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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 Student'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 ($\times10^4$/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.
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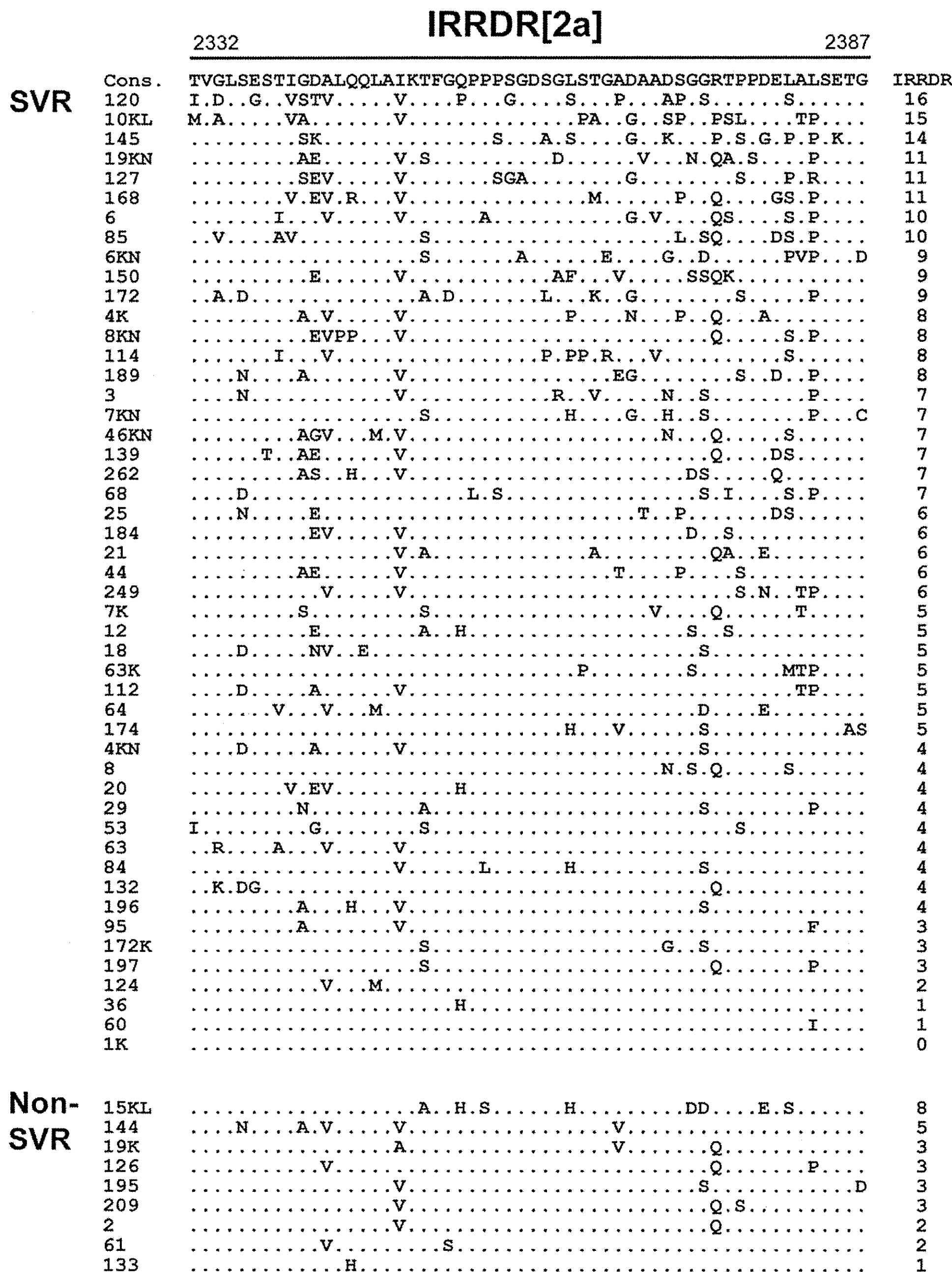

Figure 1. Sequence alignment of IRRDR[2a]. Sequences of IRRDR[2a] (interferon/ribavirin resistance-determining region of HCV-2a) obtained from SVR and non-SVR patients are aligned. The consensus sequence (Cons) is shown on the top. The numbers along the sequence indicate the aa positions. Dots indicate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

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                                         2232     ISDR/+C[2a]      2262
             2213      ISDR[2a]              2248

      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-  15KL   ................... ................. ..I........V..    2
SVR   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.

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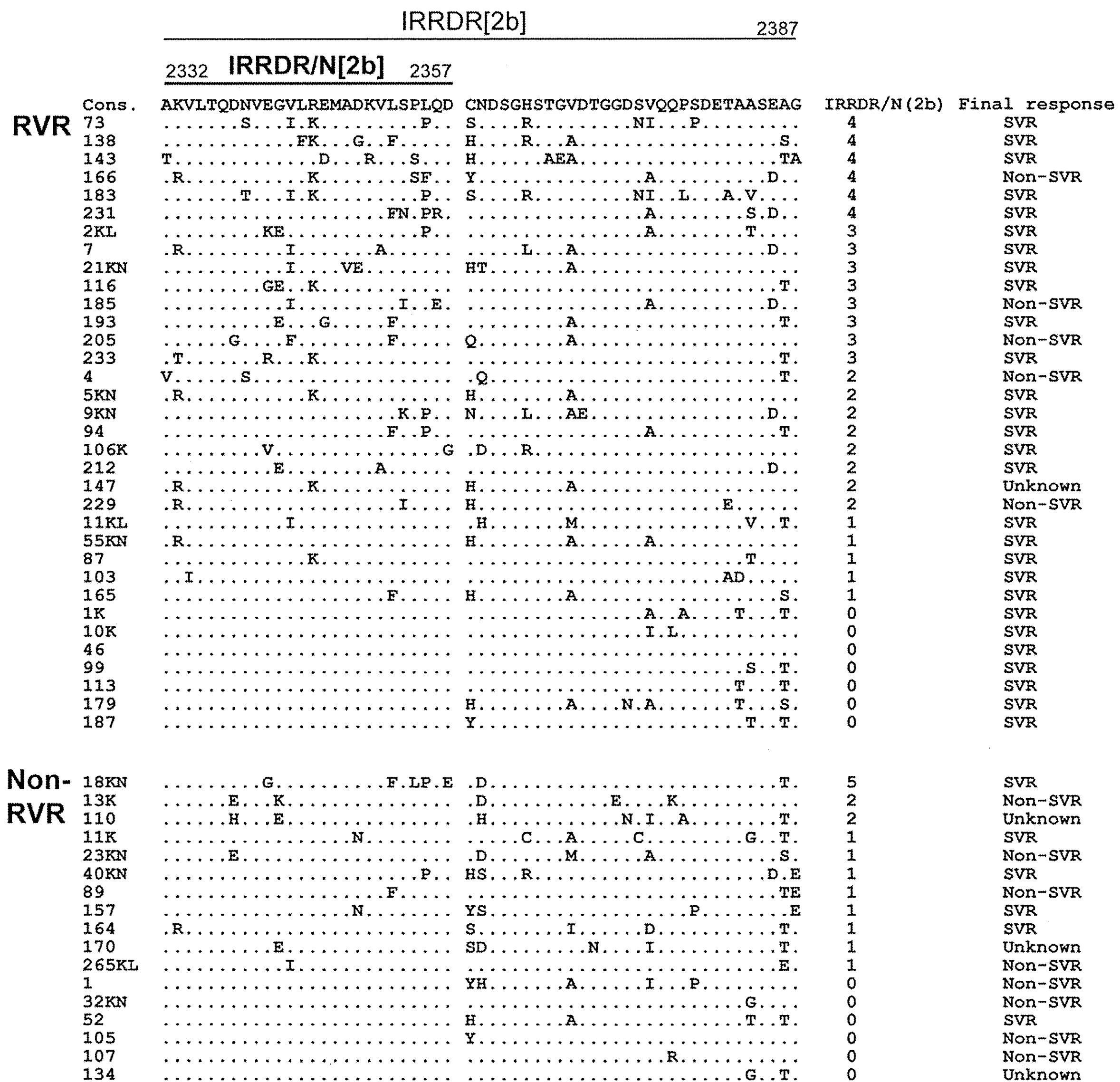

Figure 3. Sequence alignment of NS5A of HCV-2b isolates. Sequences of IRRDR/N[2b] (an N-terminal part of interferon/ribavirin resistance-determining region of HCV-2b) obtained from RVR and non-RVR patients are aligned. The consensus sequence (Cons) is shown on the top. The numbers along the sequence 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.

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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,

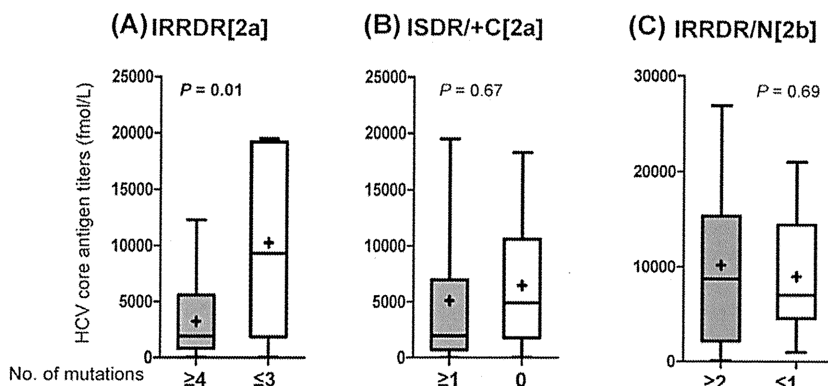

Figure 4. Correlation between NS5A sequence heterogeneity and pretreatment serum HCV core antigen titers in HCV-2a and HCV-2b infections. Pretreatment serum HCV core antigen titers of patients classified on the basis of the number of mutations in IRRDR[2a] (interferon/ribavirin resistance-determining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

```
                         IRRDR
                2334                            2379
HCV-1b   PPIPPPRRKRT-VVLTESTVSSALAELATKTFGSS--GSSAVDSGTATAPPDQASDDG---DKGSDVESYSSMPPLEGEPGDPDL
HCV-2a   T.T.....R..-.G.S...IGD..QQ..I....QPPPSGDSGL.TG.D.ADSGGRTPP-DELAL.ETG.T...............
HCV-2b   A.V.....R.A-K...QDN.EGV.R.M.D.VLSPLQDHNDSGH.TGVDTGG.SVQQPS-DETAA.EAG.L...............
HCV-3a   ..V........-IQ.DG.N..A...A..K.S.P.VNPQDENSS.SGVDTQSSTT.KVPPSPGGE..S..C...............
HCV-4a   ..V.S......-.Q....V..T......A....Q.--EP.SDRDTDL.T.TETTDSGPIVV.DA..DG.................
HCV-5a   ..V.......KP...SD.N..QV..D..HAR.KADTQSIEGQ..AVG.SSQPDS-GPEEKR.DD..AA.................
HCV-6a   T.........L-IQ.D..A..Q..QQ..D.V.VEDTST.EPSSGLGGSIAGPSSP.PTTAD.TC..AG.F...............
HCV-7a   ..V.......AVIQ....A..T......ERS.PKE---EAPPSDSAISLDSPA.N.PPSDC.Q..EI-.F...............
```

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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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 $<15 \times 10^4/\text{mm}^3$ (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 $<15 \times 10^4/\text{mm}^3$ improved NVR predictability (85.7 %), and that of IL28B major and platelets $\geq 15 \times 10^4/\text{mm}^3$ improved non-NVR (response) (97.1 %) predictability.

Conclusion IRRDR ≥6 and age <60 years were significantly associated with SVR. IL28B minor and platelets $<15 \times 10^4/\text{mm}^3$ 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 $\geq$11 g/dL (women) or $\geq$12 g/dL (men), platelet counts of $\geq 9 \times 10^4/\text{mm}^3$, HCV RNA $\geq$5.0 Log IU/mL, neutrophil count $\geq 1500/\text{mm}^3$, 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

Table 1 Univariate analysis of factors associated with SVR, relapse, and NVR

Factor	All	SVR	Relapse	NVR	*p* value		
					SVR versus non-SVR	SVR versus relapse	Response versus NVR
n	75	37	20	18	–	–	–
Age (years)	60 (30–74)	57 (33–70)	63 (30–74)	63 (40–71)	**0.0018**	**0.0071**	0.111
Sex: male/female	43/32	23/14	11/9	9/9	0.486	0.778	0.587
BMI (kg/m^2)	22.2 (15.7–37.6)	22.1 (18.3–37.6)	21.9 (15.7–30.7)	23.0 (16.6–31.3)	0.844	0.357	0.298
HCV RNA (Log IU/mL)	6.2 (5.0–7.1)	6.2 (5.0–7.1)	6.2 (5.3–6.7)	6.2 (5.3–7.1)	0.727	0.913	0.606
ALT (U/L)	38 (8–265)	37 (11–174)	37 (10–265)	41 (8–148)	0.618	0.493	0.896
γ-GTP (U/L)	32 (9–406)	32 (9–406)	25 (9–127)	44 (20–151)	0.614	0.503	**0.07**
Hemoglobin (g/dL)	14.0 (11.0–18.6)	14.4 (11.9–18.6)	14.3 (11.0–16.1)	13.2 (12.0–14.5)	**0.0049**	0.213	**0.0020**
Platelets ($\times 10^4$/mm^3)	16.3 (9.1–30.9)	16.9 (9.1–30.9)	18.9 (9.8–25.2)	12.1 (9.1–21.8)	0.124	0.802	**0.0016**
Total cholesterol (mg/dL)	176 (99–248)	181 (106–248)	164 (100–230)	182 (99–237)	0.572	0.243	0.617
PEG-IFN (μg/kg/week): <1.4/≥1.4	23/52	14/23	5/15	4/14	0.184	0.326	0.373
Ribavirin (mg/kg/day): <11.0/≥11.0	46/29	21/16	13/7	12/6	0.422	0.545	0.594
IRRDR mutations: ≤5/≥6	45/30	13/24	17/3	15/3	**0.00002**	**0.00035**	**0.027**
ISDR mutations: ≤1/≥2	57/18	27/10	18/2	12/6	0.597	0.182	0.346
HCV core aa 70: wild/mutant	51/24	30/7	12/8	9/9	**0.025**	0.117	**0.083**
HCV core aa 91: wild/mutant	53/22	27/10	14/6	12/6	0.801	1.000	0.768
IL28B genotype: major/minor	57/18	34/3	16/4	7/11	**0.0024**	0.226	**0.0000095**

Values in bold are significant

SVR sustained virological response, *NVR* null virological response, *non-SVR* relapse *plus* NVR, *Response* non-NVR (SVR *plus* relapse), *BMI* body mass index, *ALT* alanine aminotransferase, *γ-GTP* gamma-glutamyl transpeptidase, *IRRDR* interferon/ribavirin resistance-determining region, *ISDR* interferon sensitivity-determining region, *IL* interleukin, *HCV* hepatitis C virus, *PEG-IFN* pegylated interferon

by 47 % (27/57), relapse was shown by 32 % (18/57), and NVR was shown by 21 % (12/57). Although a significant correlation was observed between ISDR heterogeneity and early virological response such as RVR ($p = 0.028$) (data not shown), no significant correlation was observed between ISDR heterogeneity and late virological response such as SVR, relapse, and NVR (Table 1). In this connection, ISDR heterogeneity at a cutoff point of one mutation (ISDR ≥1 vs. ISDR = 0) was also not significantly associated with treatment outcome (data not shown).

Correlation between core mutations and treatment responses

Examination of the possible correlation of either arginine at position 70 (Arg^{70}) or leucine at position 91 (Leu^{91}) of the core protein of HCV with treatment responses [15] revealed that among 51 patients infected with HCV core aa 70 wild (Arg^{70}), SVR was achieved by 59 % (30/51), relapse was shown by 24 % (12/51), and NVR was shown by 18 % (9/51). By contrast, among 24 patients infected with HCV core aa 70 mutant (non-Arg^{70}), SVR was achieved by 29 % (7/24), relapse was shown by 33 % (8/24), and NVR was shown by 38 % (9/24). There was a significant difference in the proportion of HCV core aa 70 wild and mutant between SVR and non-SVR patients ($p = 0.025$), and between response and NVR patients ($p = 0.083$). No significant correlation was observed between HCV core aa 91 heterogeneity and virological responses (Table 1).

Correlation between the genetic variation near the IL28B gene and treatment responses

The frequency of allele rs8099917 among the patients was 76 % for TT (57/75), 4 % for TG (3/75), and 20 % for GG (15/75). Univariate analysis revealed that among patients with genotype TT (IL28B major), SVR was achieved by 60 % (34/57), relapse was shown by 28 % (16/57), and NVR was shown by 12 % (7/57). By contrast, among patients with TG or GG (IL28B minor), SVR was achieved by 17 % (3/18), relapse was shown by 22 % (4/18), and NVR was shown by 61 % (11/18). There was a significant

difference in the proportion of IL28B major and minor between SVR and non-SVR patients ($p = 0.0024$), and between response and NVR patients ($p = 0.0000095$) (Table 1).

Identification of independent predictive factors for SVR, relapse, and NVR by multivariate logistic regression analysis

Factors significantly associated with certain virological responses were identified by multivariate analysis: IRRDR ≥6 [odds ratio (OR) 11.906, $p < 0.0001$] and age <60 years (OR 0.228, $p = 0.015$) were significantly associated with SVR; IRRDR ≤5 (OR 0.070, $p = 0.0008$) and age ≥60 years (OR 5.825, $p = 0.015$) with relapse; and IL28B minor (OR 14.618, $p = 0.0019$) and platelets <15 × 10^4/mm^3 (OR 0.113, $p = 0.0096$) with NVR (Table 2).

Positive predictive values of combinations of IRRDR mutation and age for SVR

As stated above, IRRDR ≥6 predicted SVR with a positive value of 80 % (24/30) (Table 1). Assessment of the predictability of SVR by combinations of IRRDR mutation and age, the two most potent factors identified by multivariate analysis, revealed that IRRDR ≥6 and age <60 years predicted SVR with a positive value of 93.3 % (14/15) and that IRRDR ≤5 and age ≥60 years predicted non-SVR with a value of 84.0 % (21/25) (Table 3).

Positive predictive values of combinations of IL28B and platelets for NVR

Based on their significant correlation with NVR as demonstrated by multivariate analysis, combinations of IL28B genotype and platelets were examined for their positive predictive values for NVR. IL28B minor and platelets <15 × 10^4/mm^3 predicted NVR with a positive value of 85.7 % (6/7). On the other hand, IL28B major and platelets ≥15 × 10^4/mm^3 predicted viral disappearance either transiently (relapse) or sustainably (SVR), referred to as response, with a value of 97.1 % (34/35) (Table 4).

Positive predictive values of combinations of IRRDR mutation and IL28B for SVR and non-NVR (response)

Significant correlation was observed between IRRDR and IL28B ($p = 0.003768$) (data not shown). The combination of IRRDR ≥6 and IL28B major predicted SVR with a positive value of 82.1 % (23/28), and predicted non-NVR (response) with a value of 92.9 % (26/28). On the other hand, IRRDR ≤5 and IL28B minor predicted non-SVR with a value of 87.5 % (14/16) (Table 5).

Positive predictive values of combinations of IRRDR mutation and HCV core aa 70 for SVR and non-NVR (response)

Combinations of IRRDR ≥6 and HCV core aa 70 wild predicted SVR with a positive value of 82.6 % (19/23), and predicted non-NVR (response) with a value of 91.3 %

Table 2 Multivariate analysis of factors associated with SVR, relapse, and NVR

Factor	Category	SVR		Relapse		NVR	
		Odds ratio (95 % CI)	*p* value	Odds ratio (95 % CI)	*p* value	Odds ratio (95 % CI)	*p* value
IRRDR mutations	≤5	1	**<0.0001**	1	**0.0008**	NA	NA
	≥6	11.906 (3.421–41.440)		0.070 (0.015–0.331)			
Age (years)	<60	1	**0.015**	1	**0.015**	NA	NA
	≥60	0.228 (0.069–0.749)		5.825 (1.415–23.980)			
HCV core aa 70	Wild	1	0.112	NA	NA	NA	NA
	Mutant	0.358 (0.101–1.270)					
IL28B genotype	Major	NA	NA	NA	NA	1	**0.0019**
	Minor					14.618 (2.699–79.173)	
Platelets (×10^4/mm^3)	<15	NA	NA	NA	NA	1	**0.0096**
	≥15					0.113(0.022–0.588)	
γ-GTP (U/L)	<80	NA	NA	NA	NA	NA	NA
	≥80						
Hemoglobin (g/dL)	<14	NA	NA	NA	NA	1	0.105
	≥14					0.274 (0.057–1.309)	

SVR sustained virological response, *NVR* null virological response, *95 % CI* 95 % confidence interval, *γ-GTP* gamma-glutamyl transpeptidase, *IRRDR* interferon/ribavirin resistance-determining region, *NA* not applicable

Table 3 Positive predictive values of combinations of IRRDR mutation and age for SVR

IRRDR mutations	Age (years)	SVR	Non-SVR	Odds ratio (95 % CI)	*p* value
≥6	**<60**	**14/15 (93.3 %)**	1/15 (6.7 %)	73.481 (7.418–727.850)	0.0002
≥6	≥60	10/15 (66.7 %)	5/15 (33.3 %)	10.500 (2.308–47.777)	0.0024
≤5	<60	9/20 (45.0 %)	11/20 (55.0 %)	4.295 (1.075–17.167)	0.0392
≤5	**≥60**	4/25 (16.0 %)	**21/25 (84.0 %)**	1	–

SVR sustained virological response, *IRRDR* interferon/ribavirin resistance-determining region, *95 % CI* 95 % confidence interval

Table 4 Positive predictive values of combinations of IL28B genotype and baseline platelets for NVR

IL28B genotype	Platelets ($\times 10^4$/mm^3)	NVR	Response	Odds ratio (95 % CI)	*p* value
Major	**≥15**	1/35 (2.8 %)	**34/35 (97.1 %)**	1	–
Major	<15	6/22 (27.3 %)	16/22 (72.7 %)	12.750 (1.414 to 114.931)	0.023
Minor	≥15	5/11 (45.5 %)	6/11 (54.5 %)	28.333 (2.796 to 287.103)	0.0047
Minor	**<15**	**6/7 (85.7 %)**	1/7 (14.3 %)	203.999 (11.174 to >999.999)	0.0003

NVR null virological response, *Response* non-NVR (SVR *plus* relapse), *95 % CI* 95 % confidence interval

(21/23). On the other hand, IRRDR ≤5 and HCV core aa 70 mutant predicted non-SVR with a value of 88.2 % (15/17) (Table 6).

Discussion

Host factors (such as age, sex, ethnicity, platelets, liver fibrosis, and obesity) and viral factors (genotype and viral load) have been associated with the outcome of PEG-IFN/RBV therapy [6]. To date, few studies have compared the impact of viral genetic polymorphisms, such as IRRDR, ISDR, and core mutations, and IL28B polymorphisms as host genetic factors on the clinical outcome of PEG-IFN/RBV therapy. Recently, viral genetic polymorphisms including double-wild in the core region, IRRDR ≥6, and ISDR ≥2 have been described as significant predictors of SVR to PEG-IFN/RBV therapy for 48 weeks [13, 19]. IL28B major genotype (TT) and core aa 70 substitutions are independent predictors of SVR, and IL28B minor genotype is an independent predictor of NVR [20]. Also, IL28B polymorphisms and mutations in the ISDR of HCV are significant pretreatment predictors of response to PEG-IFN/RBV therapy [21]. Nonetheless, IRRDR polymorphism, which is a potent viral determiner of SVR [11–13], was not examined in these studies.

In the present study we compared the impact of IRRDR, ISDR, and core mutations as viral genetic polymorphisms, and IL28B genotype as a host genetic factor, on the clinical outcome of PEG-IFN/RBV therapy—SVR, relapse, and NVR—for CHC-1b with a high viral load. IRRDR ≥6 was identified as a viral genetic polymorphism that independently predicted SVR to PEG-IFN/RBV treatment (Tables 1, 2). Moreover, IRRDR ≤5 was identified as a viral genetic polymorphism that most effectively predicted relapse, and IL28B minor genotype (TG or GG) was identified as a host genetic factor that most effectively predicted NVR.

On the other hand, ISDR ≥2 was not significantly associated with treatment outcome in the present cohort, although it is considered a viral determiner of SVR [19, 21]. ISDR was identified as a factor showing significant correlation with RVR ($p = 0.028$) by univariate analysis (data not shown). In other words, ISDR was a factor related to only early viral dynamics.

The C-terminal region of NS5A such as IRRDR is among the most variable sequences across the different genotypes and subtypes of HCV [22, 23]. The correlation observed between IRRDR heterogeneity and PEG-IFN/RBV responsiveness might be linked to experimental observations that an HCV subgenomic RNA replicon containing NS5A of HCV-1b exerts more profound inhibitory effects on IFN activities than does its original HCV-2a replicon, and that domain swapping of a C-terminal region of NS5A including IRRDR results in a transfer of their anti-IFN activities [24]. Moreover, the C-terminal region of NS5A has been implicated as playing important roles in viral replication and particle formation [25, 26]. These clinical and experimental data thus support our hypothesis that IRRDR is involved, at least partly, in the viral strategy of evading IFN-mediated antiviral host defense mechanisms. Similarly, the aa substitutions in the core region are associated with proteins involved in resistance to IFN monotherapy, such as SOCS, which are

Table 5 Positive predictive values of combinations of IRRDR mutation and IL28B genotype for SVR and non-NVR (response)

IRRDR mutations	IL28B genotype	SVR	Non-SVR	SVR versus non-SVR		NVR	Response	NVR versus response	
				Odds ratio (95 % CI)	*p* value			Odds ratio (95 % CI)	*p* value
≥6	**Major**	**23/28 (82.1 %)**	5/28 (17.9 %)	32.200 (5.489–188.909)	0.0001	2/28 (7.1 %)	**26/28 (92.9 %)**	1	–
≥6	Minor	1/2 (50.0 %)	1/2 (50.0 %)	7.000 (0.302–162.202)	0.225	1/2 (50.0 %)	1/2 (50.0 %)	13.000 (0.572–295.204)	0.107
≤5	Major	11/29 (37.9 %)	18/29 (62.1 %)	4.278 (0.813–22.513)	0.0863	5/29 (17.2 %)	24/29 (82.8 %)	2.708 (0.480–15.294)	0.259
≤5	Minor	2/16 (12.5 %)	**14/16 (87.5 %)**	1	–	10/16 (62.5 %)	6/16 (37.5 %)	21.667 (3.733–125.766)	0.0006

SVR sustained virological response, *NVR* null virological response, *Response* non-NVR (SVR *plus* relapse), *IRRDR* interferon/ribavirin resistance-determining region, *95 % CI* 95 % confidence interval

Table 6 Positive predictive values of combinations of IRRDR mutation and HCV core aa 70 for SVR and non-NVR (response)

IRRDR mutations	HCV core aa 70	SVR	Non-SVR	SVR versus non-SVR		NVR	Response	NVR versus response	
				Odds ratio (95 % CI)	*p* value			Odds ratio (95 % CI)	*p* value
≥6	**Wild**	**19/23 (82.6 %)**	4/23 (17.4 %)	35.625 (5.730–221.504)	0.0001	2/23 (8.7 %)	**21/23 (91.3 %)**	1	–
≥6	Mutant	5/7 (71.4 %)	2/7 (28.6 %)	18.750 (2.065–170.214)	0.0092	1/7 (14.3 %)	6/7 (85.7 %)	1.750 (0.134–22.778)	0.669
≤5	Wild	11/28 (39.3 %)	17/28 (60.7 %)	4.853 (0.924–25.496)	0.062	7/28 (25.0 %)	21/28 (75.0 %)	3.500 (0.650–18.852)	0.145
≤5	**Mutant**	2/17 (11.8 %)	**15/17 (88.2 %)**	1	–	8/17 (47.1 %)	9/17 (52.9 %)	9.333 (1.6346–52.917)	0.012

SVR sustained virological response, *NVR* null virological response, *Response* non-NVR (SVR *plus* relapse), *IRRDR* interferon/ribavirin resistance-determining region, *95 % CI* 95 % confidence interval

known to inhibit IFN-α-induced activation of the Jak-STAT pathway and the expression of the antiviral proteins 2′,5′-OAS and MxA [27].

The IL28B gene encodes a cytokine distantly related to type I (α and β) IFN and to the IL10 family. IL28B, IL28A, and IL29 are three closely related cytokine genes that encode proteins known as type III IFN (IFN-λs) and form a cytokine gene cluster at chromosomal region 19q13 [28]. The three cytokines IFN-λ1, -λ2, and -λ3 are induced by viral infection and have antiviral activities [29, 30]: IFN-λ induces a steady increase in the expression of a subset of IFN-stimulated genes, whereas IFN-α induces the same genes with more rapid and transient kinetics [31].

In the present study, the prediction of response to PEG-IFN/RBV combination therapy based on these concurrent factors was highly positive: SVR was positively predicted in 93.3 % of patients with IRRDR $\geq$6 and age <60 years (Table 3), in 82.1 % of those with IRRDR $\geq$6 and IL28B major (Table 5), and in 82.6 % of those with IRRDR $\geq$6 and HCV core aa 70 wild (Table 6). Relapse was positively predicted in 73.3 % of patients with IRRDR $\leq$5 and age $\geq$60 years, and in 77.8 % of those with IRRDR $\leq$5 and HCV core aa 70 mutant (data not shown). NVR was positively predicted in 85.7 % of patients with IL28B minor and platelets $<15 \times 10^4$/mm^3 (Table 4). On the basis of these observations, new therapeutic strategies could be designed for treating chronic HCV-1b infection: patients predicted to achieve an SVR would be most eligible for standard PEG-IFN/RBV therapy for 48 weeks, those predicted to relapse could be advised to adopt an extended 72-week therapy instead of the 48-week standard therapy [30], and those predicted to have NVR could be advised to wait for a future therapy such as a combination of protease inhibitors [32, 33].

In conclusion, viral genetic polymorphisms in IRRDR ($\geq$6 or $\leq$5 mutations) and HCV core aa 70 (wild or mutant), host factors such as IL28B genotype (major or minor), age (<60 or $\geq$60 years), and platelet counts ($\geq 15 \times 10^4$/mm^3 or less), and combinations of these factors could be used to design therapeutic strategies for patients infected with HCV-1b with high viral loads. Further prospective study is needed to verify this hypothesis.

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Conflict of interest None of the authors has any conflict of interest.

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Serum metabolomics reveals γ-glutamyl dipeptides as biomarkers for discrimination among different forms of liver disease

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Background & Aims: We applied a metabolome profiling approach to serum samples obtained from patients with different liver diseases, to discover noninvasive and reliable biomarkers for rapid-screening diagnosis of liver diseases.

Methods: Using capillary electrophoresis and liquid chromatography mass spectrometry, we analyzed low molecular weight metabolites in a total of 248 serum samples obtained from patients with nine types of liver disease and healthy controls.

Results: We found that γ-glutamyl dipeptides, which were biosynthesized through a reaction with γ-glutamylcysteine synthetase, were indicative of the production of reduced glutathione, and that measurement of their levels could distinguish among different liver diseases. Multiple logistic regression models facilitated the discrimination between specific and other liver diseases and yielded high areas under receiver-operating characteristic curves. The area under the curve values in training and independent validation data were 0.952 and 0.967 in healthy controls, 0.817 and 0.849 in drug-induced liver injury, 0.754 and 0.763 in asymptomatic hepatitis B virus infection, 0.820 and 0.762 in chronic hepatitis B, 0.972 and 0.895 in hepatitis C with persistently normal alanine transaminase, 0.917 and 0.707 in chronic hepatitis C, 0.803 and 0.993 in cirrhosis type C, and 0.762 and 0.803 in hepatocellular carcinoma, respectively. Several γ-glutamyl dipeptides also manifested potential for differentiating between nonalcoholic steatohepatitis and simple steatosis.

Conclusions: γ-Glutamyl dipeptides are novel biomarkers for liver diseases, and varying levels of individual or groups of these peptides have the power to discriminate among different forms of hepatic disease.

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Introduction

Acute or chronic viral hepatitis affects populations around the world, and the disease often progresses from chronic hepatitis and cirrhosis to hepatocellular carcinoma (HCC) [1]. Accurate diagnosis at earlier stages is necessary for improved therapeutic outcome. However, the diagnostic procedures are laborious and not risk-free. Patients with suspected liver damage are initially subjected to liver function tests that include the assessment of aspartate transaminase (AST), alanine transaminase (ALT), and γ-glutamyl transpeptidase (γ-GTP) serum levels. If these levels are abnormal, patients are then subjected to diagnostic imaging, such as ultrasound and computed tomography (CT), and assays to determine the presence of antibodies against hepatitis virus. Finally, a liver biopsy may be recommended to evaluate the severity of inflammation or fibrosis and to confirm the indications for antiviral therapy.

Recently, nonalcoholic fatty liver disease (NAFLD) has become the most common liver disease in western countries. It

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