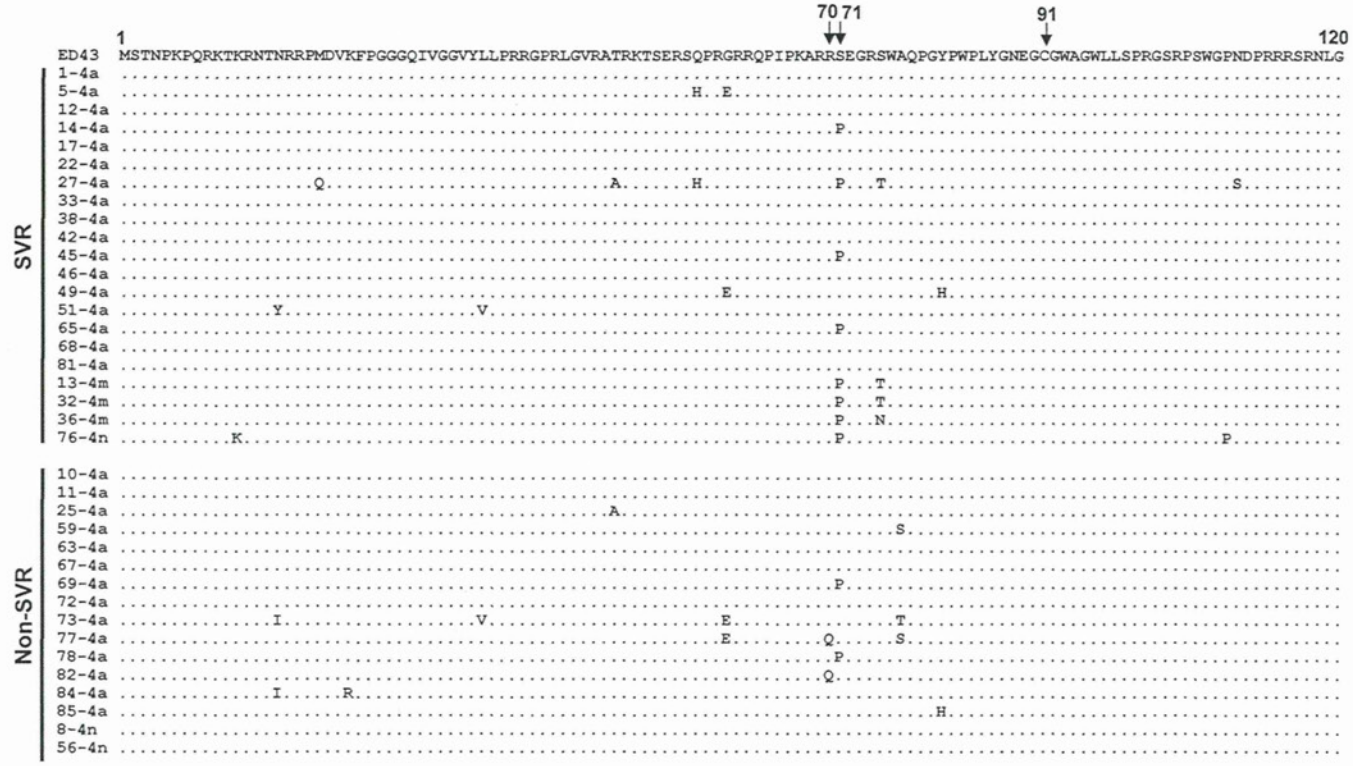

FIG 4 Sequence alignment of the core protein of HCV-4 isolates. Core protein sequences (aa 1 to 120) of HCV-4 obtained from SVR and non-SVR patients are aligned. The prototype sequence of ED43 (10) is shown on the top. The numbers along the sequence indicate the amino acid positions. Dots indicate residues identical to those of the prototype sequence.

come (4, 12). In the present study, however, we found no significant correlation between core protein polymorphism and treatment outcome in HCV-4 infection. The residue at position 70 of the core protein of all but two HCV-4 isolates analyzed in this study was Arg (Fig. 4), which is known to be associated with SVR in HCV-1b infection (4, 12). This high degree of sequence conservation at position 70 might be the reason for the lack of significant correlation between core protein polymorphism and treatment outcome in HCV-4 infection.

Single nucleotide polymorphisms (SNPs) near the IL28B region have been identified as the strongest baseline predictors of SVR to PEG-IFN/RBV in patients with HCV-1 infection. More recently, in two major studies that were carried out exclusively with HCV-4-infected patients (9, 11), the CC genotype of rs12979860 IL28B SNP was also strongly associated with SVR. It is worth noting that although the SVR rate was more than 80% among the patients with the CC genotype, these patients represented only around 40% of total SVR cases in both studies. Furthermore, the CC genotype was found in only 34% of all Egyptian patients analyzed (9). Taken together, those observations support the idea that in addition to IL28B polymorphism, there should be an additional factor(s) that influences SVR. In this context, an interplay between IRRDR and IL28B polymorphisms might explain why some patients with undesirable IL28B genotype achieve SVR and why some patients infected with HCV isolates with IRRDR[HCV-4]≥4 do not achieve SVR. Further comprehensive study is needed to validate the importance of IRRDR and IL28B polymorphisms in predicting the treatment outcome of HCV-4-infected patients.

In conclusion, the present study emphasizes the importance of IRRDR sequence heterogeneity in the prediction of PEG-IFN/RBV treatment outcome for different HCV genotype infections in

TABLE 4 Univariate and multivariate analyses for identification of independent predictive factors for SVR in HCV-4-infected patients treated with PEG-IFN/RBV therapy

Univariate analysis		Multivariate analysis	
Variable	*P* value	Odds ratio (95% CI)	*P* value
IRRDR mutations (IRRDR ≥ 4 versus IRRDR ≤ 3)	0.0004	10.5 (1.12–98.91)	0.04
Age (<42 years)	0.03		
HCV-RNA (<5,200 IU/ml)	0.08		

TABLE 5 PPV, NPV, sensitivity, and specificity of IRRDR sequence heterogeneity on the likelihood of achieving SVR and non-SVR in HCV-4 infection

Factor	PPV	NPV	Sensitivity[c]	Specificity[d]
IRRDR ≥ 4	81% (21/26)[a]		84% (21/25)	
IRRDR ≤ 3		76% (13/17)[b]		72% (13/18)

[a] $P = 0.002$.
[b] $P = 0.02$.
[c] Proportion of SVR patients who were infected with HCV isolates with IRRDR of ≥4.
[d] Proportion of non-SVR patients who were infected with HCV isolates with IRRDR of ≤3.

different ethnic groups, including Egyptian patients infected with HCV-4.

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Sequence Heterogeneity in NS5A of Hepatitis C Virus Genotypes 2a and 2b and Clinical Outcome of Pegylated-Interferon/Ribavirin Therapy

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Abstract

Pegylated-interferon plus ribavirin (PEG-IFN/RBV) therapy is a current standard treatment for chronic hepatitis C. We previously reported that the viral sequence heterogeneity of part of NS5A, referred to as the IFN/RBV resistance-determining region (IRRDR), and a mutation at position 70 of the core protein of hepatitis C virus genotype 1b (HCV-1b) are significantly correlated with the outcome of PEG-IFN/RBV treatment. Here, we aimed to investigate the impact of viral genetic variations within the NS5A and core regions of other genotypes, HCV-2a and HCV-2b, on PEG-IFN/RBV treatment outcome. Pretreatment sequences of NS5A and core regions were analyzed in 112 patients infected with HCV-2a or HCV-2b, who were treated with PEG-IFN/RBV for 24 weeks and followed up for another 24 weeks. The results demonstrated that HCV-2a isolates with 4 or more mutations in IRRDR (IRRDR[2a]≥4) was significantly associated with rapid virological response at week 4 (RVR) and sustained virological response (SVR). Also, another region of NS5A that corresponds to part of the IFN sensitivity-determining region (ISDR) plus its carboxy-flanking region, which we referred to as ISDR/+C[2a], was significantly associated with SVR in patients infected with HCV-2a. Multivariate analysis revealed that IRRDR[2a]≥4 was the only independent predictive factor for SVR. As for HCV-2b infection, an N-terminal half of IRRDR having two or more mutations (IRRDR[2b]/N≥2) was significantly associated with RVR, but not with SVR. No significant correlation was observed between core protein polymorphism and PEG-IFN/RBV treatment outcome in HCV-2a or HCV-2b infection. ***Conclusion:*** The present results suggest that sequence heterogeneity of NS5A of HCV-2a (IRRDR[2a]≥4 and ISDR/+C[2a]), and that of HCV-2b (IRRDR[2b]/N≥2) to a lesser extent, is involved in determining the viral sensitivity to PEG-IFN/RBV therapy.

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Introduction

Hepatitis C virus (HCV) is a major cause of chronic liver disease, such as chronic hepatitis, liver cirrhosis and hepatocellular carcinoma, with180 million people being currently infected with HCV worldwide. It is estimated that 70% of acute infections become persistent [1]. As a consequence of the long-term persistence of HCV infection, the number of patients with hepatocellular carcinoma is expected to increase further over the next 20 years. More than two decades have passed since the discovery of HCV, and yet therapeutic options remain limited. Standard regimens for treatment of chronic hepatitis C include pegylated interferon alpha (PEG-IFN) and ribavirin (RBV) [2]. In addition, two protease inhibitors (telaprevir and boceprevir) were approved in May 2011 by the U. S. Food and Drug Administration (FDA) for clinical use in combination with PEG-IFN/RBV to treat chronic hepatitis C patients with HCV genotype 1 [3,4].

In Japan, about 70% of HCV-infected patients are infected with HCV genotype 1b (HCV-1b) and most of the remaining patients are infected with HCV-2a (25%) or HCV-2b (5%) [5]. When treated with PEG-IFN/RBV, the sustained virological response (SVR) rate is ca. 50% in HCV-1b infection, and ca. 80% in HCV-2a and -2b infections [2,6]. The mechanism(s) underlying the different responses among patients with different HCV genotypes and subtypes is still unclear. However, this suggests that viral genetic heterogeneity could affect, at least to some extent, the sensitivity to IFN-based therapy. In this context, sequence heterogeneity of the viral NS5A protein has been widely discussed for its correlation with IFN responsiveness. Sequence variations within a region in NS5A of HCV-1b defined as the IFN sensitivity-determining region (ISDR) is correlated with IFN responsiveness [7]. In HCV-2a infection, the influence of sequence heterogeneity in and around a region corresponding to ISDR on the IFN responsiveness was also suggested [8–10]. Recently, we identified a

new region near the C-terminus of NS5A of HCV-1b, which we refer to as the IFN/RBV resistance-determining region (IRRDR) [11,12]. The degree of sequence variation within IRRDR was significantly correlated with the clinical outcome of PEG-IFN/RBV combination therapy. The significance of IRRDR of other HCV genotypes, however, has not been investigated yet.

In addition to the NS5A sequence variation, HCV core protein polymorphism was also proposed as a pretreatment predictor of poor virological response in HCV-1b-infected patients treated with PEG-IFN/RBV therapy [13]. It is not clear at this stage whether core protein polymorphism could be used to predict the treatment outcome in HCV-2a and -2b infections. In the present study, we investigated the impact of viral genetic heterogeneity in the NS5A and core regions of HCV-2a and -2b on PEG-IFN/RBV treatment outcome. To the best of our knowledge, this is the first report describing the possible correlation between PEG-IFN/RBV responsiveness and NS5A-IRRDR heterogeneity of HCV-2a and -2b.

Materials and Methods

Ethics statement

The study protocol, which conforms to the provisions of the Declaration of Helsinki, was approved beforehand by the Ethic Committees in Kobe Asahi Hospital and Kobe University, and written informed consent was obtained from each patient prior to the treatment.

Patients

A total of 112 patients seen at Kobe Asahi Hospital and Kobe University Hospital, Kobe, Japan, who were chronically infected with HCV-2a (61 patients) or HCV-2b (51 patients), were enrolled in the study. HCV subtype was determined according to the method of Okamoto et al. [14]. The patients were treated with PEG-IFN α-2b (Pegintron®; Schering-Plough, Kenilworth, NJ) (1.5 μg per kilogram body weight, once weekly, subcutaneously) and RBV (Rebetol®; Schering-Plough) (600~800 mg daily, per os), for 24 weeks according to a standard treatment protocol for Japanese patients established by a hepatitis study group of the Ministry of Health, Labour 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 [15].

Sequence analysis of the NS5A and core regions

HCV RNA was extracted from 140 μl of serum using a commercially available kit (QIAmp viral RNA kit; QIAGEN, Tokyo, Japan). The extracted RNA was reverse transcribed and amplified for NS5A and core regions using Super script III one step RT-PCR platinum Taq HiFi (Invitrogen, Tokyo, Japan). The resultant RT-PCR product was subjected to a second-round PCR by using Platinum Taq DNA polymerase high fidelity III (Invitrogen). Primers used for amplification of full-length NS5A of the HCV-2a and -2b genomes and those of the core region of HCV-2a were reported previously [16,17]. Primers for amplification of the core region of HCV-2b are as follows: C-2b/1 (5′-AGCCATAGTGGTCTGCGGAACC-3′; sense, nucleotides [nt] 136 to 157) and C-2b/4 (5′-GGAACARTTGCACTCTTGG-GTG-3′; antisense, nt 1241 to 1262) for one step RT-PCR; C-2b/2 (5′-CCACTCTATGTCCGGTCATTTGG-3′; sense, nt 208 to 230) and C-2b/3 (5′-GAGCTGCCAGGTGATGCTG-3′; antisense, nt 971 to 989) for the second round PCR. RT was performed at 45°C for 30 min and terminated at 94°C for 2 min, followed by the first-round PCR over 35 cycles, with each cycle consisting of denaturation at 94°C for 30 sec, annealing at 55°C for 30 sec and extension at 68°C for 90 sec. The second-round PCR was performed under the same condition. The sequences of the amplified fragments were determined by direct sequencing without subcloning. The amino acid (aa) sequences were deduced and aligned using GENETYX Win software version 7.0 (GENETYX Corp., Tokyo, Japan). The numbering of aa residues for HCV-2a and -2b isolates is according to the polyprotein of HCV-J6 [18] and -J8 [19], respectively.

Statistical analysis

Numerical data were analyzed by *S*tudent's *t* test while categorical data by Fisher's exact probability test [8]. To evaluate the optimal threshold of the number of aa mutations in ISDR and IRRDR for prediction of treatment outcomes, the receiver operating characteristic curve was constructed. Univariate and multivariate logistic regression analyses were performed to identify independent predictors for treatment outcomes. All statistical analyses were performed using the SPSS version 16 software (SPSS Inc., Chicago, IL). 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 with the accession numbers AB600751 through AB600834.

Results

Patients' Responses to PEG-IFN/RBV Combination Therapy in HCV-2a and HCV-2b infections

Of the 61 patients infected with HCV-2a, 46 (75%) patients cleared HCV viremia by week 4 (rapid virological response [RVR]), and all the patients (100%) by week 12 (early virological response [EVR]) and at week 24 (end-of-treatment response [ETR]) (Table 1). Likewise, of 51 patients infected with HCV-2b, 34 (67%), 51 (100%) and 50 (98%) patients achieved RVR, EVR and ETR, respectively. After the end of treatment, 105 patients (58 with HCV-2a and 47 with HCV-2b) could be followed up for another 24 weeks. At the end, SVR was achieved by 49 (84%) patients infected with HCV-2a and by 34 (72%) patients with HCV-2b. Only 9 (16%) and 13 (28%) patients with HCV-2a and -2b, respectively, were non-SVR. There was no case of null-response (continuous viremia throughout the treatment and follow up periods) since all the non-SVR patients once cleared viremia at a certain time point followed by a rebound in viremia either before or after the end of the treatment (relapse).

Comparison of the base line demographic characteristics between SVR and non-SVR patients revealed that, in HCV-2a infection, SVR patients had a significantly lower average age than that of non-SVR (Table 2). In HCV-2b infection, on the other hand, SVR patients had significantly γ-GTP levels than those of non-SVR. There was no significant difference in viremia titers between SVR and non-SVR in patients infected with HCV-2a or -2b.

Sequence Analysis of NS5A of HCV-2a and HCV-2b

The entire NS5A region of the HCV-2a and -2b genomes in pretreatment sera were sequenced, and aa sequences deduced. All the sequences obtained were aligned and the consensus sequences for HCV-2a and -2b were inferred. An N-terminal half (aa 1977 to 2196) of the consensus sequences of HCV-2a and -2b isolates were each identical to the prototype sequences, HCV-J6 [18] and

Table 1. Proportions of various virological responses of HCV-2a- and HCV-2b-infected patients treated with PEG-IFN/RBV.

Response	Proportion		
	HCV-2a	HCV-2b	All
RVR	46/61* (75%)	34/51 (67%)	80/112 (71%)
Non-RVR	15/61 (25%)	17/51 (33%)	32/112 (29%)
EVR	61/61 (100%)	51/51 (100%)	112/112 (100%)
ETR	61/61 (100%)	50/51 (98%)	111/112 (99%)
SVR	49/58 (84%)	34/47 (72%)	83/105 (79%)
Non-SVR	9/58 (16%)	13/47 (28%)	22/105 (21%)

*No. of patients/no. of total.
Abbreviations: RVR, rapid virological response; EVR, early virological response; ETR, end-of-treatment response; SVR, sustained virological response.
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HCV-J8 [19], respectively. The remaining C-terminal half (aa 2197 to 2442) of the consensus sequences were identical to those reported by Murakami et al. [8] except that His at position 2358 in the HCV-2b sequence was replaced with Cys, which was more conserved (59% of the isolates tested) than His (22%).

To investigate the impact of NS5A heterogeneity on the clinical outcome of PEG-IFN/RBV therapy, we first performed a sliding window analysis with a window size of 20 residues over the full-length NS5A sequences obtained from 23 RVR and 7 non-RVR patients infected with HCV-2a along with the consensus sequence, as described previously [8]. This analysis revealed that the number of aa mutations differed significantly between RVR and non-RVR isolates in two regions within the C-terminal half of NS5A (data not shown). The more C-terminally located one exactly matched the region that corresponded to IRRDR of HCV-1b, ranging from aa 2332 to 2387, thus being referred to as IRRDR[2a] (see Figure 1). The other region composed of a part of ISDR plus its carboxy-flanking region, ranging from aa 2232 to 2262, thus being referred to as ISDR/+C[2a] (see Figure 2). It was confirmed that the average numbers of aa mutations in IRRDR[2a] and ISDR/+C[2a] were each significantly larger in isolates from RVR than those from non-RVR patients (Table 3). More importantly, the average numbers of aa mutations in IRRDR[2a] and ISDR/+C[2a] were each significantly larger in SVR than in non-SVR. Sequences of IRRDR[2a] and ISDR/+C[2a] obtained from SVR and non-SVR patients and the number of mutations of each isolate are shown in Figures 1 and 2.

Likewise, a sliding window analysis on HCV-2b isolates (16 RVR and 6 non-RVR) identified an N-terminal part of IRRDR (aa 2332 to 2357), referred to as IRRDR/N[2b], that showed a significant difference in the number of aa mutations between RVR and non-RVR (data not shown). The average numbers of aa mutations in IRRDR/N[2b] were significantly larger in RVR than in non-RVR (Table 3). However, they did not differ significantly between SVR and non-SVR. Sequences of IRRDR[2b]/N obtained from RVR and non-RVR patients are shown in Figure 3.

Correlation between NS5A Sequence Heterogeneity and SVR or RVR in HCV-2a and HCV-2b infections

The receiver operating characteristic analysis identified the optimal thresholds of the numbers of aa mutations in IRRDR[2a] and ISDR/+C[2a] for the prediction of RVR and SVR in HCV-2a infection; four and one for IRRDR[2a] and ISDR/+C[2a], respectively (data not shown). Accordingly, we found that 86% (42/49) of SVR patients, and only 22% (2/9) of non-SVR, were infected with HCV-2a isolates having IRRDR with 4 or more mutations (IRRDR[2a]≥4) (Table 4). On the other hand, 14% (7/49) of SVR, and 78% (7/9) of non-SVR patients, were infected with isolates having IRRDR with 3 or less mutations (IRRDR[2a]≤3). These results suggested that IRRDR[2a]≥4 was significantly associated with SVR ($P = 0.0003$). Similarly, 93% (42/46) of RVR patients, and only 33% (5/15) of non-RVR, were infected with HCV-2a isolates of IRRDR[2a]≥4 while 7% (4/46) of RVR patients, and 67% (10/15) of non-RVR, were infected with HCV-2a isolates of IRRDR[2a]≤3, with the results suggesting that IRRDR[2a]≥4 was significantly associated with RVR as well ($P<0.0001$).

As for ISDR/+C[2a] heterogeneity, 71% (35/49) of SVR, and 22% (2/9) of the non-SVR patients, were infected with HCV-2a isolates with ISDR/+C having one or more mutation (ISDR/+C[2a]≥1) (Table 4). On the other hand, 29% (14/49) of SVR patients, and 78% (7/9) of the non-SVR, were infected with isolates with ISDR/+C without mutation (ISDR/+C[2a] = 0). Thus, ISDR/+C[2a]≥1 was significantly associated with SVR ($P = 0.008$).

Table 2. Demographic characteristics of HCV-2a- and HCV-2b-infected patients with SVR and non-SVR.

Factor	HCV-2a			HCV-2b		
	SVR	Non-SVR	*P* value	SVR	Non-SVR	*P* value
Age	49.78±13.67*	62.89±7.01	**0.007**	50.03±15.03	55.08±11.22	0.28
Sex (male/female)	22/27	3/6	0.72	17/17	8/5	0.53
Body weight (kg)	60.39±11.00	54.67±10.51	0.15	57.72±13.46	65.08±7.26	0.06
Platelets ($\times 10^4/mm^3$)	18.54±5.71	19.43±10.78	0.72	17.57±5.65	15.20±7.281	0.27
Hemoglobin (g/dl)	14.38±6.07	14.0±1.56	0.88	14.19±1.59	13.78±1.5	0.49
γ-GTP (IU/L)	37.66±53.25	36.83±24.82	0.97	39.68±34.33	81.30±69.11	**0.02**
ALT (IU/L)	64.75±52.45	94.38±141.3	0.28	86.35±91.95	86.85±118.7	0.98
HCV-RNA (KIU/ml)	1350±1424	1598±1464	0.63	5543±7643	7905±14210	0.47
HCV core antigen (fmol/L)	6543±6927	6105±8290	0.91	9054±6743	9390±8723	0.92

*Mean ± S.D.
Abbreviations: SVR, sustained virological response; γ-GTP, gamma glutamyl transpeptidase; ALT, alanine aminotransferase.
doi:10.1371/journal.pone.0030513.t002

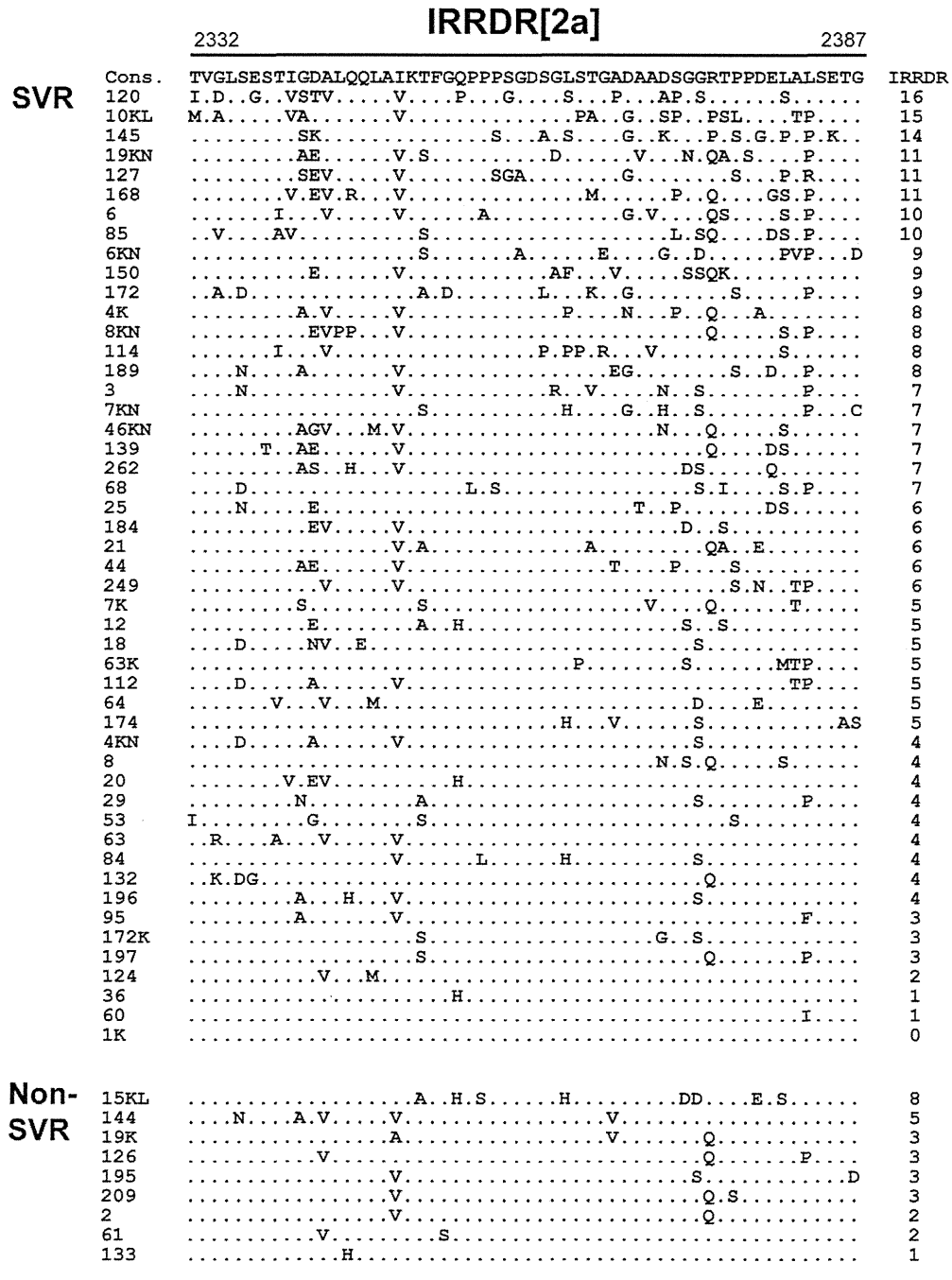
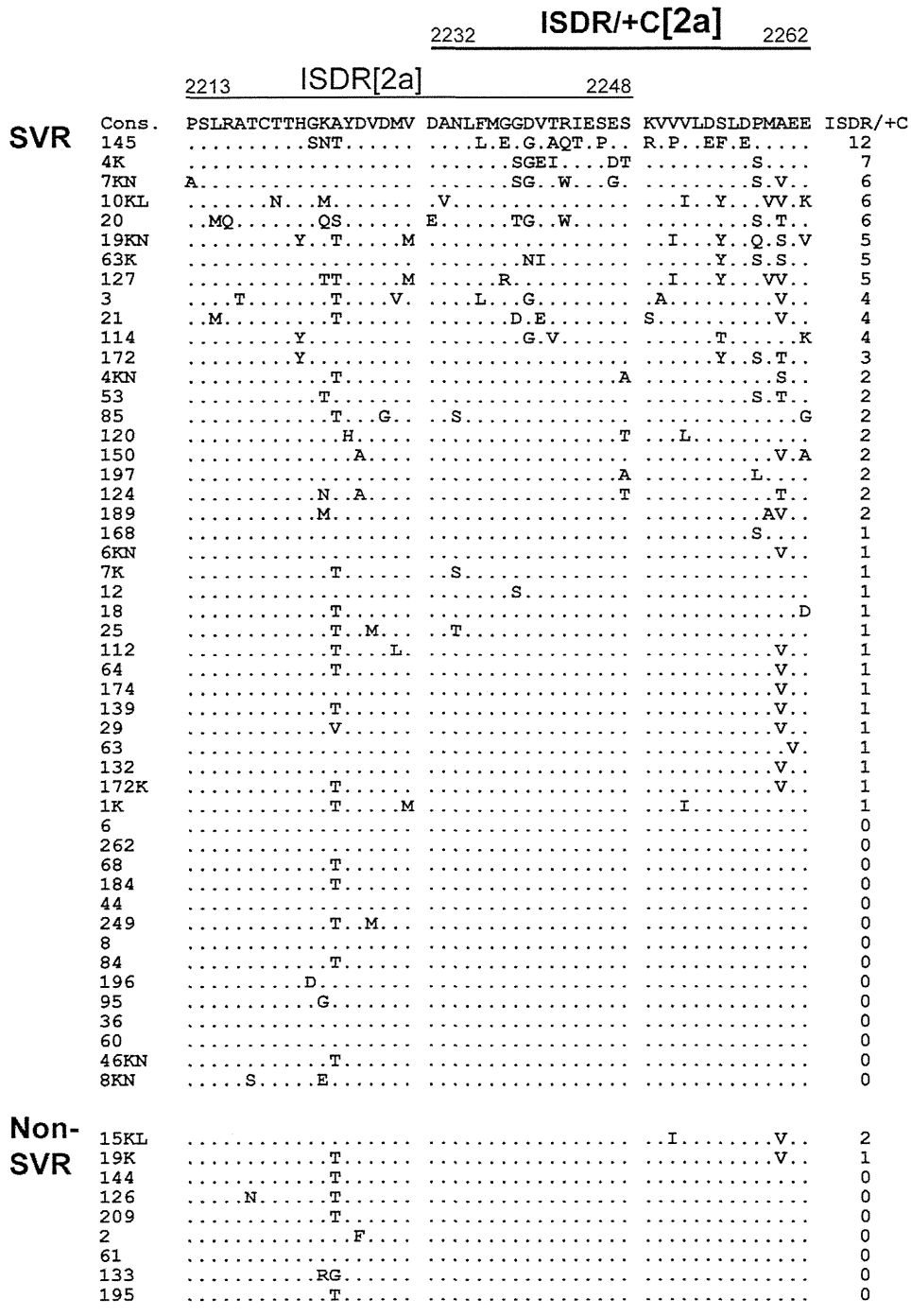


Figure 1. Sequence alignment of IRRDR[2a]. Sequences of IRRDR[2a] (interferon/ribavirin resistance-determining region of HCV-2a) obtained from SVR and non-SVR patients are aligned. The consensus sequence (Cons) is shown on the top. The numbers along the sequence indicate the aa positions. Dots indicate residues identical to those of the Cons sequence. The numbers of the mutations in IRRDR[2a] are shown on the right.
doi:10.1371/journal.pone.0030513.g001

As for HCV-2b infection, the receiver operating characteristic analysis identified "two" as the optimal threshold of the number of mutations in IRRDR/N[2b] by which to predict RVR (data not shown). Accordingly, we found that 65% (22/34) of RVR, and 18% (3/17) of non-RVR patients, were infected with HCV-2b isolates of IRRDR/N[2b]≥2 (Table 4). On the other hand, 35% (12/34) of RVR, and 82% (14/17) of the non-RVR patients, were infected with IRRDR/N[2b]≤1. These results suggested that IRRDR/N[2b]≥2 was significantly associated with RVR ($P = 0.0025$). However, no correlation, or even no tendency toward significant correlation, was observed between IRRDR/N[2b]≥2 and SVR in HCV-2b infection.

Correlation between NS5A Sequence Heterogeneity and Viremia Titers in the Serum of patients infected with HCV-2a and HCV-2b before PEG-IFN/RBV Therapy

Next, we examined the impact of IRRDR sequence heterogeneity on HCV titers in the serum before the initiation of the treatment. As shown in Figure 4A, patients infected with IRRDR[2a]≥4 had significantly lower pretreatment serum



	Cons.	PSLRATCTTHGKAYDVDMV	DANLFMGGDVTRIESES	KVVVLDSLDPMAEE	ISDR/+C
SVR	145	.....SNT.....	....L.E.G.AQT.P..	R.P..EF.E.....	12
	4K	.....	.....SGEI....DT	.....S....	7
	7KN	A.....	.....SG..W...G.	.....S.V..	6
	10KL	.....N...M.....	.V.....	...I..Y...VV.K	6
	20	..MQ.....QS.....	E.....TG..W.....	.....S.T..	6
	19KN	.....Y..T.....M	.....	..I...Y..Q.S.V	5
	63K	.....	.....NI.....	.....Y..S.S..	5
	127	.....TT.....M	.....R.....	..I...Y...VV..	5
	3	....T.....T....V.	....L...G.....	.A.....V..	4
	21	..M.....T.....	.....D.E.....	S.....V..	4
	114	.....Y.....	.....G.V.....	.....T.....K	4
	172	.....Y.....	.....	.....Y..S.T..	3
	4KN	.....T.....	.....A	.....S..	2
	53	.....T.....	.....	.....S.T..	2
	85	.....T...G..	..S.....	.....G	2
	120	.....H.....	.....T	...L.....	2
	150	.....A....	.....	.....V.A	2
	197	.....	.....A	.....L....	2
	124	.....N..A....	.....T	.....T..	2
	189	.....M.....	.....	.....AV..	2
	168	.....	.....	.....S....	1
	6KN	.....	.....	.....V..	1
	7K	.....T.....	..S.....	.....	1
	12	.....	.....S.....	.....	1
	18	.....T.....	.....	.....D	1
	25	.....T..M...	..T.....	.....	1
	112	.....T....L.	.....	.....V..	1
	64	.....T.....	.....	.....V..	1
	174	.....	.....	.....V..	1
	139	.....T.....	.....	.....V..	1
	29	.....V.....	.....	.....V..	1
	63	.....	.....	.....V.	1
	132	.....	.....	.....V..	1
	172K	.....T.....	.....	.....V..	1
	1K	.....T.....M	.....	...I.....	1
	6	.....	.....	.....	0
	262	.....	.....	.....	0
	68	.....T.....	.....	.....	0
	184	.....T.....	.....	.....	0
	44	.....	.....	.....	0
	249	.....T..M...	.....	.....	0
	8	.....	.....	.....	0
	84	.....T.....	.....	.....	0
	196	.....D.....	.....	.....	0
	95	.....G.....	.....	.....	0
	36	.....	.....	.....	0
	60	.....	.....	.....	0
	46KN	.....T.....	.....	.....	0
	8KN	.....S.....E.....	.....	.....	0
Non-SVR	15KL	.....	.....	..I.....V..	2
	19K	.....T.....	.....	.....V..	1
	144	.....T.....	.....	.....	0
	126	.....N.....T.....	.....	.....	0
	209	.....T.....	.....	.....	0
	2	.....F.....	.....	.....	0
	61	.....	.....	.....	0
	133	.....RG.....	.....	.....	0
	195	.....T.....	.....	.....	0

Figure 2. Sequence alignment of ISDR/+C[2a]. Sequences of ISDR/+C[2a] (part of interferon sensitivity determining-region plus its carboxy-flanking region of HCV-2a) obtained from SVR and non-SVR patients are aligned. The consensus sequence (Cons) is shown on the top. The numbers along the sequence indicate the aa positions. Dots indicate residues identical to those of the Cons sequence. The numbers of the mutations in ISDR/+C[2a] are shown on the right.
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HCV core antigen titers than those infected with IRRDR[2a]≤3. On the other hand, there was no significant difference in HCV viremia titers between ISDR/+C[2a]≥1 and ISDR/+C[2a] = 0 (Figure 4B). Also, in HCV-2b infection, there was no significant difference in pretreatment HCV viremia titers between IRRDR/N[2b]≥2 and IRRDR/N[2b]≤1 (Figure 4C).

Correlation between Core Protein Sequence Heterogeneity and RVR or SVR

A close correlation between core protein sequence patterns and treatment outcome has been proposed in HCV-1b infection [12,13]. To examine this hypothesis in HCV-2a and -2b infections, core regions of the virus genome were amplified from the pretreated sera, and the aa sequences deduced and aligned

Table 3. Average numbers of aa mutations within IRRDR[2a], ISDR/+C[2a] and IRRDR/N[2b] of HCV NS5A obtained form pre-treated sera of HCV-2a and -2b-infected patients with SVR, non-SVR, RVR and non-RVR.

NS5A region	No. of mutations			No. of mutations		
	SVR	Non-SVR	*P* value	RVR	Non-RVR	*P* value
IRRDR[2a] (aa 2332–2387)	6.4±3.4*	3.3±2.1	**0.01**	6.8±3.3	3.3±1.9	0.0003
ISDR/+C[2a] (aa 2232–2262)	2.0±2.4	0.3±0.7	**0.047**	2.1±2.5	0.6±0.7	0.025
IRRDR/N[2b] (aa 2332–2357)	1.8±1.5	1.4±1.3	0.45	2.0±1.4	1.0±1.2	0.01

*Mean ± S.D.

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.

doi:10.1371/journal.pone.0030513.t003

IRRDR[2b] 2387

2332 **IRRDR/N[2b]** 2357

	Cons.	AKVLTQDNVEGVLREMADKVLSPLQD	CNDSGHSTGVDTGGDSVQQPSDETAASEAG	IRRDR/N(2b)	Final response
RVR	73	.....S...I.K.....P..	S....R.....NI...P.....	4	SVR
	138	.....FK...G..F.....	H....R...A.....S.	4	SVR
	143	T.....D...R...S...	H.....AEA.....TA	4	SVR
	166	.R.....K.....SF..	Y.....A.....D..	4	Non-SVR
	183	.....T...I.K.....P..	S....R.....NI..L...A.V....	4	SVR
	231	.....FN.PR.	.....A.....S.D..	4	SVR
	2KL	.....KE.....P..	.....A.....T....	3	SVR
	7	.R.....I.....A.....	.....L...A.....D..	3	SVR
	21KN	.....I....VE.....	HT.....A.....	3	SVR
	116	.....GE..K.....	.....T.	3	SVR
	185	.....I.....I..E.	.....A.....D..	3	Non-SVR
	193	.....E...G.....F.....	.....A.....T.	3	SVR
	205	.....G....F.....F.....	Q.....A.....	3	Non-SVR
	233	.T.....R...K.....	.....T.	3	SVR
	4	V.....S.....	.Q.....T.	2	Non-SVR
	5KN	.R.....K.....	H.....A.....	2	SVR
	9KN	.....K.P..	N....L...AE.....D..	2	SVR
	94	.....F..P..	.....A.....T.	2	SVR
	106K	.....V.....G	.D...R.....	2	SVR
	212	.....E.....A.....	.....D..	2	SVR
	147	.R.....K.....	H.....A.....	2	Unknown
	229	.R.....I....	H.....E.....	2	Non-SVR
	11KL	.....I.....	.H.....M.....V..T.	1	SVR
	55KN	.R.....	H.....A.....A.....	1	SVR
	87	.....K.....	.....T....	1	SVR
	103	..I.....	.....AD.....	1	SVR
	165	.....F.....	H.....A.....S.	1	SVR
	1K	.....	.....A..A....T...T.	0	SVR
	10K	.....	.....I.L.....	0	SVR
	46	.....	.....	0	SVR
	99	.....	.....S..T.	0	SVR
	113	.....	.....T...T.	0	SVR
	179	.....	H.....A....N.A.....T...S.	0	SVR
	187	.....	Y.....T..T.	0	SVR
Non-RVR	18KN	.....G.....F.LP.E	.D.....T.	5	SVR
	13K	.....E...K.....	.D.....E....K.....	2	Non-SVR
	110	.....H...E.....	.H.....N.I..A.....T.	2	Unknown
	11K	.....N.....	.....C...A.....C.....G..T.	1	SVR
	23KN	.....E.....	.D.....M.....A.....S.	1	Non-SVR
	40KN	.....P..	HS...R.....D.E	1	SVR
	89	.....F.....	.....TE	1	Non-SVR
	157	.....N.....	YS.....P.....E	1	SVR
	164	.R.....	S.....I.....D.....T.	1	SVR
	170	.....E.....	SD.....N....I.....T.	1	Unknown
	265KL	.....I.....	.....E.	1	Non-SVR
	1	.....	YH.....A.....I...P.....	0	Non-SVR
	32KN	.....	.....G....	0	Non-SVR
	52	.....	H.....A.....T..T.	0	SVR
	105	.....	Y.....	0	Non-SVR
	107	.....	.....R.....	0	Non-SVR
	134	.....	.....G..T.	0	Unknown

Figure 3. Sequence alignment of NS5A of HCV-2b isolates. Sequences of IRRDR/N[2b] (an N-terminal part of interferon/ribavirin resistance-determining region of HCV-2b) obtained from RVR and non-RVR patients are aligned. The consensus sequence (Cons) is shown on the top. The numbers along the sequence indicate the aa positions. Dots indicate residues identical to those of the Cons sequence. The numbers of the mutations in IRRDR/N[2b] and the final treatment outcome of each patient are shown on the right.

doi:10.1371/journal.pone.0030513.g003

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.
doi:10.1371/journal.pone.0030513.t004

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,

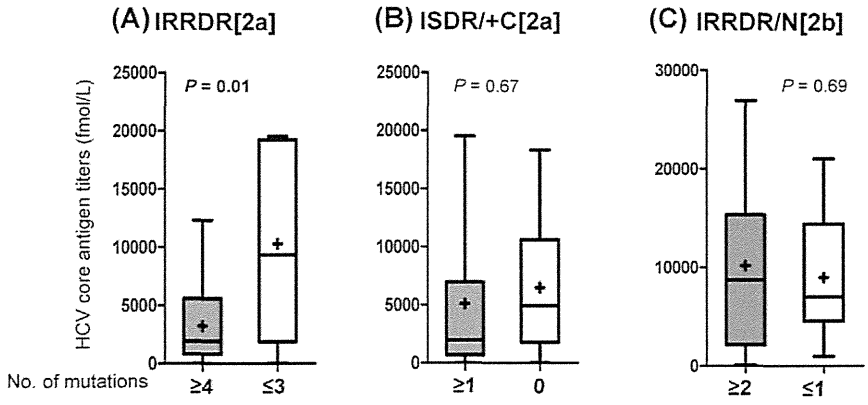

Figure 4. Correlation between NS5A sequence heterogeneity and pretreatment serum HCV core antigen titers in HCV-2a and HCV-2b infections. Pretreatment serum HCV core antigen titers of patients classified on the basis of the number of mutations in IRRDR[2a] (interferon/ribavirin resistance-determining 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.
doi:10.1371/journal.pone.0030513.g004

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

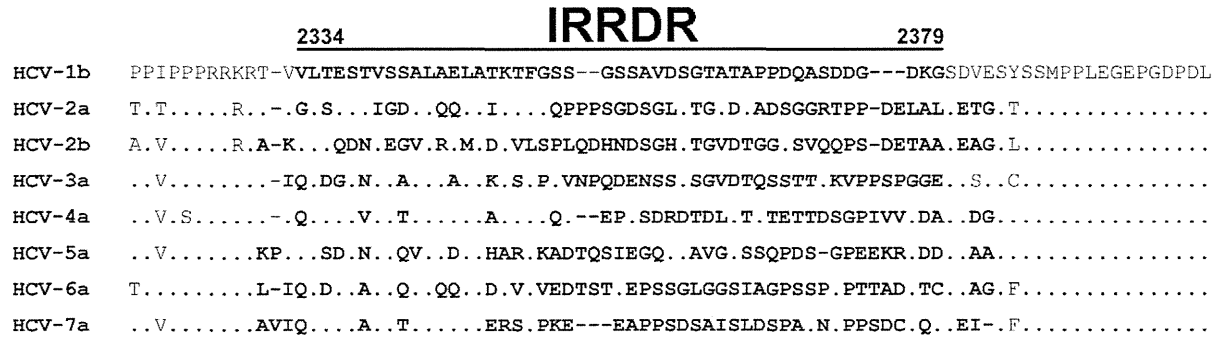

Figure 5. Sequence alignment of IRRDR (interferon/ribavirin resistance-determining region) and its upstream and downstream sequences of different HCV genotypes. The residues in the region that corresponds to IRRDR of HCV-1b [11] are written in boldface letters. Dots indicate residues identical to the 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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Mutations in non-structural 5A and rapid viral response to pegylated interferon-α-2b plus ribavirin therapy are associated with therapeutic efficacy in patients with genotype 1b chronic hepatitis C

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Abstract. For patients chronically infected with hepatitis C virus (HCV), mutations in the non-structural 5A (NS5A) gene are important predictive factors for the response to interferon (IFN) therapy. In the present study, factor analysis of the therapeutic response of patients following pegylated IFN and ribavirin combination therapy was assessed in a multicenter study. Chronic HCV-infected patients with genotype 1b and high viral load (n=96, mean age 56.5 years; 59 males, 68 females) treated with pegylated IFN-α-2b and ribavirin combination therapy were enrolled. This study was conducted at Kobe University Hospital and 25 affiliated hospitals in Hyogo prefecture. Sixty-five patients (68%) completed treatment with both pegylated IFN and ribavirin at >80% of the weight-based scheduled dosages. Patients who reduced or terminated therapy were frequently aged women (mean age 60.8 years; 11 males, 17 females). Overall, a sustained viral response (SVR) was achieved in 42 (44%) patients out of 96. Based on per-protocol-based (PPB) analysis, the SVR rate in patients with ≥6 amino acid (aa) mutations in the IFN resistance-determining region (IRRDR) (75%) or ≥1 aa mutation in the IFN sensitivity-determining region (ISDR) (61%) was significantly higher than that in patients with <5 aa mutations in IRRDR (30%) or no mutation in ISDR (29%). Multivariate analysis revealed that rapid viral response (RVR) (odds ratio, 18.1) and mutations of ≥6 in IRRDR (odds ratio, 15.5) were significantly associated with SVR. In conclusion, mutations in the NS5A region, particularly in patients with ≥6 aa mutations in IRRDR were strongly associated with a therapeutic response to pegylated IFN and ribavirin combination therapy.

Introduction

Hepatitis C virus (HCV) is a major cause of chronic liver disease, with an estimated 170 million people infected worldwide. In Japan, the carrier rate is estimated to be approximately 1% of the general population. This rate increases depending on age and reaches approximately 5% in individuals over 70 years of age. The main goal of treatment for chronic hepatitis C is prevention of cirrhosis and hepatocellular carcinoma by eradication of the virus. Interferon (IFN)-based therapy was initiated in 1992, and efficacy of treatment regimens has

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Key words: chronic hepatitis C, pegylated IFN and ribavirin therapy, non-structural 5A

improved year by year. Although the HCV viral eradication rate is approximately 5% following 24 weeks of treatment with conventional IFN therapy, the therapeutic result of combined pegylated IFN and ribavirin is ~55%. However, approximately half of patients treated with pegylated-IFN do not achieve a sustained viral response (1-3).

Due to the numerous side effects and the high cost of treatment, it is important to understand the individual mechanisms involved in non-response to treatment and to predict therapeutic efficacy prior to treatment. It has been reported that various viral and host factors are associated with the therapeutic response.

The role of amino acid (aa) mutations within the functional regions of non-structural 5A (NS5A) in relation to therapeutic response has been reported by several researchers. In 1996, it was reported that a high number of mutations in the IFN-sensitivity-determining region (ISDR) (aa 2209-2248) was strongly related to the sustained viral response (SVR) to IFN monotherapy in genotype 1b Japanese patients (4,5). In 2008, high mutations in the IFN-ribavirin resistance-determining region (IRRDR) (aa 2334-2379) were also related to the SVR to combined pegylated-IFN and ribavirin therapy (6). The significance of these mutations was also confirmed by studies carried out in different populations in different countries (7).

Based on previous studies, factor analysis and determination of NS5A viral mutations in relation to SVR of patients treated with pegylated-IFN and ribavirin combination therapy for HCV genotype 1b and a high viral load was carried out in a collaborative study in Kobe, Japan.

Materials and methods

Sample collection. Serum samples were collected from chronic hepatitis C patients with genotype 1b and a high viral load. A total of 96 patients (age 57.7±8.3 years; 45 males, 51 females) who were treated by subcutaneous injections of pegylated-IFN-α-2b once every week (1.5 μg/kg) (Pegintron; Schering-Plough, Innishannon, Country Cork, Ireland) in combination with oral ribavirin (400-800 mg) daily for 48 weeks between September, 2006 and June, 2008 were enrolled. HCV-RNA in serum samples was examined at 4 weeks, at the end of treatment and 6 months after the end of treatment. Serum samples were collected and stored at -80˚C until virological examination. The rapid virological response (RVR) was defined as undetectable HCV-RNA at 4 weeks. Patients who had persistent undetectable serum HCV-RNA and normal serum alanine aminotransferase (ALT) levels 6 months after the end of treatment were considered to have an SVR.

The standard dosage of PEG-IFN (1.5 μg/kg) and ribavirin (12 mg/kg) was determined depending on the weight-based dose. Patients treated with >80% of the standard dosage were considered as high drug adherence and patients treated with at least one drug at <80% of the standard dosage were categorized as a low drug adherence group.

This study was conducted by Kobe University Hospital and 25 affiliated hospitals in Hyogo prefecture. The study protocol was approved by the Ethics Committee of Kobe University Hospital, and written informed consent was obtained from each patient before treatment.

Table I. Comparison of the base characteristics of the SVR and the non-SVR groups.

Factor	SVR	Non-SVR	P-value
No. of patients (%)	42 (44%)	54 (56%)	
Age, years	55.1±8.6	59.7±7.5	0.005
Males:Females	22:20	23:31	
BMI (kg/m^2)	24.0±3.4	23.2±3.4	0.85
ALT (IU/l)	72.3±69.4	75.8±61.8	0.66
PLT (x10^4/mm^3)	17.7±4.9	17.0±5.3	0.68
RVR	15/38	3/49	<0.001
PPB/ITT	30/41 (73%)	25/54 (46%)	0.03

SRV, sustained viral response; BMI, body mass index; PLT, platelets; ALT, alanine aminotransferase; RVR, rapid viral response; PPB, per-protocol-based analysis; ITT, intention-to-treat analysis.

Table II. Drug adherence of patients to pegylated-interferon and ribavirin therapy.

	High drug adherence	Low drug adherence	P-value
No. of patients (%)	65 (68%)	31 (32%)	
Age, years	57.4±8.2	59.3±7.2	0.25
Male:Female	33:32	13:18	
BMI (kg/m^2)	23.6±2.8	23.5±4.3	NS
ALT (IU/l)	78.2±54.5	72.7±68.5	0.7
PLT (x10^4/mm^3)	16.3±5.6	16.7±4.6	0.8
SVR	30/65 (46%)	11/31 (35%)	NS
ISDR ≥1	26/50 (52%)	12/26 (46%)	NS
IRRDR ≥6	18/50 (36%)	11/26 (42%)	NS

BMI, body mass index; ALT, alanine aminotransferase; PLT, platelets; SRV, sustained viral response; ISDR, IFN sensitivity-determining region; IRRDR, IFN resistance-determining region.

NS5A sequence analysis. HCV-RNA was extracted from 140 μl serum using a commercial kit according to the manufacturer's protocol (QIAmp Viral RNA kit; Qiagen, Tokyo, Japan). The NS5A region of the HCV genome was amplified and sequenced by nested RT-PCR using primer sets (6). The aa sequences were deduced and aligned using GENETYX Win software version 7.0 (Genetyx Corp., Tokyo, Japan).

Statistical analysis. Differences in parameters, including all available patient demographic, biochemical, hematological, and virological data, as well as ISDR and IRRDR sequence variations factors, were determined between the different patient groups by the Student's t-test for numerical variables, and Fisher's exact probability test for categorical variables.

Subsequently, univariate and multivariate logistic analyses were performed to identify variables that independently predict SVR. The odds ratios (OR) and 95% confidence intervals

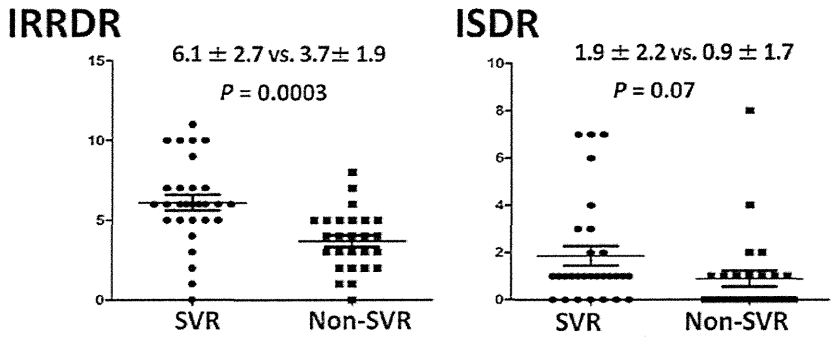

Figure 1. The number of mutations in IRRDR and ISDR. The number of mutations in IRRDR and ISDR was higher in the SVR group than in the non-SVR group.

Table III. Number of mutations in the NS5A region in relation to sustained viral response (SVR).

NS5A	Factor	SVR n (%)	Non-SVR n (%)	P-value
IRRDR	≥6	9/15 (60)[a]	3/17 (18)[a]	0.02[a]
	≥4	13/15 (87)	9/17 (53)	0.05
ISDR	≥4	3/15 (20)	1/17 (6)	0.25
	≥2	5/15 (33)	3/17 (18)	0.22
	≥1	11/15 (73)	7/17 (41)	0.06

[a] Statistically significant result. ISDR, IFN sensitivity-determining region; IRRDR, IFN resistance-determining region.

Table IV. Univariate and multivariate analyses in relation to the sustained viral response (SVR).

Univariate analysis		Multivariate analysis	
Factor	P-value	Odds ratio (95% CI)	P-value
IRRDR (IRRDR ≥6 vs. IRRDR ≤5)	0.000	18.1 (3.5-94.4)	0.001
ISDR (ISDR ≥1 vs. ISDR =0)	0.000		
RVR	0.017	15.5 (1.3-179.1)	0.028
LVR	0.001		
HCV-RNA titer (≥1000 vs. <1000)	0.099		
Age (≥60 vs. <60)	0.072		
Gender (male)	1.000		
PLT (≥15 vs. <15)	0.427		

ISDR, IFN sensitivity-determining region; IRRDR, IFN resistance-determining region; RVR, rapid viral response; LVR, late viral response.

(CIs) were also calculated. Positive and negative predictive values of SVR were computed, and their significance levels were evaluated using the sign test. All statistical analyses were performed using the SPSS version 16 software (SPSS Inc., Chicago, IL). Unless otherwise stated, a P-value of <0.05 was considered to indicate a statistically significant result.

Results

Baseline characteristics and on-treatment response in association with SVR. Baseline characteristics and on-treatment response are summarized in Table I. Overall, 42 cases out of 96 (44%) achieved an SVR. SVR patients were significantly younger in age and had a higher rate of RVR than the non-SVR patients. The prevalence of high drug adherence in SVR patients (73%) was significantly higher than that in non-SVR patients (46%) (P=0.03).

Drug adherence to pegylated interferon and ribavirin therapy. Due to various side effects, 31 patients were not treated with a sufficiently high dosage. Table II summarizes the patient groups with low and high drug adherence. Sixty-five (68%)

patients had high drug adherence to the therapy. Older age women tended to require dose reductions. The SVR rate (35%) in patients with low drug adherence was significantly lower than those (46%) with high drug adherence.

Mutations in the NS5A region and predictive indicators for SVR. Factor analysis in association with the SVR was performed by per-protocol-based (PPB) analysis. The average number of mutations in IRRDR was significantly higher in the SVR group (6.1±2.7) than that in the non-SVR group (3.7±1.9) (P=0.0003). The average number of mutations in ISDR was also higher in the SVR group (1.9±2.2) than that in the non-SVR group (0.9±1.7), but this difference did not achieve statistical significance (Fig. 1). The SVR group and the non-SVR group were compared based on the number of mutations in the NS5A region. The prevalence of patients with ≥6 aa mutations within IRRDR in the SVR group (60%) was significantly higher than that in the non-SVR group (18%) (P=0.02). Similarly, the prevalence of patients with ≥1 aa mutation within ISDR in the SVR group (73%) was higher than that in the non-SVR group (41%), but this difference was not statistically significant (P=0.06). All patients with ≥6 aa mutations in IRRDR and ≥1 aa mutation in ISDR achieved an SVR (Table III). The positive predictive values of SVR in patients with ≥6 aa mutations in IRRDR was 78%. The sensitivity and specificity were 64 and 86%, respectively.

Factor analysis in association with the SVR. Univariate and multivariate analyses are summarized in Table IV. Univariate analysis showed that ≥6 aa mutations in IRRDR and ≥1 aa mutation in ISDR were strongly associated with an SVR. In addition, RVR and LVR were also significant between the two groups. Multivariate analysis revealed that ≥6 aa mutations in IRRDR (odds ratio 18.1) and RVR (odds ratio 15.5) were significantly related to the SVR.

Discussion

Pegylated-IFN and ribavirin combination therapy has been a standard treatment for patients with chronic hepatitis C. However, HCV genotype 1 is more resistant to IFN treatment than genotypes 2 or 3. In Japan, genotype 1b is the most prevalent and it is important to predict the therapeutic response for these patients prior to therapy (7-9). In general, approximately 50% of patients with genotype 1b do not achieve SVR even when using a combination of pegylated-IFN plus ribavirin treatment (10). In the present study, the overall SVR rate was 44% and this value was slightly lower than that in a previous study (8). The reason for this is possibly related to the patient age and drug adherence. The present study showed that age, drug adherence and RVR in the SVR group were significantly different than these values in the non-SVR group. The SVR rate in patients younger than 65 years was 52% and was significantly higher than that in patients over 65. In addition, the SVR rate (46%) in patients with high drug adherence was higher than that (35%) in patients with low drug adherence. There is no doubt that elder patients have difficulties continuing therapy and are forced to reduce the dosage or terminate treatment because of side effects. In the present study, the percentage of patients having low drug adherence was 32%, and the majority of patients in this group were aged women. Physically and mentally, it is frequently difficult to continue therapy for elder patients. The average age of patients in Japan is older than that in most other European countries and this is one of the important reasons for the therapeutic difference among Japanese studies and those carried out in other countries.

On-treatment response is an important factor for predicting SVR; RVR 4 weeks following the initiation of treatment has been reported to be a good predictor of SVR (11-13). In this study, RVR was an important factor for predicting SVR by multivariate analysis. The positive predictive value was 82% and RVR was confirmed to be a good predictor in this study. However, even when patients are predicted as good responders for IFN/RBV therapy, they do not always achieve SVR as side effects result in dose reduction or termination of the planned IFN/RBV treatment. It was also reported that drug adherence is related to SVR (14). In this study, 3 patients relapsed after achieving RVR. The first case was over 65 years of age, the second case had low drug adherence, and the third was an older patient over 65 years with low drug adherence. Incomplete treatment is an important factor contributing to the failure of achieving SVR. This result suggests the necessity for prolonged therapy or therapeutic modification in patients with RVR receiving a dosage reduction.

Mutations in several amino acids in the NS5A protein have been described and are thought to play an important role in response to IFN treatment. It has been reported that a high number of mutations in ISDR and IRRDR are significantly associated with SVR (6). In the present study, patients with ≥1 aa mutation in ISDR and ≥6 aa mutations in IRRDR tended to achieve SVR, which was supported by previous data (6). For ISDR, the mutation results are similar to previous studies (4,5). Compared with ISDR, IRRDR was more strongly associated with SVR in this study. Based on the multivariate analysis, only IRRDR was associated with an SVR. Patients with more than 6 IRRDR mutations had a higher SVR rate and it was the same as previous studies (6). The positive predictive value and sensitivity was >80%, suggesting it to be a good predictive marker. All patients with ≥6 aa mutations and ≥1 aa mutation in ISDR achieved SVR following pegylated-IFN and ribavirin combination therapy. The importance of the NS5A mutation is still controversial. It has been reported that a mutation in NS5A is not related to the IFN response in European and American HCV strains (15-18). However, the importance of NS5A was reported in Asian HCV strains including Taiwan and Chinese strains (19,20). To date, this inconsistency is unclear but is partly related to the fact that HCV strains are different depending on geographic distribution (21). Meta-analysis revealed that the prevalence of a mutation in ISDR was 44.1% in Japanese and 24.8% in European patients, respectively (21). Mutational studies are sometimes inconsistent even among Japanese studies, suggesting that mutations in the NS5A region vary based on different geographical regions even in Japan.

The NS5A protein has a transcriptional activation function and represses IFN-induced gene expression (22). In addition, the NS5A protein interacts with antiviral protein PKR resulting in suppressed PKR activity (23). It is possible that mutations in the NS5A protein may affect the structural and/or biological functions of NS5A and inhibit IFN activity (23,24).

Mutations in E2-PePHD (aa 659-670), PKRBD (aa 2209-2274) and NS5A-V3 (aa 2356-2379) are also reported to be associated with IFN sensitivity (24,25).

Recent studies have shown that SNPs in the IL28B region are strongly associated with response to IFN therapy (26). In this study, genomic factors in the host were not analyzed due to the pre-treatment study design and informed consent. Therapeutic prediction can be more accurate upon examination of host factors as well as viral factors. In the near future, new drug therapies such as protease and polymerase inhibitors called new direct-acting antivirals (DAAs) will become available (27). Standard therapy for hepatitis C virus will include combination therapies using DAAs and pegylated-IFN plus ribavirin. However, the SVR rate by telaprevir-based pegylated-IFN plus ribavirin combination therapy (REALIZE study; phase III, randomized, double blind, placebo-controlled study) was found to be as high as 31% in patients who were non-responders to prior treatment (28). The viral response to pegylated-IFN and ribavirin combination therapy is important for the development of future combination therapies.

In conclusion, mutations in the NS5A region, particularly in patients with more than 6 aa mutations in the IRRDR region are strongly associated with the therapeutic response to pegylated-IFN and ribavirin combination therapy.

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ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

Prediction of response to pegylated interferon/ribavirin combination therapy for chronic hepatitis C genotype 1b and high viral load

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Abstract
Background This study explores pretreatment predictive factors for ultimate virological responses to pegylated interferon-α (1.5 μg/kg/week) and ribavirin (600–1000 mg/day) (PEG-IFN/RBV) combination therapy for patients infected with hepatitis C virus (HCV)-1b and a high viral load.
Methods A total of 75 patients underwent PEG-IFN/RBV combination therapy for 48 weeks. HCV amino acid (aa) substitutions in non-structural protein 5a, including those in the IFN/RBV resistance-determining region (IRRDR) and the IFN sensitivity-determining region and the core regions, as well as the genetic variation (rs8099917) near the interleukin 28B (IL28B) gene (genotype TT) were analyzed.
Results Of the 75 patients, 49 % (37/75) achieved a sustained virological response (SVR), 27 % (20/75) showed relapse, and 24 % (18/75) showed null virological response (NVR). Multivariate logistic regression analysis identified IRRDR with 6 or more mutations (IRRDR ≥6) [odds ratio (OR) 11.906, $p < 0.0001$] and age <60 years (OR 0.228, $p = 0.015$) as significant determiners of SVR and IL28B minor (OR 14.618, $p = 0.0019$) and platelets $<15 \times 10^4/mm^3$ (OR 0.113, $p = 0.0096$) as significant determiners of NVR. A combination of IRRDR ≥6 and age <60 years improved SVR predictability (93.3 %), and that of IRRDR ≤5 and age ≥60 years improved non-SVR predictability (84.0 %). Similarly, a combination of IL28B minor and platelets $<15 \times 10^4/mm^3$ improved NVR predictability (85.7 %), and that of IL28B major and platelets $\geq 15 \times 10^4/mm^3$ improved non-NVR (response) (97.1 %) predictability.
Conclusion IRRDR ≥6 and age <60 years were significantly associated with SVR. IL28B minor and platelets $<15 \times 10^4/mm^3$ were significantly associated with NVR. Certain combinations of these factors improved SVR and NVR predictability and could, therefore, be used to design therapeutic strategies.

Keywords IRRDR · IL28B · SVR · Relapse · NVR

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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 (CHC), the number of patients with hepatocellular carcinoma is expected to increase over the next 20 years [2]. To reduce the impact of this worldwide health problem, efficient treatment is required. Currently, combination therapy with pegylated

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interferon-α and ribavirin (PEG-IFN/RBV) is the standard treatment for CHC. The therapy is sometimes not easily tolerated, however, and sustained virological response (SVR) is achieved in only ~50 % of patients, with SVR rarely being achieved in those infected with the most resistant genotypes—HCV-1a and HCV-1b involving high viral loads [3]. In Japan, the most common genotype is HCV-1b. Given the considerable side effects of the PEG-IFN/RBV therapy, the possibility of its discontinuation, and its high cost, being able to predict treatment outcome is desirable. A wide range of predictors would assist clinicians and patients in more accurately assessing the likelihood of SVR and thus in making more informed treatment decisions [4]. One of the most reliable methods of predicting response is to monitor the early drop in serum HCV RNA levels during treatment [5]; however, there is no established method of predicting such an outcome before treatment [6].

Although host factors including age, sex, ethnicity, platelets, liver fibrosis, obesity, and viral factors including genotype and viral load have been associated with the outcome of PEG-IFN/RBV therapy [6], little was known until recently about host genetic factors and viral genetic polymorphisms within a given genotype of HCV that might be associated with response to the therapy. Recent reports have revealed factors associated with response to PEG-IFN/RBV therapy: single nucleotide polymorphisms, as host genetic factors, located in interleukin (IL) 28B (rs8105790, rs11881222, rs8103142, rs28416813, rs4803219, rs8099917, rs7248668, and rs12979860) on chromosome 19 [7–10] and amino acid (aa) substitutions in non-structural protein 5a (NS5A), especially those in the IFN/RBV resistance-determining region (IRRDR) [11–13] and the IFN sensitivity-determining region (ISDR) [14], and the core region of HCV [15, 16], as viral genetic polymorphisms.

In this study, we compare the impact of host genetic factors such as IL28B and viral genetic polymorphisms including those in IRRDR, ISDR, and core mutations of HCV, as pretreatment predictive factors of PEG-IFN/RBV treatment outcome, and aim at establishing a rational strategy for the treatment of CHC patients infected with HCV-1b with high viral loads.

Methods

Patients

A total of 75 patients (43 men and 32 women; median age 60 years; range 30–74) who completed PEG-IFN/RBV combination therapy for 48 weeks were enrolled in the study. They were seen at Kobe Asahi Hospital in Kobe, Japan, and diagnosed with chronic HCV-1b infection on the basis of the presence of anti-HCV antibodies and HCV RNA. Informed consent in writing was obtained from each patient, and the study protocol, conforming to ethical guidelines, was approved by the Ethics Committee of Kobe Asahi Hospital. The HCV genotype was determined according to the method of Okamoto et al. [17]. The inclusion and exclusion criteria for the 75 patients in this study were as follows: patients were required to have hemoglobin levels of ≥11 g/dL (women) or ≥12 g/dL (men), platelet counts of $\geq 9 \times 10^4/mm^3$, HCV RNA ≥5.0 Log IU/mL, neutrophil count $\geq 1500/mm^3$, and thyroid-stimulating hormone levels within normal limits. Patients were excluded if they had human immunodeficiency virus (HIV) or hepatitis B coinfection, creatinine clearance <50 mL/min, cause of liver disease other than CHC, evidence of advanced liver disease, preexisting psychiatric conditions, or a history of severe psychiatric disorder. Patients were treated with PEG-IFN α-2b (1.5 μg/kg body weight, once a week subcutaneously) and RBV (600–1000 mg daily, per os) for 48 weeks, according to the standard treatment protocol for Japanese patients established by a hepatitis study group of the Ministry of Health, Labor and Welfare, Japan. Serum samples were collected from the patients at intervals of 4 weeks before, during, and after the treatment, and tested for HCV RNA based on the COBAS TaqMan HCV test (Roche Diagnostics, Basel, Switzerland).

Sequence analysis of HCV NS5A and HCV core regions

HCV RNA was extracted from 140 μL of serum with the use of a commercially available kit (QIAmp viral RNA kit; QIAGEN, Tokyo, Japan). Amplification of full-length NS5A and the core regions of the HCV genome was carried out as described [11, 12, 18]. The sequences of the amplified fragments of NS5A and the core regions were determined by direct sequencing without subcloning. The aa sequences were deduced and aligned with the use of GENETYX Win software version 7.0 (GENETYX., Tokyo, Japan).

Genetic variation near the IL28B gene

Genetic polymorphism rs8099917 around the IL28B gene was determined by real-time polymerase chain reaction (PCR) with the TaqMan assay [7]. We defined the IL28B major allele as homozygous for the major sequence (TT) and the IL28B minor allele as homozygous (GG) or heterozygous (TG) for the minor sequence.

Statistical analysis

Statistically significant differences in treatment responses according to patient baseline parameters of age, sex, body mass index (BMI), HCV RNA load, alanine aminotransferase (ALT), γ-glutamyl transpeptidase (γ-GTP), hemoglobin, platelets, total cholesterol, and drug doses of PEG-IFN and RBV were determined by the Wilcoxon two-sample test for numerical variables and Fisher's exact probability test for categorical variables. Likewise, statistically significant differences in treatment responses according to NS5A and core mutations and genetic variation near the IL28B gene (genotype TT) were determined by Fisher's exact probability test. Variables with a p value of <0.1 in univariate analysis were included in stepwise multivariate logistic regression analysis. Variables with a p value of <0.05 in multivariate analysis were considered statistically significant. The odds ratio was also calculated. All statistical analyses were carried out with SAS software version 9.2 (SAS, Chicago, IL, USA).

Nucleotide sequence accession numbers

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

Results

Patient responses to PEG-IFN/RBV combination therapy

Among the 75 patients enrolled in this study, rapid virological response (RVR) at week 4 was achieved by 13 % (10/75), complete early virological response (cEVR) at week 12 by 60 % (45/75), end-of-treatment response (ETR) by 72 % (54/75), and SVR by 49 % (37/75). SVR was seen in 90 % (9/10), 76 % (34/45), and 69 % (37/54) of the RVR, cEVR, and ETR patients, respectively (data not shown). Continuous viremia throughout the observation period (72 weeks), referred to as null virological response (NVR), was observed in 24 % (18/75), while transient disappearance of serum HCV RNA at a certain point in time followed by a rebound in viremia either before or after the end of the treatment course, referred to as a relapse, was observed in 27 % (20/75).

The numbers of patients who received ≥1.4 μg/kg/week of the dose of PEG-IFN were 23 of 37 in SVR, 15 of 20 in relapse, and 14 of 18 in NVR. Similarly, the numbers of patients who received ≥11.0 mg/kg/day of the dose of RBV were 16 of 37 in SVR, 7 of 20 in relapse, and 6 of 18 in NVR.

Correlation between patient demographic characteristics and treatment responses

The baseline characteristics and the clinical responses of the patients are shown in Table 1. By univariate analysis, sex, BMI, HCV RNA, ALT, total cholesterol levels, and drug doses of PEG-IFN and RBV showed no significant difference between SVR and non-SVR (relapse *plus* NVR) patients. SVR patients were significantly younger ($p = 0.0018$) with a higher level of hemoglobin ($p = 0.0049$) than non-SVR patients. Relapse patients were significantly older ($p = 0.0071$) than SVR patients. NVR patients had a significantly higher level of γ-GTP ($p = 0.07$) and lower level of hemoglobin ($p = 0.0020$) with fewer platelets ($p = 0.0016$) than response (SVR plus relapse) patients (Table 1).

Correlation between the number of NS5A mutations and treatment responses

Using receiver operating characteristic curve analysis, the optimal cutoff number of mutations in IRRDR for predicting SVR has been estimated at 6 [12, 13]. By univariate analysis, examination of a possible correlation between IRRDR mutations and treatment responses revealed that among 30 patients infected with HCV isolates involving 6 or more IRRDR mutations (IRRDR ≥6), SVR was achieved by 80 % (24/30), relapse was shown by 10 % (3/30), and NVR was shown by 10 % (3/30). By contrast, among 45 patients infected with HCV isolates involving 5 or fewer mutations (IRRDR ≤5), SVR was achieved by 29 % (13/45), relapse was shown by 38 % (17/45), and NVR was shown by 33 % (15/45). There was a significant difference in the proportion of HCV isolates involving IRRDR ≥6 and those involving IRRDR ≤5 between SVR and non-SVR patients ($p = 0.00002$), between SVR and relapse patients ($p = 0.00035$), and between response and NVR patients ($p = 0.027$) (Table 1). Notably, among the 30 patients infected with HCV isolates of IRRDR ≥6, 24 (80 %) achieved SVR, suggesting that IRRDR ≥6 could predict SVR with a positive predictive value of 80 %.

Examination of the possible correlation between treatment response and ISDR mutation at a cutoff point of 2 mutations, a newly proposed ISDR criterion for PEG-IFN/RBV responsiveness [14], revealed that among 18 patients infected with HCV isolates involving 2 or more ISDR mutations (ISDR ≥2), SVR was achieved by 56 % (10/18), relapse was shown by 11 % (2/18), and NVR was shown by 33 % (6/18). By contrast, among 57 patients infected with HCV isolates involving ISDR ≤1, SVR was achieved