%)		12/34 (35%)	14/17 (82%)	

*No. of isolates with a given factor/total no. of SVR or RVR.
Abbreviations: SVR, sustained virological response; RVR, rapid virological response; IRRDR[2a], interferon/ribavirin resistance-determining region of HCV-2a; ISDR/+C[2a], part of interferon sensitivity determining-region plus its carboxy-flanking region of HCV-2a; IRRDR/N[2b], an N-terminal part of interferon/ribavirin resistance-determining region of HCV-2b.
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with the prototype sequences (HCV-J6 [18] and HCV-J8 [19]). The residues at positions 70 and 91, which were reported to be associated with the treatment outcome in HCV-1b infection [13], were both well conserved among HCV-2a and -2b isolates and, therefore, no correlation with treatment outcome was expected for these residues (Figures S1 and S2). In this connection, the residues at positions 48 and 110 of HCV-2a isolates showed certain degrees of variation. However, there was no significant correlation between the sequence patterns and the treatment outcome.

Identification of Independent Predictive Factors for SVR and RVR in HCV-2a and HCV-2b infections

In order to identify significant independent predictors of SVR in HCV-2a and HCV-2b infections, univariate and multivariate logistic regression analyses were carried out using all available data of baseline patients' parameters and viral genetic polymorphic factors. Univariate analysis identified 3 factors that were significantly associated with SVR in HCV-2a infection; the heterogeneity of IRRDR[2a] (≥4 vs. ≤3), ISDR/+C[2a] (≥1 vs. = 0) and patients' age (<55 years) (Table 5). Subsequently, these factors were entered in multivariate regression analysis. The result obtained revealed that the IRRDR[2a] heterogeneity was the only independent predictive factor for SVR in HCV-2a infection ($P = 0.001$). The IRRDR[2a] heterogeneity was also the independent predictive factor for RVR (Table S1).

As for HCV-2b infection, univariate analysis identified two host factors that were significantly, or almost significantly, associated with SVR; γ-GTP levels (<30 IU/L) and body weight (<65 kg) (Table 5). No viral factor was identified in this analysis. In subsequent multivariate analysis, γ-GTP levels was identified as an independent predictive factor for SVR in HCV-2b infection. In this connection, the heterogeneity of IRRDR/N[2b], a viral factor, was identified to be significantly associated with RVR in HCV-2b infection (Table S1).

Discussion

The clinical outcome of PEG-IFN/RBV therapy for HCV infection is influenced by a number of host and viral factors [20]. It has recently been reported that host genetic polymorphisms near or within the IL28B gene on the chromosome 19 show a critical impact on the treatment outcome of patients infected with HCV-1a and -1b [21–23]. Also, HCV genetic polymorphisms have been known to contribute to differences in the treatment outcome, as demonstrated by the observations that SVR rates for patients infected with HCV genotypes 2 and 3 are higher than those for patients infected with HCV genotype 1 [2,6]. Moreover,

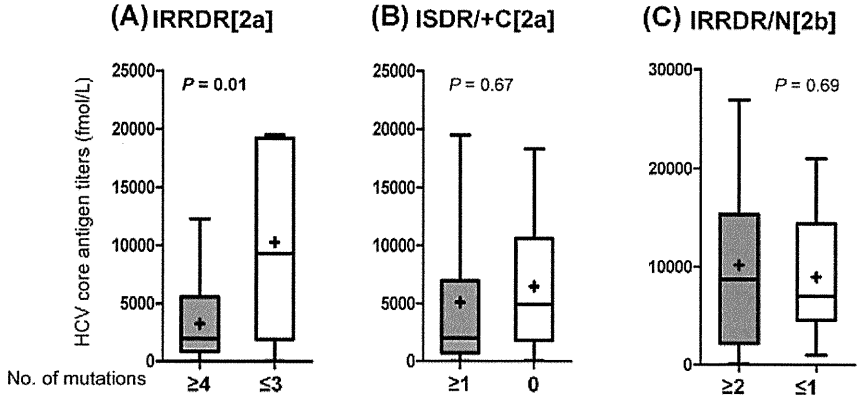

Figure 4. Correlation between NS5A sequence heterogeneity and pretreatment serum HCV core antigen titers in HCV-2a and HCV-2b infections. Pretreatment serum HCV core antigen titers of patients classified on the basis of the number of mutations in IRRDR[2a] (interferon/ribavirin resistance-determining region of HCV-2a) (≥4 vs. ≤3) (A), ISDR/+C[2a] (part of interferon sensitivity determining-region plus its carboxy-flanking region of HCV-2a) (≥1 vs. = 0) (B) and IRRDR/N[2b] (≥2 vs. ≤1) (an N-terminal part of interferon/ribavirin resistance-determining region of HCV-2b) (C) are depicted. Maximum and minimum values are indicated by the upper and lower bars, respectively. Distribution ranges are displayed as boxes. Mean and median values are also indicated inside the boxes as+and horizontal bars, respectively.
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Table 5. Univariate and multivariate analyses for identification of independent predictive factors for SVR in HCV-2a- and -2b-infected patients treated with PEG-IFN/RBV therapy.

Genotype	Variable	Univariate		Multivariate	
		Odds ratio (95% CI)	*P* value	Odds ratio (95% CI)	*P* value
HCV-2a	IRRDR[2a] mutations	21.0 (3.6–122.5)	0.0003	21.0 (3.6–122.5)	**0.001**
	ISDR/+C[2a] mutations	8.8 (1.6–47.4)	0.008		
	Age (<55 years)	9.8 (1.1–84.7)	0.026		
HCV-2b	γ-GTP (<30 IU/L)	26.0 (1.3–504.7)	0.004	6.2 (1.1–36.2)	0.04
	Body weight (<65 kg)	3.8 (1.0–13.9)	0.06		

Abbreviations: SVR, sustained virological response; IRRDR[2a], interferon/ribavirin resistance-determining region of HCV-2a; ISDR/+C[2a], part of interferon sensitivity determining-region plus its carboxy-flanking region of HCV-2a; γ-GTP, gamma glutamyl transpeptidase.

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polymorphisms of NS5A and core regions of a given HCV genotype, in particular HCV-1b, have been linked to the difference in SVR rates [7,8,11–13,17]. It should be noted that the significant link between polymorphisms of NS5A and core regions of HCV-1b and treatment outcome was inferred mostly from studies carried out on patients in Asian countries, in particular Japan, and that somewhat controversial results were obtained from studies carried out on patients infected with HCV-1a or -1b in non-Asian countries [24–31]. However, we would like to point out that most of these publications focused mainly on ISDR and core mutations, but not on IRRDR. In addition, the impact of viral genetic variation on treatment outcome in non-HCV-1 infection, either in Asian or non-Asian countries, is still unclear.

In our previous study, we identified IRRDR in NS5A of HCV-1b as a significant determinant for PEG-IFN/RBV treatment outcome; EVR and, more importantly, SVR [11,12]. Consistent with the previous observation, we have demonstrated in the present study that sequence heterogeneity within IRRDR is closely correlated with the treatment responses in HCV-2a and -2b infections. In HCV-2a infection, IRRDR[2a]≥4 was closely associated with RVR (Table S1) and SVR (Table 5). In HCV-2b infection, the sequence heterogeneity within an N-terminal part of IRRDR (IRRDR/N[2b]) was significantly associated with RVR (Table S1). Furthermore, both IRRDR[2a]≥4 and ISDR/+C[2a]≥1 showed remarkable positive predictive values (95%) for SVR prediction (Table S2), suggesting the clinical usefulness of these markers to encourage those patients to receive PEG-IFN/RBV treatment. On the other hand, their negative predictive values for non-SVR were rather low (50% and 33%). This suggests the possible involvement of another factor(s) that determines non-SVR and may limit the clinical usefulness of these markers to accurately predict non-SVR.

The present results were dependent upon the small number of non-SVR patients due to the high response rates of HCV-2a and -2b. In spite of this, the parallels between the RVR/non-RVR and the SVR/non-SVR analyses, especially in HCV-2a infection, support the possibility that the sequences presented in this study are truly representative of the viruses in general circulation.

The clinical correlation between IRRDR sequence heterogeneity and virological responses of IFN-based therapy in HCV infection can be linked to a recent experimental observation by Tsai et al. [32] that an HCV subgenomic RNA replicon containing NS5A of HCV-1b exerted more profound inhibitory effects on IFN activities than the original HCV-2a replicon, and that domain swapping between NS5A sequences of HCV-1b and -2a in the V3 and/or a C-terminal region including IRRDR resulted in a transfer of their anti-IFN activities. Also, it is worthy to note that IRRDR is among the most variable sequences across the different genotypes and subtypes of HCV [33] whereas its upstream and downstream sequences show a higher degree of sequence conservation (Figure 5). This may suggest that whereas the upstream and downstream sequences have a conserved function(s) across all the HCV genotypes, IRRDR sequences have a genotype-dependent or even a strain-dependent function(s). Indeed, the upstream sequences, especially a Pro-rich motif, play key roles in multiple stages of viral replication [34] while the downstream sequence in viral particle assembly and production [35]. Therefore, the sequence heterogeneity of IRRDR and its significant correlation with IFN-responsiveness imply the possibility that IRRDR is involved, at least partly, in the viral strategy to evade IFN-mediated antiviral host defense mechanisms. Its possible molecular mechanism, however, is yet to be elucidated. The IRRDR sequence heterogeneity also suggests genetic flexibility of this region and, indeed, the C-terminal portion of NS5A was shown to tolerate sequence insertions and deletions [36]. This flexibility might play an important role in modulating the interaction with various host systems, including IFN-induced antiviral machineries. It is also possible that the genetic flexibility of IRRDR is accompanied by compensatory changes elsewhere in the viral genome and that these compensatory changes affect overall viral fitness and responses to IFN-based therapy [37].

The relapse rate was higher in HCV-2b infection than in HCV-2a (Table 1). It should be noted that while the sequence heterogeneity within IRRDR[2a] was significantly correlated with both RVR and SVR in HCV-2a infection, IRRDR/N[2b] was correlated only with RVR in HCV-2b infection. These observations might be linked to an intrinsic difference in IFN- and/or RBV-sensitivity between HCV-2a and -2b isolates [8,38]. We assume that HCV-2b is considered between HCV-1b and HCV-2a in terms of resistance to PEG-IFN/RBV treatment and that an extended treatment for a total of 36~48 weeks would be needed to prevent relapse in HCV-2b infection, especially for patients who have risk factors that do not fit the SVR or RVR prediction criteria (Table 5 and Table S1).

A mutation at position 70 of the core protein of HCV-1b has been reported to be correlated with PEG-IFN/RBV treatment outcome [12,13]. In the present study, however, we found no significant correlation between core protein polymorphism and treatment outcome in HCV-2a or -2b infections. The residue at position 70 of the core protein of HCV-2a and -2b isolates was Arg, which is known to be associated with SVR in HCV-1b infection [12,13], and was well conserved in all the isolates tested in the present study (Figures S1 and S2). The observed sequence

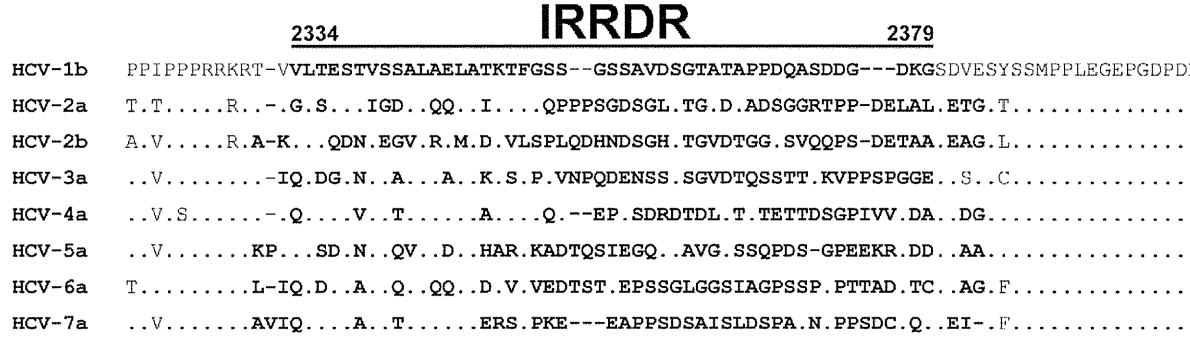

Figure 5. Sequence alignment of IRRDR (interferon/ribavirin resistance-determining region) and its upstream and downstream sequences of different HCV genotypes. The residues in the region that corresponds to IRRDR of HCV-1b [11] are written in boldface letters. Dots indicate residues identical to the HCV-1b sequence. References of aligned sequences are: HCV-1b, El-Shamy et al. [11]; HCV-2a and -2b, Murakami et al. [8]; HCV-3a, X76918; HCV-4a, Y11604; HCV-5a, AF064490; HCV-6a, D84262; HCV-7a, EF108306.
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conservation at position 70 might be the reason for the lack of significant correlation between core protein polymorphism and treatment outcome in HCV-2a or -2b infections. On the other hand, Thr at position 110 of the core protein of HCV-2a has recently been reported to be significantly associated with SVR [10]. In the present study, Thr at position 110 was found in 35% (14/40) and 14% (1/6) of SVR and non-SVR cases, respectively (Figure S1). Similarly, Thr at position 48 was found in 35% (14/40) of SVR cases, but not in non-SVR cases (0/6). The observed differences between SVR and non-SVR, however, were not statistically significant due possibly to the small number of samples tested. A larger-scale study would be needed to determine the possible importance of those residues.

We preliminarily analyzed a host genetic factor, the single nucleotide polymorphism (SNP) at rs8099917 near the IL28B gene [21–23], of a portion of the patients examined in the present study. The result showed that the minor genotypes (T/G and G/G) were found in 5.1% (2/39) and 15.4% (2/13) of RVR and non-RVR patients, respectively, and 2.8% (1/36) and 20.0% (2/10) of SVR and non-SVR patients, respectively (Kim et al., unpublished observation). Although the differences were not statistically significant due probably to the small number of the patients tested, the minor genotypes showed a trend toward being associated with non-SVR, and with non-RVR to a lesser extent, in HCV-2a and -2b infections, as has been reported for HCV-1a and -1b infections [21–23]. The impact of the IL28B SNP, however, appeared to be weaker in HCV-2a and -2b infections than that seen in HCV-1a and -1b infections, and also weaker than that of the most powerful viral factor, IRRDR[2a]≥4, in HCV-2a infection. In this context, we found that, of the four patients with the minor IL28B genotypes, two patients (nos. 2 and 105), who underwent unfavorable treatment response (non-RVR and non-SVR), were infected with HCV isolates of IRRDR[2a]≤3 or IRRDR/N[2b]≤1 while the other two patients (no. 63 and 106), who achieved favorable treatment response (SVR and/or RVR), were infected with HCV isolates of IRRDR[2a]≥4. This might imply the possibility that, in HCV-2 infection, the combination of the minor IL28B genotypes and a low degree of IRRDR sequence heterogeneity has a strong power to predict unfavorable treatment responses whereas a high degree of IRRDR sequence heterogeneity has a dominant predictive power for favorable treatment responses regardless the IL28B genotype. Analysis in a large-scale multicenter study is needed to clarify this issue.

In conclusion, our data suggest that the sequence heterogeneity of NS5A, i.e., IRRDR[2a]≥4, and ISDR/+C[2a]≥1 to a lesser extent, would be a useful predictive marker for SVR in HCV-2a infection. Also, IRRDR/N[2b]≥2 is significantly associated with RVR in HCV-2b infection. These results further emphasize the importance of NS5A, a viral factor, in determining the responsiveness to PEG-IFN/RBV therapy.

Supporting Information

Figure S1 Sequence alignment of the core protein of HCV-2a isolates. Core protein sequences (aa 1 to 120) of HCV-2a obtained from SVR and non-SVR patients are aligned. Prototype sequence of HCV-J6 [18] is shown on the top. The numbers along the sequence indicate the aa positions. Dots indicate residues identical to those of the prototype sequence.
(TIF)

Figure S2 Sequence alignment of the core protein of HCV-2b isolates. Core protein sequences (aa 1 to 120) of HCV-2b obtained from SVR and non-SVR patients are aligned. Prototype sequence of HCV-J8 [19] is shown on the top. The numbers along the sequence indicate the aa positions. Dots indicate residues identical to those of the prototype sequence.
(TIF)

Table S1 Univariate and multivariate analyses for identification of independent predictive factors for RVR in HCV-2a- and -2b-infected patients treated with PEG-IFN/RBV therapy.
(DOC)

Table S2 Positive and negative predictive values (PPV and NPV) of NS5A polymorphic factors for SVR prediction.
(DOC)

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Author Contributions

Conceived and designed the experiments: AE SRK HH. Performed the experiments: AE IS YI LD. Analyzed the data: AE IS YI LD SI SY TF ST YY YS TA HH. Contributed reagents/materials/analysis tools: SRK SI SY TF ST YY YS TA. Wrote the paper: AE SRK HH. Obtained permissions from the Ethics Committees: AE SRK HH.

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Polymorphisms of Hepatitis C Virus Non-Structural Protein 5A and Core Protein and Clinical Outcome of Pegylated-Interferon/Ribavirin Combination Therapy

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Key Words
Hepatitis C virus · Non-structural protein 5A · Interferon/ribavirin resistance-determining region · Interferon sensitivity-determining region · Core protein · Sustained virological response · Prediction

Abstract
Objective: Hepatitis C virus (HCV genome) polymorphisms are thought to influence the outcome of pegylated-interferon/ribavirin (PEG-IFN/RBV) therapy. This study aimed to examine non-structural protein 5A (NS5A) polymorphisms, e.g. IFN/RBV resistance-determining region (IRRDR) and IFN sensitivity-determining region (ISDR), and core protein polymorphism as predictive therapeutic markers. ***Methods:*** Pretreatment sequences of NS5A and core regions were analyzed in 68 HCV-1b-infected patients treated with PEG-IFN/RBV. ***Results:*** Of 24 patients infected with HCV having an IRRDR with 6 or more mutations (IRRDR≥6), 18 (75%) patients achieved sustained virological response (SVR), whereas only 11 (25%) of 44 patients infected with HCV having IRRDR≤5 did. IRRDR≥6 was significantly associated with SVR ($p < 0.0001$). On the other hand, ISDR≥2 was significantly associated with relapse (either before [breakthrough] or after end-of-treatment response [ETR-relapse]) ($p < 0.05$) and a point mutation of the core protein from Arg to Gln at position 70 (Gln^{70}) was significantly associated with null-response ($p < 0.05$). Multivariate analysis identified IRRDR≥6 as the only viral genetic factor that independently predicted SVR. ***Conclusion:*** NS5A (IRRDR and ISDR) and core protein polymorphisms are associated with the outcome of PEG-IFN/RBV therapy for chronic hepatitis C. In particular, IRRDR≥6 is a useful marker for prediction of SVR.

Introduction

Hepatitis C virus (HCV) is the major cause of chronic liver diseases worldwide [1]. As a consequence of the long-term persistence of chronic hepatitis C, the number of patients with hepatocellular carcinoma is expected to increase further over the next 20 years [2]. To reduce the impact of this worldwide health problem, efficient treatment is required. Currently, a combination therapy of pegylated-interferon-α and ribavirin (PEG-IFN/RBV) is a

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standard treatment for chronic hepatitis C [3]. However, this therapy is sometimes difficult to tolerate and results in a sustained virological response (SVR) in only ~50% of patients, especially those infected with the most resistant genotypes, HCV-1a and HCV-1b [3]. Given the considerable side effects, the possibility of discontinuation and the high cost of this treatment, prediction of treatment outcome is needed. An expanded range of predictors may assist clinicians and patients in more accurately assessing the likelihood of an SVR and thus in making more informed treatment decisions [4].

Since the HCV genotype is one of the major factors affecting the IFN-based therapy response, IFN resistance is, at least partly, genetically encoded by HCV itself [5]. In this context, non-structural protein 5A (NS5A) has been widely discussed for its correlation with IFN responsiveness. Enomoto et al. [6] proposed that sequence variations within a region in NS5A spanning from amino acids (aa) 2,209 to 2,248, called the IFN sensitivity-determining region (ISDR), is correlated with IFN responsiveness. Recently, we identified a new region near the C-terminus of NS5A spanning from aa 2,334 to 2,379, which we referred to as the IFN/RBV resistance-determining region (IRRDR) [7]. The degree of sequence variation within IRRDR was significantly associated with the clinical outcome of PEG-IFN/RBV combination therapy. On the other hand, prediction of SVR by aa substitutions within the core protein in Japanese patients infected with HCV-1b has also been proposed [8, 9]. In multivariate analysis, the criterion of double-wild core, presence of Arg at position 70 and Leu at position 91 (Arg^{70}/Leu^{91}), was identified as an independent SVR predictor.

This study aimed to examine NS5A polymorphisms, including those in IRRDR and ISDR, and core polymorphism as predictive markers for HCV treatment outcome. The core protein with Arg^{70}/Leu^{91} was defined as wild-core while the other patterns as non-wild-core. The possible correlation of either Arg^{70} alone or Leu^{91} alone with the clinical outcome of PEG-IFN/RBV therapy was also examined.

Patients and Methods

Patients

A total of 68 patients seen at Kobe Asahi Hospital in Kobe, Japan, who were chronically infected with HCV-1b, with diagnoses based on anti-HCV antibody detection and HCV-RNA detection, were enrolled in the study. HCV subtype was determined as according to the method of Okamoto et al. [10]. Patients were treated with PEG-IFNα-2b (Pegintron®; Schering-Plough, Kenilworth, N.J., USA) (1.5 μg/kg b.w., once weekly, s.c.) and RBV (Rebetol®; Schering-Plough) (600–800 mg daily, per os), according to a standard treatment protocol for Japanese patients established by a hepatitis study group of the Ministry of Health, Labor and Welfare, Japan. All patients received >80% of scheduled dosage of PEG-IFN and RBV. Serum samples were collected from the patients at intervals of 4 weeks before, during and after the treatment, and tested for HCV RNA and core antigen titers as reported previously [11].

The study protocol was approved beforehand by the Ethic Committee in Kobe Asahi Hospital, and written informed consent was obtained from each patient prior to the treatment.

Sequence Analysis of HCV NS5A and Core

HCV RNA was extracted from 140 μl of serum using a commercially available kit (QIAmp viral RNA kit; Qiagen, Tokyo, Japan). Amplification of full-length NS5A and core regions of the HCV genome were performed as described elsewhere [7, 11, 12]. The sequences of the amplified fragments of NS5A and core regions were determined by direct sequencing. The aa sequences were deduced and aligned using Genetyx Win software version 7.0 (Genetyx Corp., Tokyo, Japan).

Statistical Analysis

Statistical differences in the patients' baseline parameters according to the degree of IRRDR polymorphism were determined by Student's t test for numerical variables and Fisher's exact probability test for categorical variables. Likewise, statistical differences in treatment responses according to NS5A and core polymorphisms were determined by Fisher's exact probability test. Kaplan-Meier HCV survival curve analysis was performed based on serum HCV-RNA positivity data during the treatment period (48 weeks) according to NS5A and core polymorphisms. The data obtained were evaluated by the log-rank test. Uni- and multivariate logistic analyses were performed to identify variables that independently predicted the treatment outcome. Variables with a p value of <0.1 in univariate analysis were included in a multivariate logistic regression analysis. The odds ratios and 95% confidence intervals (95% CI) were also calculated. All statistical analyses were performed using SPSS version 16 software (SPSS Inc., Chicago, Ill., USA). Unless otherwise stated, a p value <0.05 was considered as statistically significant.

Nucleotide Sequence Accession Numbers

The sequence data reported in this paper have been deposited in the DDBJ/EMBL/GenBank nucleotide sequence databases under the accession numbers AB285035 through AB285081, AB354116 through AB354118, and AB518774 through AB518861.

Results

Patients' Responses to PEG-IFN/RBV Combination Therapy

Among 68 patients enrolled in this study, HCV-RNA negativity was achieved by 8 (12%) patients at week 4 (rapid virological response [RVR]), 36 (53%) patients at week 12 (early virological response [EVR]), 47 (69%) patients at

Table 1. Proportions of various virological responses of patients treated with PEG-IFN/RBV

Virological response	Proportion, patients	
	n/total	%
RVR	8/68	12
EVR	36/68	53
ETR	47/68	69
SVR	29/68	43
Non-SVR	39/68	57
Null-response	17/68	25
ETR-relapse	18/68	26
Breakthrough	4/68	6

PEG-IFN/RBV = Pegylated-interferon/ribavirin; RVR = rapid virological response; EVR = early virological response; ETR = end-of-treatment response; SVR = sustained virological response.

week 48 (end-of-treatment response [ETR]) and 29 (43%) patients at week 72 (SVR) (table 1). A total of 39 patients (57%) failed to achieve SVR and they were referred to as non-SVR. Non-SVR can be further divided into three categories: (i) null-response, which is defined by continued presence of serum HCV RNA during the entire period of the treatment and follow-up; (ii) breakthrough, defined as transient disappearance of HCV RNA followed by its reappearance before the end of the 48-week treatment, and (iii) ETR-relapse, defined by reappearance of HCV RNA after ETR has been achieved. Seventeen (25%) patients were null-response while 18 (26%) and 4 (6%) patients were ETR-relapse and breakthrough, respectively (table 1).

Correlation between NS5A Polymorphism and Treatment Responses

Using a receiver operating characteristic curve analysis, 6 mutations in IRRDR were previously estimated as an optimal cutoff number of mutations for SVR prediction [7]. Initially the correlation between the patients' demographical, hematological, biochemical and virological baseline parameters and the degree of IRRDR polymorphism was examined. This analysis revealed that patient's sex was the only factor that significantly correlated to the degree of IRRDR polymorphism since 49% (17/35) of males were infected with HCV isolates having IRRDRs with 6 mutations or more (IRRDR≥6) compared to 21% (7/33) of females ($p = 0.02$) (table 2). HCV-RNA titers or HCV core antigen titers did not differ significantly between patients infected with HCV isolates of IRRDR≥6 and those of IRRDR≤5.

Next, the possible correlation between IRRDR polymorphism and the ultimate treatment responses was examined. Among 24 patients infected with HCV isolates of IRRDR≥6, 18 (75%), 6 (25%), 3 (12.5%) and 3 (12.5%) patients were SVR, non-SVR, null-response and relapse (ETR-relapse *plus* breakthrough), respectively (table 3). By contrast, among 44 patients infected with HCV isolates of IRRDR≤5, 11 (25%), 33 (75%), 14 (32%) and 19 (43%) patients were SVR, non-SVR, null-response and relapse (ETR-relapse *plus* breakthrough), respectively. The proportions of different treatment responses among HCV isolates with IRRDR≥6 and IRRDR≤5 were significantly different. Furthermore, patients infected with HCV isolates with Ala at position 2360 (Ala^{2360}) in IRRDR had a more significant likelihood of SVR than those infected with HCV isolates with non-Ala^{2360}, who tended to be non-SVR, in particular null-response (table 3; fig. 1).

As the IRRDR polymorphism was closely correlated with the ultimate treatment responses, it was also significantly correlated with the on-treatment responses, in particular EVR and ETR (table 4). However, there was no significant correlation between the IRRDR polymorphism and RVR. Also, the presence of Ala^{2360} was correlated significantly with ETR.

Regarding the analysis of ISDR polymorphism and its correlation to the treatment responses, first, the criterion of ISDR with 4 mutations or more (ISDR≥4), the initial criterion of IFN responsiveness proposed by Enomoto et al. [6] was tested. Since the prevalence of ISDR≥4 was only 9% (6/68) of all isolates analyzed, this criterion did not significantly correlate with the treatment responses (data not shown). Next, the correlation between the treatment responses and ISDR mutations at a cutoff point of 2 mutations, a newly proposed ISDR criterion of PEG-IFN/RBV responsiveness [13, 14] was tested. Although there was no significant difference in the proportions of SVR and non-SVR between HCV isolates with ISDR of 2 mutations or more (ISDR≥2) and those of ISDR≤1, a small but significant difference in the proportions of SVR and relapse (ETR-relapse *plus* breakthrough) was observed between ISDR≥2 and ISDR≤1 (table 3). Interestingly, ISDR polymorphism was the only virological factor examined in this study that showed a significant correlation with RVR (table 4). However, this correlation disappeared when further time points of treatment course, such as EVR and ETR, were considered.

Table 2. Correlation between IRRDR polymorphism and patients' demographic characteristics

Factor	IRRDR≥6	IRRDR≤5	p value
Age, mean ± SD	58.71 ± 8.44	59.61 ± 10.30	0.71
Sex, male/female	17/7	18/26	0.02
Body weight, kg	59.87 ± 9.56	58.20 ± 11.92	0.56
Platelets, $\times 10^4/mm^3$	17.22 ± 5.5	14.96 ± 4.71	0.16
Hemoglobin, g/dl	14.25 ± 1.48	13.55 ± 1.77	0.11
γ-GTP, IU/l	49.50 ± 44.29	55.60 ± 65.60	0.69
GPT, IU/l	47.54 ± 33.09	49.33 ± 34.78	0.84
HCV-RNA, KIU/ml	2,070.21 ± 1,720.27	2,038.57 ± 1,963.05	0.95
HCV core antigen, fmol/l	6,750.87 ± 6,859.82	9,320.52 ± 10,636.48	0.30

IRRDR = Interferon/ribavirin resistance-determining region; γ-GTP = γ-guanosine triphosphate; GPT = glutamic pyruvate transaminase.

Table 3. Correlation between NS5A and core protein polymorphisms and ultimate virological responses of patients treated with PEG-IFN/RBV

Protein	Factor	Total[a]	SVR[b]	Non-SVR	Null-response	Relapse (ETR-relapse *plus* breakthrough)	p value		
							SVR vs. non-SVR	SVR vs. null-response	SVR vs. relapse (ETR-relapse *plus* breakthrough)
NS5A	IRRDR≥6	24	18 (75)[c]	6 (25)	3 (12.5)	3 (12.5)	<0.0001	0.005	0.0006
	IRRDR≤5	44	11 (25)	33 (75)	14 (32)	19 (43)			
	Ala^{2360}	18	12 (67)	6 (33)	1 (5)	5 (28)	0.026	0.016	0.2
	Non-Ala^{2360}	50	17 (34)	33 (66)	16 (32)	17 (34)			
	ISDR≥2	18	10 (56)	8 (44)	6 (33)	2 (11)	0.27	1.0	0.048
	ISDR≤1	50	19 (38)	31 (62)	11 (22)	20 (40)			
Core	Wild-core (Arg^{70}/Leu^{91})	33	18 (55)	15 (45)	5 (15)	10 (30)	0.1	0.07	0.27
	Non-wild-core	35	11 (31)	24 (69)	12 (34)	12 (34)			
	Gln^{70}	21	5 (24)	16 (76)	8 (38)	8 (38)	0.06	0.04	0.19
	Non-Gln^{70}	47	24 (51)	23 (49)	9 (19)	14 (30)			
	Met^{91}	19	7 (37)	12 (63)	5 (26)	7 (37)	0.59	0.74	0.75
	Non-Met^{91}	49	22 (45)	27 (55)	12 (24)	15 (31)			

SVR = Sustained virological response; ETR = end-of-treatment response; IRRDR = interferon/ribavirin resistance-determining region; Ala^{2360} = alanine at position 2360; ISDR = interferon sensitivity-determining region; Arg^{70} = arginine at position 70; Leu^{91} = leucine at position 91; Gln^{70} = glutamine at position 70; Met^{91} = methionine at position 91.

[a] Total number of isolates with a given factor.

[b] Number of SVR, non-SVR, null-response or relapse (ETR-relapse *plus* breakthrough) cases with a given factor.

[c] Values in parentheses are percentages.

Correlation between Core Polymorphism and Treatment Responses

Recently, it was reported that polymorphism at positions 70 and/or 91 of the core protein of HCV-1b correlates with and predicts the treatment outcome of Japanese patients treated with PEG-IFN/RBV combination therapy [8, 9]. We aimed to test the consistency of this observation among our patient cohort. The result revealed that among 33 patients infected with HCV isolates of wild-core (Arg^{70}/Leu^{91}), 18 (55%), 15 (45%), 5 (15%) and 10 (30%) patients were SVR, non-SVR, null-response and relapse (ETR-relapse *plus* breakthrough), respectively (table 3; fig. 1). On

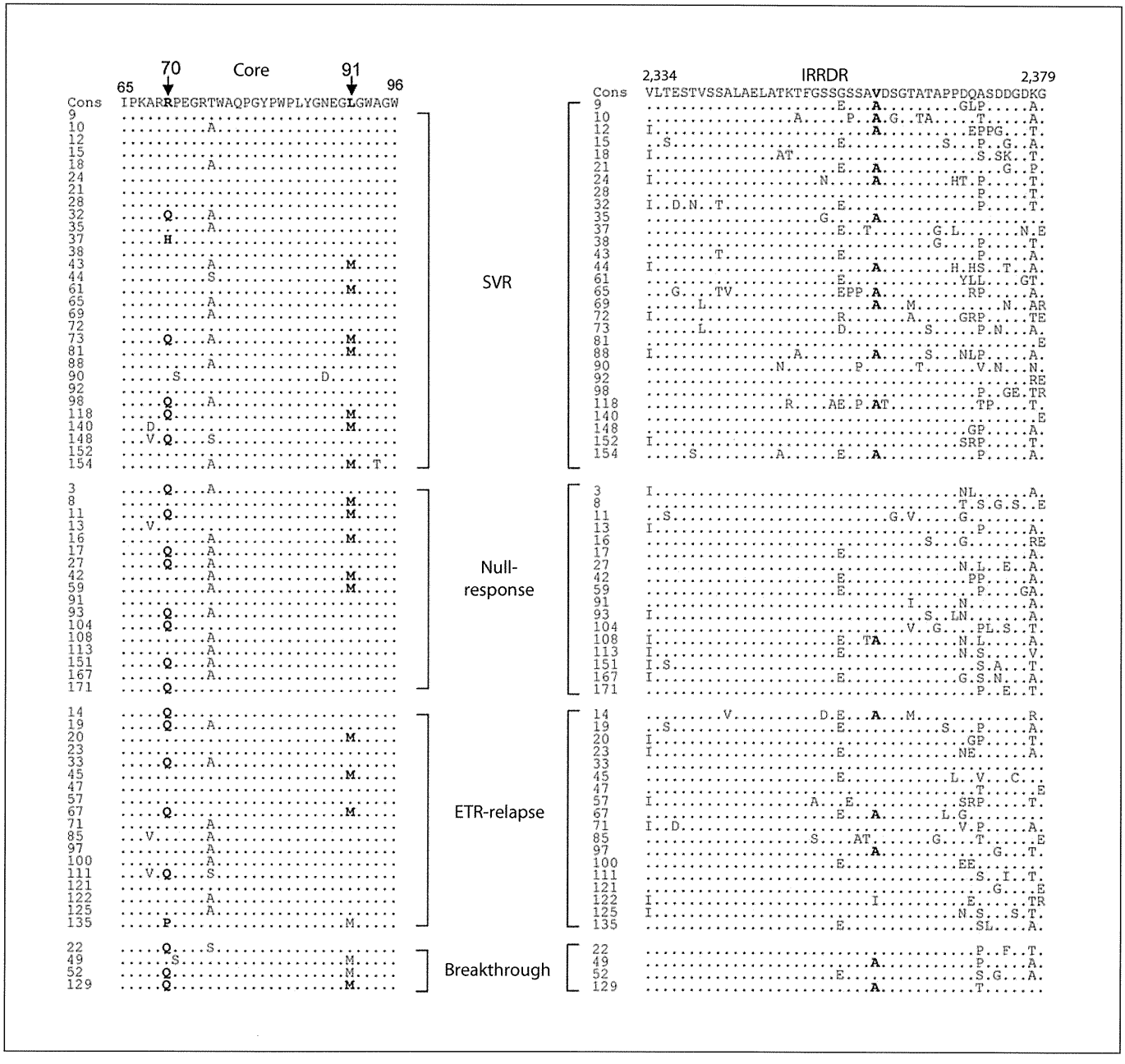

Fig. 1. Sequence alignment of the core protein (aa 65–96) and IRRDR of NS5A obtained from pretreated sera in patients infected with HCV-1b. The consensus (Cons) sequence is shown at the top. Amino acids at positions 70 and 91 of the core protein, and position 2360 of NS5A are shown in boldface.

the other hand, of 35 patients infected with HCV isolates of non-wild-core, 11 (31%), 24 (69%), 12 (34%) and 12 (34%) patients were SVR, non-SVR, null-response and relapse (ETR-relapse *plus* breakthrough), respectively. Thus, there was no significant correlation between wild-core and SVR or non-SVR (p = 0.1). However, a single mutation at position 70 (Gln^{70} vs. non-Gln^{70}) was significantly correlated with treatment outcome (SVR vs. null-response; p = 0.04).

As for the on-treatment responses, wild-core (Arg^{70}/Leu^{91}) was significantly correlated with EVR and ETR, whereas Gln^{70} was correlated with non-EVR and non-ETR (table 4).

Table 4. Correlation between NS5A and core protein polymorphisms and on-treatment virological responses of patients treated with PEG-IFN/RBV

Protein	Factor	Total[a]	RVR[b]	Non-RVR	EVR	Non-EVR	ETR	Non-ETR	p value		
									RVR vs. non-RVR	EVR vs. non-EVR	ETR vs. non-ETR
NS5A	IRRDR≥6	24	5 (21)[c]	19 (79)	17 (71)	7 (29)	21 (87)	3 (13)	0.12	0.04	0.026
	IRRDR≤5	44	3 (7)	41 (93)	19 (43)	25 (57)	26 (59)	18 (41)			
	Ala^{2360}	18	4 (22)	14 (78)	13 (72)	5 (28)	16 (89)	2 (11)	0.19	0.1	0.04
	Non-Ala^{2360}	50	4 (8)	46 (92)	23 (46)	27 (54)	31 (62)	19 (38)			
	ISDR≥2	18	6 (33)	12 (67)	9 (50)	9 (50)	11 (61)	7 (39)	0.003	0.79	0.39
	ISDR≤1	50	2 (4)	48 (96)	27 (54)	23 (46)	36 (72)	14 (28)			
Core	Wild-core (Arg^{70}/Leu^{91})	33	5 (15)	28 (85)	23 (70)	10 (30)	28 (85)	5 (15)	0.47	0.009	0.009
	Non-wild-core	35	3 (9)	32 (91)	13 (37)	22 (63)	19 (54)	16 (46)			
	Gln^{70}	21	2 (10)	19 (90)	6 (29)	15 (71)	10 (48)	11 (52)	1.0	0.009	0.02
	Non-Gln^{70}	47	6 (13)	41 (87)	30 (64)	17 (36)	37 (79)	10 (21)			
	Met^{91}	19	2 (11)	17 (89)	8 (42)	11 (58)	11 (58)	8 (42)	1.0	0.29	0.25
	Non-Met^{91}	49	6 (12)	43 (88)	28 (57)	21 (43)	36 (73)	13 (27)			

RVR = Rapid virological response; EVR = early virological response; ETR = end-of-treatment response; IRRDR = interferon/ribavirin resistance-determining region; Ala^{2360} = alanine at position 2360; ISDR = interferon sensitivity-determining region; Arg^{70} = arginine at position 70; Leu^{91} = leucine at position 91; Gln^{70} = glutamine at position 70; Met^{91} = methionine at position 91.

[a] Total number of isolates with a given factor. [b] Number of RVR, non-RVR, EVR, non-EVR, ETR or non-ETR cases with a given factor. [c] Values in parentheses are percentages.

Table 5. Correlation between NS5A and core protein polymorphisms

Factor	% (number of subjects/number of subtotal)[a]		p value
	IRRDR≥6	IRRDR≤5	
Ala^{2360}	50 (12/24)	14 (6/44)	0.003
Non-Ala^{2360}	50 (12/24)	86 (38/44)	
ISDR≥2	42 (10/24)	18 (8/44)	0.047
ISDR≤1	58 (14/24)	82 (36/44)	
Wild-core (Arg^{70}/Leu^{91})	67 (16/24)	39 (17/44)	0.04
Non-wild-core	33 (8/24)	61 (27/44)	
Gln^{70}	21 (5/24)	36 (16/44)	0.27
Non-Gln^{70}	79 (19/24)	64 (28/44)	

IRRDR = Interferon/ribavirin resistance-determining region; Ala^{2360} = alanine at position 2360; ISDR = interferon sensitivity-determining region; Arg^{70} = arginine at position 70; Leu^{91} = leucine at position 91; Gln^{70} = glutamine at position 70.

[a] Number of isolates with a certain factor/total number of HCV isolates with IRRDR≥6 or IRRDR≤5.

Correlation between NS5A and Core Polymorphisms

We then examined the possible correlation among the polymorphic factors in NS5A and core proteins. A significant correlation was observed between IRRDR≤5 and non-Ala^{2360} as the majority (86%) of HCV isolates with IRRDR≤5 had non-Ala^{2360} ($p = 0.003$) (table 5). Also, a significant correlation was obtained between IRRDR≤5 and ISDR≤1 since 82% of IRRDR≤5 were ISDR≤1 ($p = 0.047$). When IRRDR and core polymorphisms were compared, IRRDR≥6 was significantly correlated with wild-core (Arg^{70}/Leu^{91}) ($p = 0.04$). On the other hand, there was no significant correlation between IRRDR≥6 and non-Gln^{70}, or IRRDR≤5 and Gln^{70}, although the majority (79%) of IRRDR≥6 were non-Gln^{70}.

Influence of NS5A and Core Polymorphisms on HCV Clearance Kinetics during PEG-IFN/RBV Combination Therapy

To investigate the influence of NS5A and core polymorphisms on HCV-RNA kinetics during the entire course of PEG-IFN/RBV combination therapy, Kaplan-Meier HCV survival curve analysis was carried out based on HCV-RNA positivity according to NS5A and core

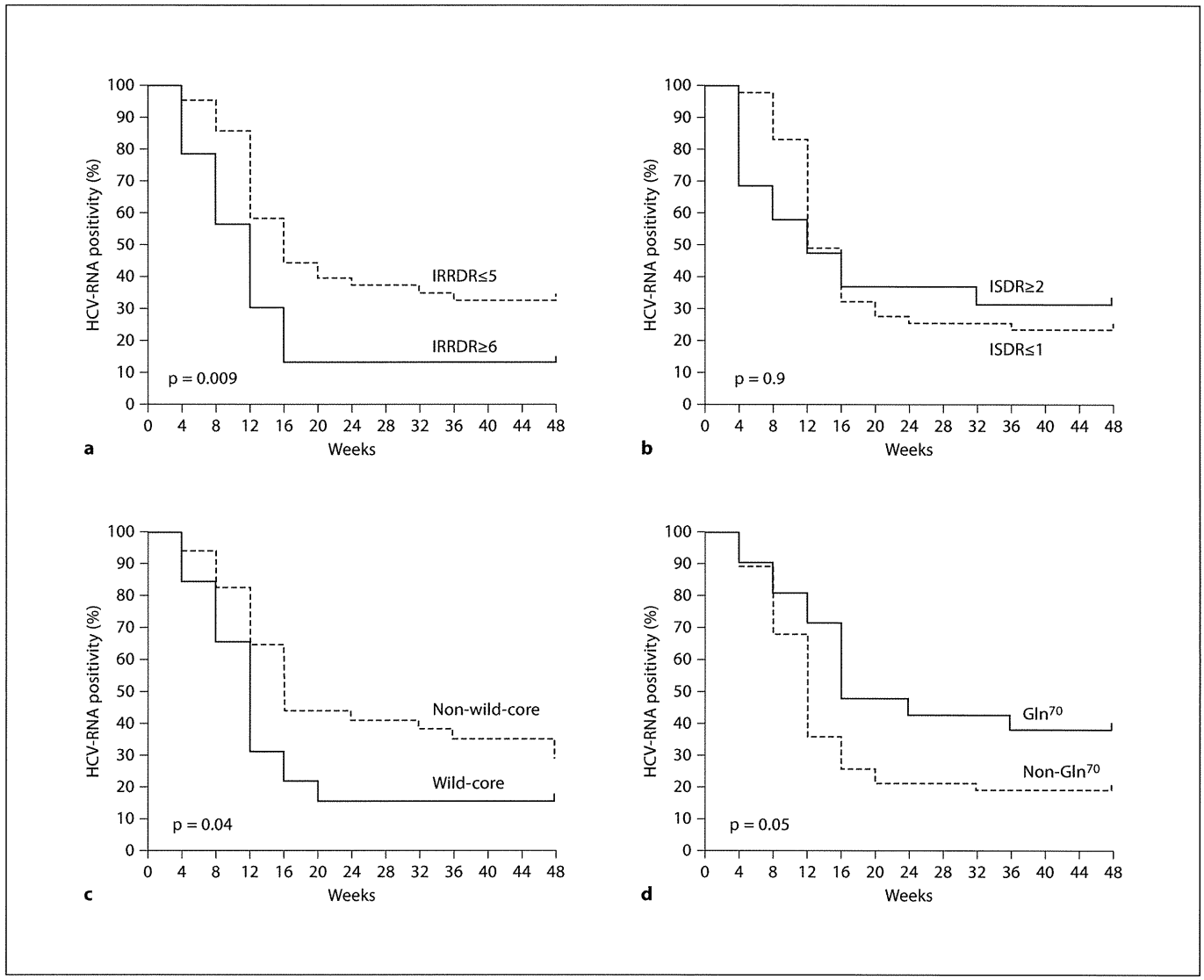

Fig. 2. Kaplan-Meier HCV survival curve analysis based on HCV-RNA positivity during the whole treatment course according to NS5A (**a**, **b**) and the core protein (**c**, **d**) polymorphisms. The difference between the analyzed groups was measured by the log-rank test.

polymorphisms. The result showed that HCV isolates of IRRDR≥6 were cleared from patients' sera more rapidly than those with IRRDR≤5 (fig. 2a). On the other hand, HCV-RNA clearance kinetics did not differ significantly between HCV isolates of ISDR≥2 and those of ISDR≤1 (fig. 2b). As for the core polymorphism, HCV isolates of non-wild-core or Gln^{70} persisted in patients' sera for longer periods of time than those of wild-core (Arg^{70}/Leu^{91}) or non-Gln^{70} (fig. 2c, d).

Next, HCV clearance kinetics during the very early stages of the treatment course, e.g., 24 h, 1, 2 and 4 weeks after initiation of PEG-IFN/RBV therapy was examined. For this purpose, a possible correlation between the degree of IRRDR, ISDR and core polymorphisms and the proportion of patients who achieved significant reduction (1 log after 24 h, 1 log after 1 week, 1.5 log after 2 weeks, and 2 log after 4 weeks) of core antigen titers was analyzed. Interestingly, IRRDR≥6 was significantly associated with reduction and/or disappearance of serum HCV core antigen titers at 24 h, 1, 2 and 4 weeks after initiation of the treatment (table 6). Again, there was no significant correlation between ISDR sequence variation

Table 6. Correlation between the proportions of patients with rapid reduction of HCV core antigen titers and degree of NS5A and core protein polymorphisms

Protein	Criteria	Number of patients with significant reduction of HCV core antigen titers/number of total							
		24 h[a] (≥1 log)[b]	p value	1 week (≥1 log)	p value	2 weeks (≥1.5 log)	p value	4 weeks (≥2 log)	p value
NS5A	IRRDR≥6	20/23	0.0006	18/23	0.004	17/23	0.018	19/23	0.008
	IRRDR≤5	17/40		16/40		16/40		19/40	
	ISDR≥2	10/19	1.0	11/19	0.59	10/19	1.0	11/19	1.0
	ISDR≤1	24/44		21/44		22/44		27/44	
Core	Wild core (Arg^{70}/Leu^{91})	23/31	0.01	22/31	0.02	20/31	0.13	24/31	0.005
	Non-wild-core	13/32		13/32		14/32		13/32	
	Gln^{70}	6/19	0.03	5/19	0.01	6/19	0.06	6/19	0.004
	Non-Gln^{70}	28/44		27/44		26/44		32/44	

Note: Patients Nos. 108, 111, 129, 135 and 152 were excluded from this analysis because their core antigen titers at certain time points were missing.

IRRDR = Interferon/ribavirin resistance-determining region; ISDR = interferon sensitivity-determining region; Arg^{70} = arginine at position 70; Leu^{91} = leucine at position 91; Gln^{70} = glutamine at position 70.

[a] Period after initiation of IFN/RBV therapy.

[b] Criteria of significant reduction of HCV core antigen titers.

(ISDR≥2 and ISDR≤1) and reduction of HCV core antigen titers during the very early stages of PEG-IFN/RBV therapy. On the other hand, non-wild-core or Gln^{70} were significantly associated with slow reduction and/or persistence of HCV core antigen in the patients' sera (table 6).

Identification of Independent Predictive Factors for SVR by Uni- and Multivariate Logistic Regression Analyses

Finally, in order to identify significant independent predictive factors of PEG-IFN/RBV treatment outcome, first, all available data of baseline patients' parameters, on-treatment responses and NS5A and core polymorphisms were entered in a univariate logistic analysis. This analysis yielded 11 factors that were correlated or nearly correlated with the treatment outcome; IRRDR mutations categorized as IRRDR≥6 and IRRDR≤5, Ala^{2360} and non-Ala^{2360}, core protein polymorphism categorized as wild-core (Arg^{70}/Leu^{91}) and non-wild-core, Gln^{70} and non-Gln^{70}, RVR and non-RVR, EVR and non-EVR, ETR and non-ETR, HCV core antigen titers, age, platelets count and hemoglobin levels (table 7). Subsequently, these 11 factors were entered in multivariate logistic regression analysis. This analysis yielded IRRDR mutations ($p = 0.005$), EVR ($p = 0.0001$) and age ($p = 0.02$) as independent predictive factors of PEG-IFN/RBV treatment outcome (table 7).

Discussion

Both host and viral genetic polymorphisms influence the outcome of PEG-IFN/RBV therapy for HCV-infected patients [15]. It has recently been reported that host genetic polymorphisms near or within the IL28B gene on chromosome 19 show a significant impact on the treatment outcome for patients infected with HCV genotype 1 (HCV-1a and -1b) [16–18]. Also, HCV genetic polymorphisms have been known to contribute to differences in the treatment outcome, as demonstrated by the observations that SVR rates for patients infected with HCV genotypes 2 and 3 are higher than those for patients infected with HCV genotype 1 [15]. Moreover, viral genetic polymorphisms, especially in the NS5A (ISDR and IRRDR) and the core regions, among HCV isolates of a given genotype have been linked to the difference in SVR rates [6–9, 19, 20]. In the present study, we compared the impact of IRRDR, ISDR and core polymorphisms of HCV-1b isolates on the clinical outcome of PEG-IFN/RBV therapy. Our results suggest that the degree of IRRDR mutations is more dominant than that of ISDR mutations and core polymorphism for predicting the anti-HCV treatment outcome.

IRRDR corresponds to a region near the C-terminus of NS5A. The obtained result that the IRRDR polymorphism influences the clinical outcome of IFN-based anti-HCV therapy can be linked to a recent experimental observation by Tsai et al. [21]. They reported that an HCV

Table 7. Uni- and multivariate logistic regression analyses to identify independent predictive factors for success of PEG-IFN/RBV combination therapy

Univariate		Multivariate	
variable	p value	odds ratio (95% CI)	p value
IRRDR mutations (IRRDR≥6 vs. IRRDR≤5)	<0.0001	14.33 (2.24–91.65)	0.005
Ala^{2360}	0.01	1.75 (0.19–15.36)	0.62
Core polymorphism (wild-core vs. non-wild-core)	0.06	0.41 (0.05–3.28)	0.34
Gln^{70}	0.04		
RVR	<0.0001		
EVR	<0.0001	41.83 (6.12–285.68)	0.0001
ETR	<0.0001		
HCV core antigen, fmol/l	0.05		
Age	0.01	0.91 (0.84–0.99)	0.02
Platelets, $\times 10^4/mm^3$	0.07		
Hemoglobin, g/dl	0.006		

IRRDR = Interferon/ribavirin resistance-determining region; Ala^{2360} = alanine at position 2360; Gln^{70} = glutamine at position 70; RVR = rapid virological response; EVR = early virological response; ETR = end-of-treatment response.

subgenomic RNA replicon containing NS5A of HCV-1b exerted more profound inhibitory effects on IFN activity than the original HCV-2a replicon, and that domain swapping between NS5A sequences of HCV-1b and -2a in the V3 and/or a C-terminus region including IRRDR resulted in a transfer of their anti-IFN activity. Since the C-terminal region of NS5A is among the most variable sequences across the different genotypes and subtypes of HCV [22], the difference in IFN responsiveness among different strains of a given HCV subtype could also be attributable, at least partly, to the genetic polymorphism within this region. The molecular mechanism underling the possible involvement of IRRDR in IFN responsiveness of the virus is still unknown. The significant difference in IRRDR sequence pattern may suggest genetic flexibility of this region and, indeed, the C-terminal portion of NS5A was shown to tolerate sequence insertions and deletions [23, 24]. This means that the C-terminal portion of NS5A is not essential for virus replication in cultured cells. It does not exclude the possibility, however, that the same region plays an important role in modulating the interaction with various host systems, including IFN responsiveness. It is also possible that the genetic flexibility of this region, especially IRRDR, is accompanied by compensatory changes elsewhere in the viral genome and that these compensatory changes affect overall viral fitness and responses to IFN therapy [25].

While we observed significant correlation between the overall number of mutations in IRRDR and PEG-IFN/RBV responsiveness, we also found a particular aa mutation, Ala^{2360}, that was significantly associated with SVR (tables 3, 7; fig. 1). It is possible that Ala or Val at this position confers a certain advantage for interaction between NS5A and the other viral or host proteins, which might affect IFN-induced antiviral responses. This issue needs to be elucidated in further studies.

The ISDR polymorphism was the only virological factor examined that showed a significant correlation with RVR (table 4), with the result being consistent with a recent report by other investigators [26]. This significant correlation, however, disappeared as the treatment went on. In contrast, the IRRDR polymorphism did not correlate significantly with RVR, however, it was the dominant viral genetic factor that was correlated with SVR (tables 3, 7). Interestingly, the combination of IRRDR and ISDR polymorphisms (IRRDR≥6 *plus* ISDR≥2) was significantly correlated with RVR and SVR (p = 0.0001 and 0.01, respectively; data not shown). This suggests a possible integrated influence of IRRDR and ISDR polymorphisms, or NS5A as a whole, on the treatment outcome. Further study is needed to clarify the issue.

The core protein polymorphisms (wild-core vs. non-wild-core, and Gln^{70} and non-Gln^{70}) were significantly correlated with the on-treatment HCV clearance kinetics

(fig. 2c, d; tables 4, 6). However, this significant correlation became blurred thereafter and eventually no significant correlation was observed between wild-core (Arg^{70}/Leu^{91}) and the final treatment outcomes (table 3). On the other hand, Gln^{70} was significantly associated with null-response, and almost significantly with non-SVR. This result is consistent, at least partly, with previous reports, including a recent multicenter study in Japan, that identified Gln^{70} as a predictive factor for poor responses to PEG-IFN/RBV treatment [8, 9, 14].

Recently, it was reported that the C-terminal region of NS5A plays a critical role in regulating the early phase of HCV particle formation [27, 28]. Moreover, sequence alteration within this region affected the degree of interaction between NS5A and core protein, which in turn affected the efficiency of progeny virus production [29]. In the present study, we observed a significant correlation between the degree of IRRDR mutations (IRRDR≥6) and the core polymorphism (table 5). Therefore, it would be interesting to investigate the degree of interaction between NS5A with IRRDR of high or low degrees of sequence variation and the wild-type (Arg^{70}/Leu^{91}) or non-wild-type of core protein, and also the impact of these interactions on progeny virus production and IFN sensitivity of the virus.

The present study identified the IRRDR polymorphism as the only viral genetic factor that independently predicted PEG-IFN/RBV treatment outcome (table 7). On the other hand, HCV is likely to utilize an alternative mechanism(s) by which to escape IFN actions through its various structural and non-structural proteins [30]. Also, a different lineage(s) of HCV-1b strains that relies more on the alternative mechanism than on IRRDR may prevail in other regions of the world. It is possible, therefore, that the impact of the IRRDR polymorphism differs with different cohorts. Analysis in a large-scale multicenter study is needed to clarify this issue.

In conclusion, NS5A (IRRDR and ISDR) and core protein polymorphisms are useful viral markers for predicting the outcome of PEG-IFN/RBV therapy for chronic hepatitis C. In particular, IRRDR≥6 is a useful marker for prediction of SVR.

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ORIGINAL ARTICLE

Sequence heterogeneity of NS5A and core proteins of hepatitis C virus and virological responses to pegylated-interferon/ribavirin combination therapy

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ABSTRACT

Both host and viral factors have been implicated in influencing the response to pegylated-interferon/ribavirin (PEG-IFN/RBV) therapy for hepatitis C virus (HCV) infection. Among the viral factors, sequence heterogeneity within NS5A and core regions has been proposed. This study aimed to clarify the relationship between virological responses to PEG-IFN/RBV therapy and sequence heterogeneity within NS5A, including the IFN/RBV resistance-determining region (IRRDR), the interferon sensitivity-determining region (ISDR) and the core region. Pretreatment sequences of NS5A and the core regions were analyzed in 57 HCV-1b-infected patients who were to be treated with PEG-IFN/RBV. Of 40 patients infected with HCV having an IRRDR with four or more mutations (IRRDR $\geq$ 4), 28 (70%) patients achieved a sustained virological response (SVR). On the other hand, only 4 (24%) of 17 patients infected with HCV having an IRRDR with three or fewer mutations (IRRDR $\leq$ 3) achieved a SVR ($P = 0.001$). Similarly, 22 (71%) of 31 patients infected with HCV and having an ISDR with one or more mutations (ISDR $\geq$ 1) achieved a SVR while 10 (38%) of 26 patients infected with HCV and having an ISDR without any mutations (ISDR = 0) achieved a SVR ($P = 0.014$). As for the core region, there was significant correlation between a single mutation at position 70 (Gln^{70}) and non-SVR ($P = 0.02$). Notably, Gln^{70} was more prominently associated with the null response ($P = 0.0007$). In conclusion, sequence heterogeneity within the IRRDR and ISDR, and a single point mutation at position 70 of the core region of HCV-1b are likely to be correlated with virological responses to PEG-IFN/RBV therapy.

Key words Core, interferon/ribavirin resistance-determining region, interferon sensitivity-determining region, pegylated-interferon/ribavirin.

Hepatitis C virus is a major cause of chronic liver diseases worldwide. Approximately 180 million people, ~3% of the world's population, are infected with HCV. Seventy percent of acute infections become persistent, and 50–75%

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List of Abbreviations: aa, amino acids; Arg^{70}, arginine at position 70 of core protein; AUC, area under the curve; CI, confidence intervals; ETR, end-of-treatment response; EVR, early virological response; Gln^{70}, glutamine at position 70 of core protein; γ-GTP, γ-glutamyl transpeptidase; HCV, hepatitis C virus; IFN, interferon; IRRDR, interferon/ribavirin resistance-determining region; ISDR, interferon sensitivity-determining region; Leu^{91}, leucine at position 91 of core protein; Met^{91}, methionine at position 91 of core protein; NS5A, nonstructural protein 5A; PEG-IFN/RBV, pegylated-interferon/ribavirin; PKR, double-stranded RNA-activated protein kinase; RBV, ribavirin; ROC, receiver operating characteristic; RVR, rapid virological response; SVR, sustained virological response.

of patients with chronic HCV infection progress to hepatocellular carcinoma (1–5). Therefore, HCV infection is a major global health problem. Although more than two decades have passed since the discovery of HCV, therapeutic options remain limited. Current standard treatment of chronic HCV infection consists of PEG-IFN and RBV, which leads to a SVR in approximately half of treated patients, especially those infected with the most resistant genotypes, HCV-1a and HCV-1b (6, 7). Given the considerable side effects and high cost of this treatment, which result in discontinuation of treatment by some patients, reliable prediction of treatment outcome is needed. An expanded range of predictors may assist clinicians and patients to more accurately assess the likelihood of an SVR and thus to make more reliably informed treatment decisions (8).

Because the SVR rate to PEG-IFN/RBV therapy depends on viral genotypes, it is generally considered that HCV genetics affect the treatment response (9). In this context, NS5A has been widely discussed because of its known correlation with IFN responsiveness. Initially, in the era of IFN monotherapy, it was proposed that sequence variations within a region in NS5A spanning from aa 2209 to 2248, called the ISDR, were correlated with IFN responsiveness (10). Subsequently, in the era of combination therapy with PEG-IFN/RBV, we identified a new region near the C-terminus of NS5A spanning from aa 2334 to 2379, which we referred to as the IRRDR (11). The degree of sequence variations within the IRRDR was significantly associated with the clinical outcome of PEG-IFN/RBV combination therapy. On the other hand, prediction of SVR by aa substitutions at positions 70 and 91 of the core protein in Japanese patients infected with HCV-1b has also been proposed (12–14). More recently, we investigated the impact of NS5A polymorphisms, including those in IRRDR and ISDR, and core polymorphism on virological responses to PEG-IFN/RBV therapy among HCV-1b-infected patients in Hyogo Prefecture, Japan. The criterion of six or more mutations in the IRRDR (IRRDR $\geq$ 6) was identified as the most powerful viral genetic factor that independently predicted SVR (15). In another study curried out on a patient cohort in Yamagata Prefecture, Japan, we proposed that polymorphism in the secondary structure of the N-terminal region of NS3 of HCV-1b influences virological responses to PEG-IFN/RBV therapy, and that virus grouping based on NS3 polymorphism can also be used to predict the outcome of the therapy (16). In the present study, we further analyzed the Yamagata cohort for a possible relationship between heterogeneity of NS5A and the core regions of the HCV genome and virological responses to PEG-IFN/RBV therapy.

MATERIALS AND METHODS

Patients

Fifty-seven patients who were chronically infected with HCV-1b, their diagnoses being based on detection of anti-HCV antibody and HCV RNA, and who had been seen at Yamagata University Hospital in Yamagata, Japan, were enrolled in the study. Their HCV subtypes were determined according to the method of Okamoto *et al.* (17). Patients were treated with PEG-IFNα-2b (Pegintron; Schering-Plough, Kenilworth, NJ, USA) (1.5 μg per kilogram of body weight, once weekly, subcutaneously) and RBV (Rebetol; Schering-Plough) (600~800 mg daily, orally), according to a standard treatment protocol for Japanese patients established by a Hepatitis Study Group of the Ministry of Health, Labor and Welfare, Japan. All patients received >80% of the scheduled doses of PEG-IFN and RBV. Serum samples were collected from the patients before treatment and at intervals of 4 weeks during the whole observation period (72 weeks), and tested for HCV RNA titers as reported previously (18).

The study protocol was approved beforehand by the Ethics Committee at Yamagata University Hospital, and written informed consent for study participation was obtained from each patient prior to treatment. Also, the study protocol conforms to the provisions of the Declaration of Helsinki.

Sequence analysis of hepatitis C virus NS5A and the core regions of the hepatitis C virus genome

Hepatitis C virus RNA was extracted from 140 μL of serum using a commercially available kit (QIAmp viral RNA kit; Qiagen, Tokyo, Japan). Amplification of full-length NS5A and the core regions of the HCV genome were performed as described elsewhere (11, 18, 19). The sequences of the amplified fragments of NS5A and core regions were determined by direct sequencing without subcloning. The aa sequences were deduced and aligned using GENETYX Win software version 7.0 (Genetyx, Tokyo, Japan).

Statistical analysis

To evaluate the optimal threshold of the IRRDR and ISDR mutations for SVR prediction, we constructed an ROC curve and calculated the AUC, sensitivity and specificity (11). Statistical differences in treatment responses according to NS5A and core sequence heterogeneity were determined by the χ^2 test. Likewise, statistical differences in the patients' baseline variables according to the degree of IRRDR polymorphism were determined by Student's *t*

test for numerical variables and the χ^2 probability test for categorical variables. Univariate and multivariate logistic analyses were performed to identify variables that were independently correlated with the treatment outcome. Variables with a P value of <0.1 in univariate analysis were further included in a multivariate logistic regression analysis. The odds ratios and 95% CI were also calculated. All statistical analyses were performed using SPSS version 16 software (SPSS, Chicago, IL, USA). Unless otherwise stated, a P value of <0.05 was considered statistically significant.

Nucleotide sequence accession numbers

The sequence data reported in this paper have been deposited in the DDBJ/EMBL/GenBank nucleotide sequence databases under the accession numbers AB601987 through AB602043.

RESULTS

Patients' responses to pegylated-interferon/ribavirin combination therapy

Among the 57 patients enrolled in this study, 8 (14%), 36 (63%), 42 (74%) and 32 (56%) patients were negative for HCV-RNA at week 4 (RVR), week 12 (EVR), week 48 (ETR) and week 72 (SVR), respectively (Table 1). SVR was achieved by all (100%) of RVR, 30 (83%) of 36 EVR, and 32 (76%) of 42 ETR patients. Non-SVR patients represented 44% (25/57) of total cases. Twenty-six percent (15/57) of the patients had continuous viremia during the whole observation period (72 weeks), referred to as a null response; whereas 18% (10/57) had transient disappearance of serum HCV RNA at a certain time point followed by a rebound in viremia either before, or after the end of, the treatment course, referred to as a relapse.

Table 1. Proportions of various virological responses of patients treated with PEG-IFN/RBV

Virological response	Proportion
RVR	14% (8/57)†
EVR	63% (36/57)
ETR	74% (42/57)
SVR	56% (32/57)
Non-SVR	44% (25/57)
Null response	26% (15/57)
Relapse	18% (10/57)

†, number of patients in the relevant category /total number of patients.

Correlation between interferon/ribavirin resistance-determining region polymorphism and treatment responses

The degree of sequence variation within the IRRDR has been proposed as a useful predictor of HCV treatment outcome (11, 15, 20, 21). We performed ROC curve analysis to estimate the optimal cutoff number of IRRDR mutations that differentiated between a SVR and non-SVR in the present patient cohort. Based on the results obtained, we estimated four mutations as the optimal number of IRRDR mutations since this provided the highest sensitivity (88%) and good specificity (52%) with an

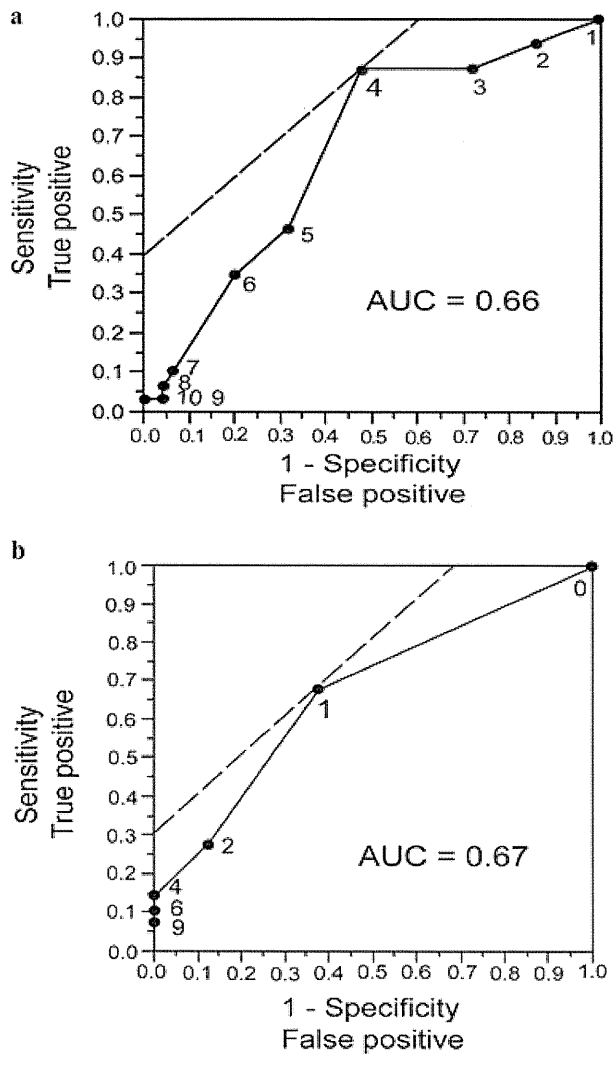

Fig. 1. ROC curve analysis of (a) IRRDR and (b) ISDR sequence heterogeneity for SVR prediction. The curves depicted by solid lines shows the AUC. Solid circles with numerals plotted on the curve represent different numbers of IRRDR and ISDR mutations analyzed. The dashed lines touch the optimal number of IRRDR and ISDR mutations for SVR prediction.

Table 2. Correlation between NS5A and core protein polymorphisms and virological responses of patients treated with PEG-IFN/RBV

Protein	Factor	Total †	SVR ‡	Non-SVR	Null response	Relapse	*P* value		
							SVR vs non-SVR	SVR vs null response	SVR vs relapse
NS5A	IRRDR ≥ 4	40	28 (70%)	12 (30%)	7 (17.5%)	5 (12.5%)	**0.001**	**0.003**	**0.01**
	IRRDR ≤ 3	17	4 (24%)	13 (76%)	8 (47%)	5 (29%)			
	ISDR ≥ 1	31	22 (71%)	9 (29%)	5 (16%)	4 (13%)	**0.014**	**0.02**	0.1
	ISDR = 0	26	10 (38%)	16 (62%)	10 (38%)	6 (24%)			
Core	Wild-core (Arg^{70}/Leu^{91})	24	15 (63%)	9 (37%)	5 (21%)	4 (16%)	0.4	0.4	0.7
	Non-wild-core	33	17 (52%)	16 (48%)	10 (30%)	6 (18%)			
	Gln^{70}	14	4 (29%)	10 (71%)	9 (64%)	1 (7%)	**0.02**	**0.0007**	0.8
	Non- Gln^{70}	43	28 (65%)	15 (35%)	6 (14%)	9 (21%)			
	Met^{91}	22	10 (46%)	12 (54%)	6 (27%)	6 (27%)	0.2	0.6	0.1
	Non- Met^{91}	35	22 (63%)	13 (37%)	9 (26%)	4 (11%)			

†, total number of isolates with a given factor; ‡, number of SVR, non-SVR, null-response or relapse cases with a given factor. *P* values indicating statistically significant difference are written in bold.

AUC of 0.66 (Fig. 1a). In this study, therefore, we used the criteria of four or more mutations in the IRRDR (IRRDR ≥ 4) and IRRDR ≤ 3. In this connection, it should be stated that the criteria of IRRDR ≥ 6 and IRRDR ≤ 5 which were used on different patient cohorts in Hyogo Prefecture (11, 15) were not selected by the ROC curve analysis in this study because of their low sensitivity (34%), although they had higher specificity (80%) than that of IRRDR ≥ 4 (52%). This difference was probably due to the low prevalence of HCV isolates with IRRDR ≥ 6 (28%) in the present patient cohort.

We found that 70%, 30%, 17.5% and 12.5% of patients infected with HCV isolates with IRRDR ≥ 4 were SVR, non-SVR, null response and relapse cases, respectively (Table 2 and Fig. 2). By contrast, 24%, 76%, 47% and 29% of patients infected with HCV isolates with IRRDR ≤ 3 were SVR, non-SVR, null response and relapse cases, respectively. Thus, the proportions of SVR, non-SVR, null response and relapse cases were significantly different among HCV isolates with IRRDR ≥ 4 and IRRDR ≤ 3.

Interestingly, while IRRDR polymorphism was correlated with the final treatment outcome, it was also closely correlated with all the responses during treatment, represented by RVR, EVR and ETR (Table 3).

Next, we investigated the correlations between the patients' demographic, hematological, biochemical and virological baseline variables and the degree of IRRDR polymorphism. This analysis revealed that patient age was the only factor that was significantly correlated with the degree of IRRDR polymorphism, patients who were infected with HCV isolates of IRRDR ≥ 4 being significantly younger on average than patients infected with HCV isolates with IRRDR ≤ 3 ($P = 0.035$) (Table 4).

Correlation between interferon sensitivity-determining region polymorphism and treatment responses

Based on ROC curve analysis, we estimated one mutation in the ISDR as an optimal cut-off number of mutations for SVR prediction since it had the highest sensitivity (69%) combined with the highest specificity (64%) and yielded an AUC of 0.67 (Fig. 1b). Seventy-one percent, 29%, 16% and 13% of patients infected with HCV isolates with one or more mutations in the ISDR (ISDR ≥ 1) were SVR, non-SVR, null response and relapse cases, respectively (Table 2 and Fig. 2). By contrast, 38%, 62%, 38% and 24% of patients infected with HCV isolates with no mutation in the ISDR (ISDR = 0) were SVR, non-SVR, null response and relapse cases, respectively. Thus, the proportions of SVR, non-SVR and null response cases were significantly different among HCV isolates with ISDR ≥ 1 and ISDR = 0.

ISDR polymorphism and the on-treatment responses had significant correlation only with EVR, since 77% of patients infected with HCV isolates with ISDR ≥ 1 were EVR whereas 54% of patients infected with HCV isolates with ISDR = 0 were non-EVR ($P = 0.01$, Table 3).

Correlation between core polymorphism and treatment responses

Recently, it was reported that polymorphism at positions 70 and/or 91 of the core protein of HCV-1b are useful negative markers for the treatment outcome of Japanese patients treated with PEG-IFN/RBV combination therapy (12–14). We have investigated the impact of various sequences patterns of both positions on treatment responses. We found that 63%, 37%, 21% and 16%

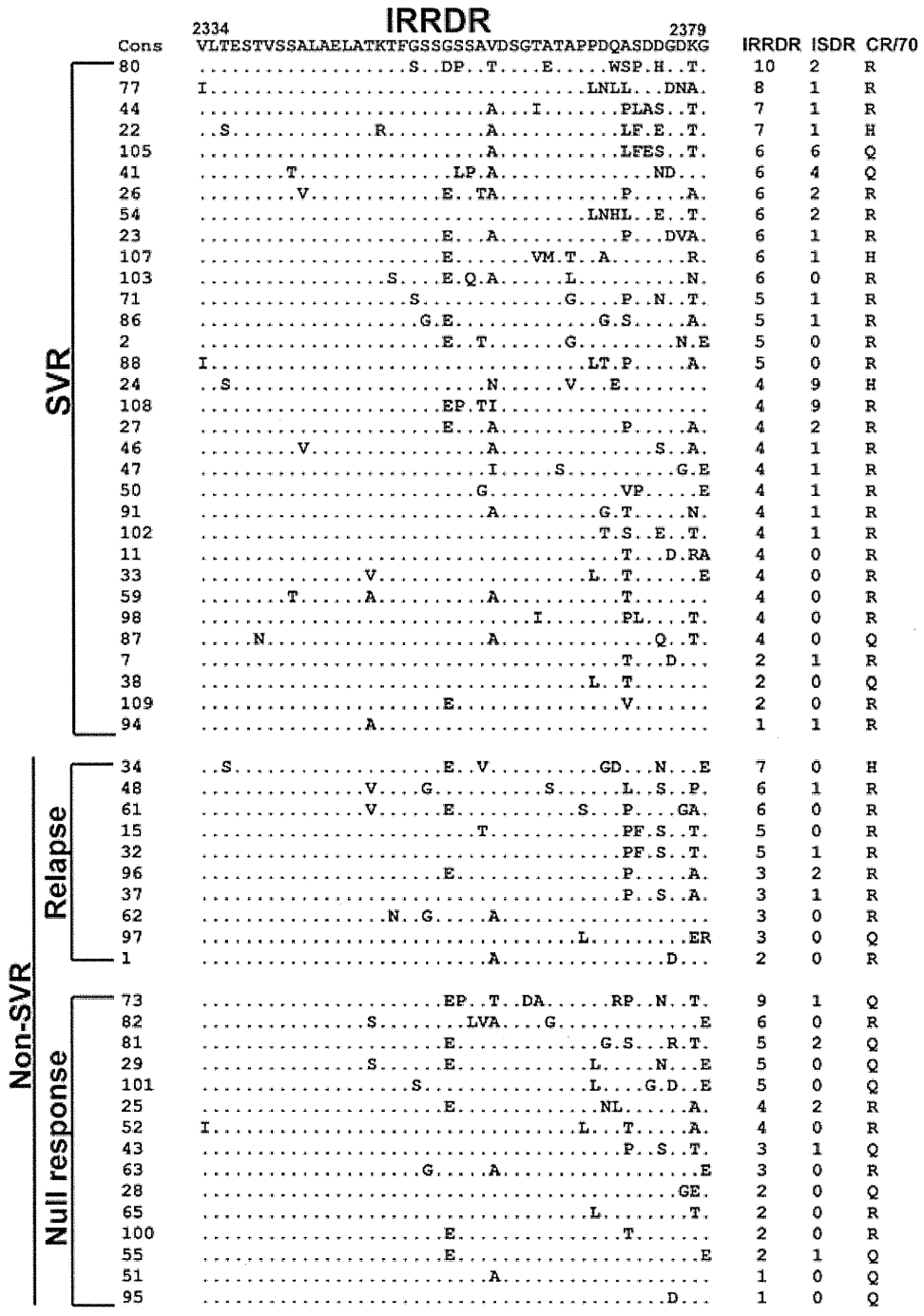

Fig. 2. Sequence alignment of IRRDR of NS5A of HCV-1b obtained from pretreatment sera. The consensus sequence is shown at the top (Cons). Dots indicate residues identical to those of the consensus sequence. Number of IRRDR and ISDR mutations, as well as the sequence pattern at aa 70 of the core protein, are shown on the right. The number of ISDR mutations was determined by comparing with the consensus sequence reported by Enomoto *et al.* (10).

of patients infected with HCV isolates with wild-core (Arg^{70}/Leu^{91}) were SVR, non-SVR, null response and relapse cases, respectively, compared to 52%, 48%, 30% and 18% of patients infected with HCV isolates with non-wild-core (Table 2). Thus, there was no significant correlation between wild-core and SVR or non-SVR ($P = 0.4$). However, the presence of a single point mutation at position 70 (Gln^{70} vs non- Gln^{70}) was significantly associated with