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

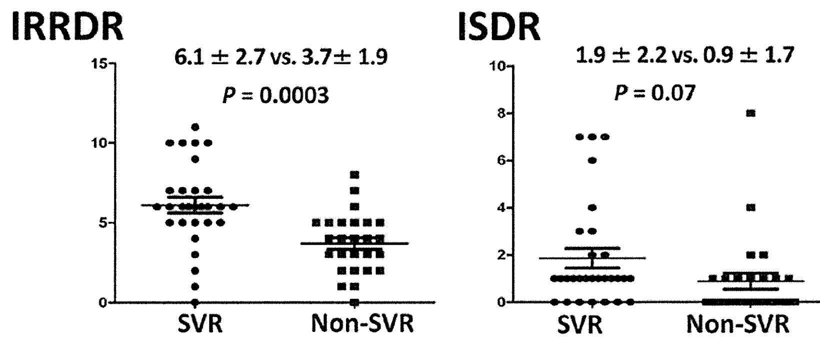

Figure 1. The number of mutations in IRRDR and ISDR. The number of mutations in IRRDR and ISDR was higher in the SVR group than in the non-SVR group.

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 $\geq$11 g/dL (women) or $\geq$12 g/dL (men), platelet counts of $\geq 9 \times 10^4/\mathrm{mm}^3$, HCV RNA $\geq$5.0 Log IU/mL, neutrophil count $\geq 1500/\mathrm{mm}^3$, 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 $\geq$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 $\geq$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 $\geq$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 $\leq$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 $\geq$6 and those involving IRRDR $\leq$5 between SVR and non-SVR patients ($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 $\geq$6, 24 (80 %) achieved SVR, suggesting that IRRDR $\geq$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 $\geq$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 $\leq$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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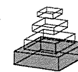

Molecular mechanism of hepatitis C virus-induced glucose metabolic disorders

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Hepatitis C virus (HCV) infection causes not only intrahepatic diseases but also extrahepatic manifestations, including metabolic disorders. Chronic HCV infection is often associated with type 2 diabetes. However, the precise mechanism underlying this association is still unclear. Glucose is transported into hepatocytes via glucose transporter 2 (GLUT2). Hepatocytes play a crucial role in maintaining plasma glucose homeostasis via the gluconeogenic and glycolytic pathways. We have been investigating the molecular mechanism of HCV-related type 2 diabetes using HCV RNA replicon cells and HCV J6/JFH1 system. We found that HCV replication down-regulates cell surface expression of GLUT2 at the transcriptional level. We also found that HCV infection promotes hepatic gluconeogenesis in HCV J6/JFH1-infected Huh-7.5 cells. HCV infection transcriptionally up-regulated the genes for phosphoenolpyruvate carboxykinase (PEPCK) and glucose 6-phosphatase (G6Pase), the rate-limiting enzymes for hepatic gluconeogenesis. Gene expression of PEPCK and G6Pase was regulated by the transcription factor forkhead box O1 (FoxO1) in HCV-infected cells. Phosphorylation of FoxO1 at Ser319 was markedly diminished in HCV-infected cells, resulting in increased nuclear accumulation of FoxO1. HCV NS5A protein was directly linked with the FoxO1-dependent increased gluconeogenesis. This paper will discuss the current model of HCV-induced glucose metabolic disorders.

Keywords: HCV, diabetes, gluconeogenesis, GLUT2, FoxO1, JNK, NS5A

INTRODUCTION

Hepatitis C virus (HCV) is a positive-sense, single stranded RNA virus that belongs to the genus *Hepacivirus* of the family *Flaviviridae*. The approximately 9.6-kb HCV genome encodes a unique open reading frame that is translated into a polyprotein of about 3,000 amino acids, which is cleaved by cellular signalases and viral proteases to generate at least 10 viral proteins, such as core, envelope 1 (E1) and E2, p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B (Choo et al., 1991; Lemon et al., 2007).

Hepatitis C virus is the main cause of chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma. More than 170 million people worldwide are chronically infected with HCV (Poynard et al., 2003). Persistent HCV infection causes not only liver diseases but also extrahepatic manifestations. It is well established that HCV perturbs the glucose metabolism, leading to insulin resistance and type 2 diabetes in predisposed individuals. Several epidemiological, clinical, and experimental data suggested that HCV infection serves as an additional risk factor for the development of diabetes (Mason et al., 1999; Negro and Alaei, 2009; Negro, 2011). HCV-related glucose metabolic changes and insulin resistance and diabetes have significant clinical consequences, such as accelerated fibrogenesis, increased incidence of hepatocellular carcinoma, and reduced virological response to interferon (IFN)-α-based therapy (Negro, 2011). Therefore, it is very important to clarify the molecular mechanism of HCV-related diabetes. However, the precise mechanisms are poorly understood.

Experimental data suggest a direct interference of HCV with the insulin signaling pathway. Transgenic mice expressing HCV core gene exhibit insulin resistance (Shintani et al., 2004; Koike, 2007). In this transgenic mice model, both tyrosine phosphorylation of the insulin receptor substrate (IRS)-1 and IRS-2 are decreased. These decreases are recovered when the proteasome activator PA28γ is deleted, suggesting that the HCV core protein suppresses insulin signaling through a PA28γ-dependent pathway (Miyamoto et al., 2007). Several other reports also showed a link of the HCV core protein with insulin resistance (Kawaguchi et al., 2004; Pazienza et al., 2007).

Hepatocytes play a crucial role in maintaining plasma glucose homeostasis by adjusting the balance between hepatic glucose production and utilization via the gluconeogenic and glycolytic pathways, respectively. Gluconeogenesis is mainly regulated at the transcriptional level of the glucose 6-phosphatase (G6Pase) and phosphoenolpyruvate carboxykinase (PEPCK) genes, whereas glycolysis is mainly regulated by glucokinase (GK). Gluconeogenesis and glycolysis are coordinated so that one pathway is highly active within a cell while the other is relatively inactive. It is well known that increased hepatic glucose production via gluconeogenesis is a major feature of type 2 diabetes (Clore et al., 2000).

To identify a novel mechanism of HCV-related diabetes, we have been investigating the effects of HCV on glucose production in hepatocytes using HCV RNA replicon cells (Lohmann et al., 1999) and HCV J6/JFH1 cell culture system (Lindenbach et al., 2005; Wakita et al., 2005; Bungyoku et al., 2009). We previously reported that HCV replication suppresses cellular glucose uptake through down-regulation of cell surface expression of glucose transporter 2 (GLUT2; Kasai et al., 2009). Furthermore, we

recently reported that HCV promotes hepatic gluconeogenesis via an NS5A-mediated, forkhead box O1 (FoxO1)-dependent pathway, resulting in increased cellular glucose production in hepatocytes (Deng et al., 2011). This paper discusses our current model for HCV-induced glucose metabolic disorders.

HCV REPLICATION DOWN-REGULATES CELL SURFACE EXPRESSION OF GLUT2

The uptake of glucose into cells is conducted by the facilitative glucose carrier, glucose transporters (GLUTs). GLUTs are integral membrane proteins that contain 12 membrane-spanning helices. To date, a total of 14 isoforms have been identified in the GLUT family (Wu and Freeze, 2002; Macheda et al., 2005; Godoy et al., 2006). Glucose is transported into hepatocytes by GLUT2. We previously reported that HCV J6/JFH1 infection suppresses hepatocytic glucose uptake through down-regulation of surface expression of GLUT2 in human hepatoma cell line, Huh-7.5 cells (Kasai et al., 2009). We also demonstrated that GLUT2 expression in hepatocytes of the liver tissues from HCV-infected patients was significantly lower than in those from patients without HCV infection. Our data suggest that HCV infection down-regulates GLUT2 expression at transcriptional level. We are currently analyzing transcriptional control of human GLUT2 promoter in HCV replicon cells as well as in HCV J6/JFH1-infected cells.

HCV INFECTION PROMOTES HEPATIC GLUCONEOGENESIS

Then we analyzed hepatic glucose production and expression of transcription factors using HCV replicon cells and HCVcc system in order to clarify a role of HCV infection in glucose metabolic changes. Hepatic glucose production is usually regulated by gluconeogenesis and glycolysis. Therefore, we examined whether HCV infection induces gluconeogenesis or glycolysis. We found that the PEPCK and G6Pase genes were transcriptionally up-regulated in J6/JFH1-infected cells (**Figure 1**). On the other hand, the GK gene was transcriptionally down-regulated in HCV-infected cells. We obtained similar data in HCV replicon cells (both in subgenomic replicon cells and full-genomic replicon cells). When HCV replication was suppressed by IFN treatment, the up-regulation of PEPCK and G6Pase gene expression as well as the down-regulation of GK gene expression were canceled. From these results, HCV infection selectively up-regulates PEPCK and G6Pase genes, whereas HCV infection down-regulates GK gene (Deng et al., 2011).

Both HCV replicon cells and HCV-infected cells produced greater amounts of glucose than the control cells. IFN treatment canceled the enhanced glucose production in HCV replicon cells as well as in HCV-infected cells. G6P is an important precursor molecule that is converted to glucose in the gluconeogenesis pathway (**Figure 1**). Our metabolite analysis showed that a significantly higher level of G6P was accumulated in HCV-infected cells than in the control cells, suggesting that HCV indeed promotes hepatic gluconeogenesis to cause hyperglycemia. There is a trend toward an increase in gluconeogenesis in HCV-infected cells (**Figure 1**).

HCV SUPPRESSES FoxO1 PHOSPHORYLATION AT Ser319, LEADING TO THE NUCLEAR ACCUMULATION OF FoxO1

It has been reported that G6Pase, PEPCK, and GK are regulated by certain transcription factors, including FoxO1 (Hirota et al., 2008), hepatic nuclear factor 4α (HNF-4α; Hirota et al.,

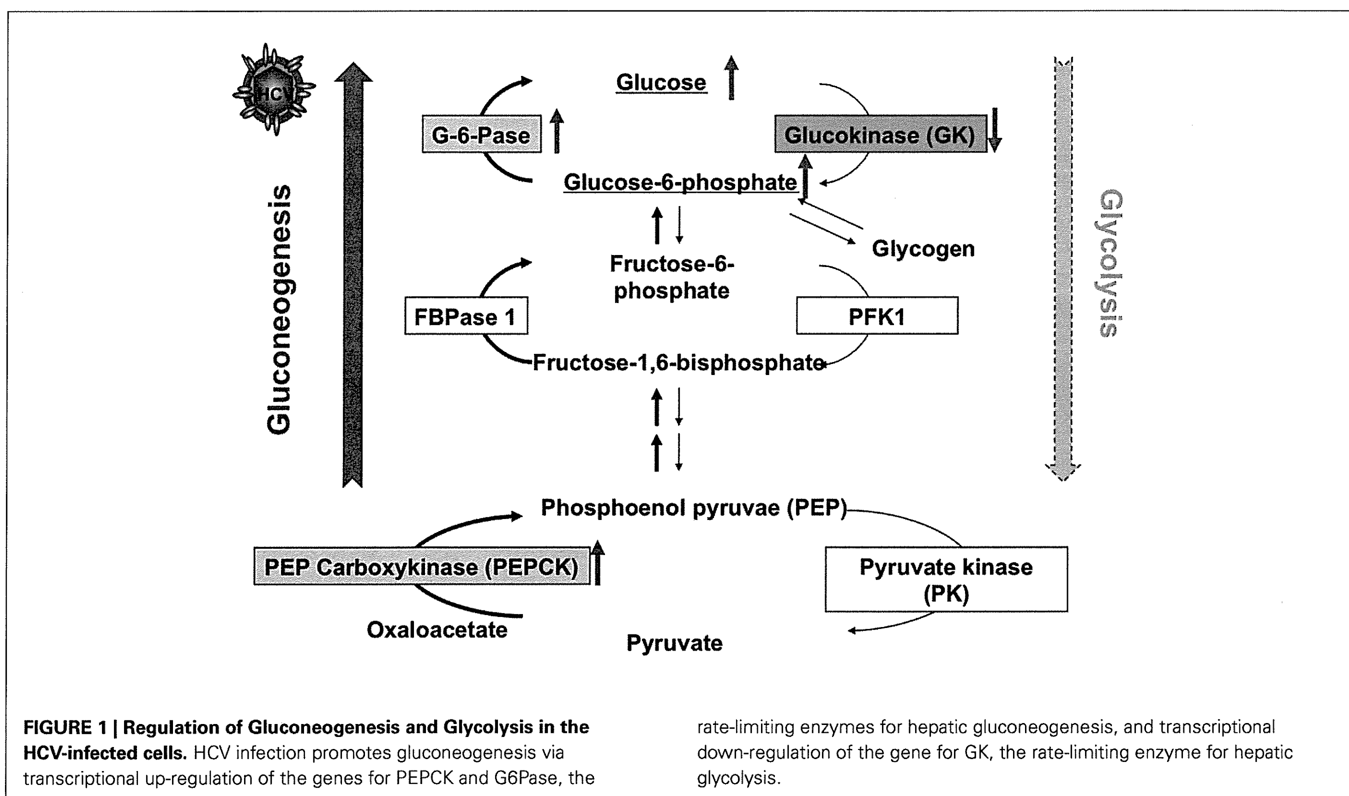

FIGURE 1 | Regulation of Gluconeogenesis and Glycolysis in the HCV-infected cells. HCV infection promotes gluconeogenesis via transcriptional up-regulation of the genes for PEPCK and G6Pase, the rate-limiting enzymes for hepatic gluconeogenesis, and transcriptional down-regulation of the gene for GK, the rate-limiting enzyme for hepatic glycolysis.

2008), Krüppel-like factor 15 (KLF15; Takashima et al., 2010), and cyclic AMP (cAMP) response element binding protein (CREB; Rozance et al., 2008). While we were analyzing these factors in both HCV replicon cells and HCV J6/JFH1-infected cells, we found the involvement of the FoxO1 in the transcriptional activation of G6Pase and PEPCK (Deng et al., 2011). It is known that the FoxO1 enhances gluconeogenesis through the transcriptional activation of various genes, including G6Pase and PEPCK (Gross et al., 2008). The function of FoxO1 is regulated by post-translational modifications, including phosphorylation, ubiquitylation, and acetylation (Tzivion et al., 2011). The phosphorylated form of FoxO1 is exported from the nucleus to the cytosol, resulting in loss of its transcriptional activity (**Figure 2**). Phosphorylation status of FoxO1 at Ser319 is critical for FoxO1 nuclear exclusion (Zhao et al., 2004). Although the total amounts of FoxO1 protein were unchanged, FoxO1 phosphorylation at Ser319 was markedly suppressed in HCV-infected cells compared to that in the mock-infected cells. It is known that the FoxO1 is phosphorylated by the protein kinase Akt and is exported from the nucleus to the cytosol, resulting in loss of its transcriptional activity (Tzivion et al., 2011). The majority of FoxO1 was accumulated in the nuclear fraction in HCV-infected cells, whereas in control cells FoxO1 was distributed in both the nuclear and cytoplasmic fractions. Akt phosphorylation was enhanced in HCV-infected cells, although the protein levels of total Akt protein were comparable, which is consistent with the report by Burdette et al. (2010). Our findings suggest an interesting scenario in which the HCV-mediated suppression in FoxO1 phosphorylation is caused by an unknown mechanism independent of Akt activity.

HCV-INDUCED JNK ACTIVATION IS INVOLVED IN THE SUPPRESSION OF FoxO1 PHOSPHORYLATION

It is known that the stress-sensitive serine/threonine kinase JNK regulates FoxO at multiple levels (van der Horst and Burgering, 2007; Karpac and Jasper, 2009). We demonstrated that HCV infection induces phosphorylation and activation of JNK in a time-dependent manner, which is similar to that observed for the suppression of FoxO1 phosphorylation. As a result, c-Jun, a key substrate for JNK, got phosphorylated and activated in HCV-infected cells. The JNK inhibitor SP600125 clearly prevented the phosphorylation of c-Jun, and concomitantly recovered the suppression of FoxO1 phosphorylation in HCV-infected cells, suggesting that HCV activates the JNK/c-Jun signaling pathway, resulting in the nuclear accumulation of FoxO1 by reducing its phosphorylation status. The detailed mechanisms of HCV-induced suppression of FoxO1 phosphorylation via the JNK/c-Jun signaling pathway remain to be explored. There are at least two possibilities. The JNK/c-Jun signaling pathway (1) suppresses a protein kinase, or (2) activates a protein phosphatase to reduce phosphorylation of FoxO1.

HCV-INDUCED MITOCHONDRIAL REACTIVE OXYGEN SPECIES PRODUCTION IS INVOLVED IN INCREASED GLUCOSE PRODUCTION THROUGH JNK ACTIVATION

Hepatitis C virus infection increases mitochondrial reactive oxygen species (ROS) production (Deng et al., 2008). *N*-acetyl cysteine (NAC; a general antioxidant) clearly prevented the phosphorylation of JNK, and concomitantly canceled the suppression of FoxO1 phosphorylation in HCV-infected cells, suggesting that

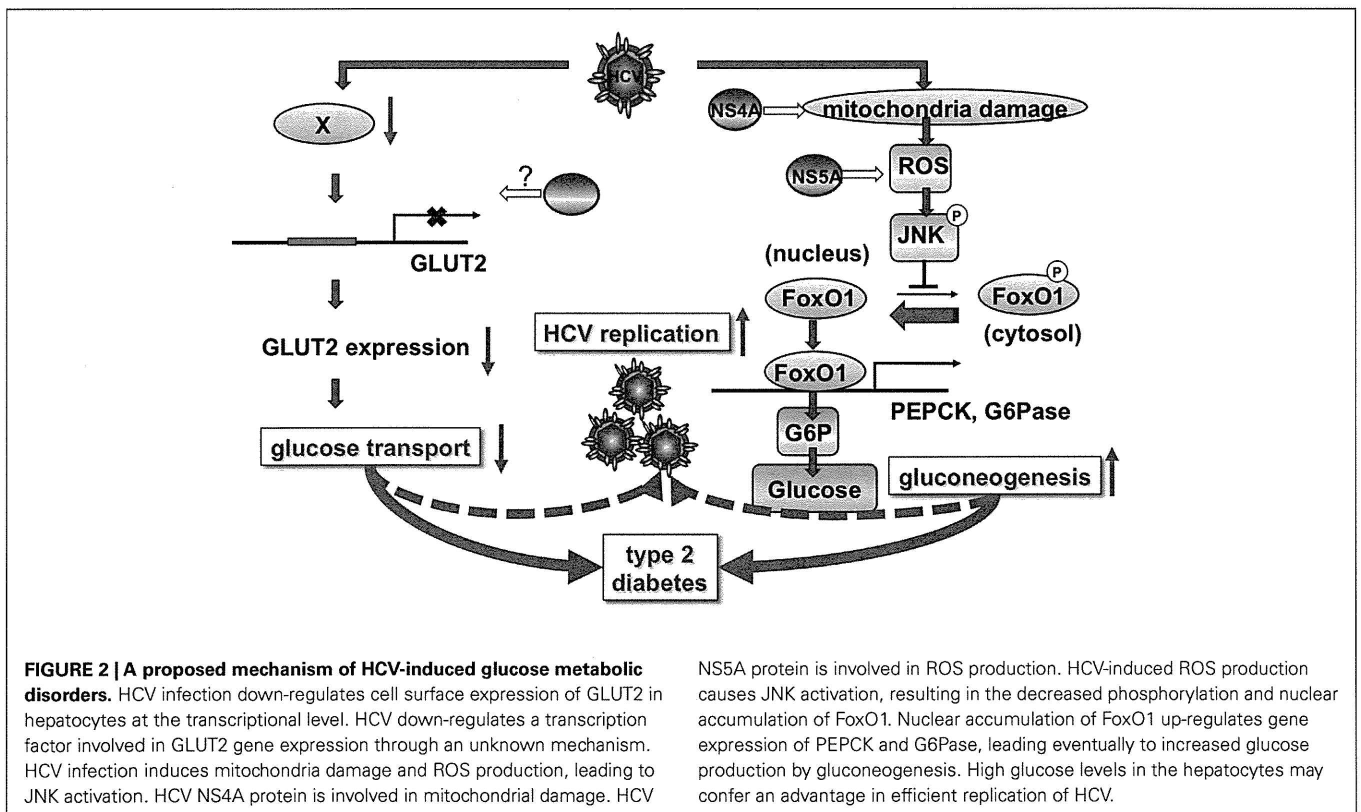

FIGURE 2 | A proposed mechanism of HCV-induced glucose metabolic disorders. HCV infection down-regulates cell surface expression of GLUT2 in hepatocytes at the transcriptional level. HCV down-regulates a transcription factor involved in GLUT2 gene expression through an unknown mechanism. HCV infection induces mitochondria damage and ROS production, leading to JNK activation. HCV NS4A protein is involved in mitochondrial damage. HCV NS5A protein is involved in ROS production. HCV-induced ROS production causes JNK activation, resulting in the decreased phosphorylation and nuclear accumulation of FoxO1. Nuclear accumulation of FoxO1 up-regulates gene expression of PEPCK and G6Pase, leading eventually to increased glucose production by gluconeogenesis. High glucose levels in the hepatocytes may confer an advantage in efficient replication of HCV.

HCV-induced ROS production is involved in the JNK activation. There was no significant difference in HCV RNA replication or infectious virus release between SP600125- or NAC-treated HCV-infected cells and non-treated HCV-infected cells. These results suggest that ROS-mediated JNK activation plays a key role in the suppression of FoxO1 phosphorylation, nuclear accumulation of FoxO1, and enhancement of glucose production in HCV-infected cells (Deng et al., 2011).

HCV NS5A IS INVOLVED IN THE ENHANCEMENT OF GLUCOSE PRODUCTION

Then we sought to determine which HCV protein(s) is involved in the enhancement of glucose production. Transient expression of NS5A protein in Huh-7.5 cells significantly promoted the gene expression levels of G6Pase and PEPCK determined by real time quantitative RT-PCR. Promoter assay revealed that the level of PEPCK promoter activity was significantly higher in NS5A-expressing cells than in the control cells. Our results suggest that NS5A activate both the PEPCK promoter and the G6Pase promoter, leading to an increase in glucose production (Deng et al., 2011). The study by Banerjee et al. (2010) suggests that the HCV core protein modulates FoxO1 and FoxA2 activation and affects insulin-induced metabolic gene regulation in human hepatocytes. Our results, however, suggest that the HCV core protein is not significantly involved in the increased gluconeogenesis (Deng et al., 2011). The difference between these two studies needs to be explored.

There were previous reports suggesting that ROS production is induced in NS5A-expressing cells (Dionisio et al., 2009) or in hepatocytes of NS5A transgenic mice (Wang et al., 2009). We therefore sought to determine whether NS5A contributes to increased hepatic gluconeogenesis through the induction of ROS production. NS5A-expressing cells displayed a much stronger signal of ROS than in control cells. NS5A-expressing cells promoted phosphorylation level at Ser63 of c-Jun and suppressed FoxO1 phosphorylation at Ser319, suggesting that NS5A mediates JNK/c-Jun activation and FoxO1 phosphorylation suppression. These results suggest that NS5A play a role in the HCV-induced enhancement of hepatic gluconeogenesis through JNK/c-Jun activation and FoxO1 phosphorylation suppression.

CONCLUSION AND FUTURE PERSPECTIVES

Taken together, we propose a model of HCV-induced glucose metabolic disorders as shown in **Figure 2.** HCV infection down-regulates cell surface expression of GLUT2 in hepatocytes at the transcriptional level. HCV down-regulates a transcription factor involved in GLUT2 gene expression through an unknown mechanism. As GLUT2 is a facilitative GLUT, it ensures large bidirectional fluxes of glucose in and out the cell due to its low affinity and high capacity (Leturque et al., 2009). Down-regulated cell surface expression of GLUT2 results in disruption of bidirectional transport of glucose in hepatocytes. Even in the fasting state, down-regulation of GLUT2 may result in low glucose uptake of hepatocytes, causing hyperglycemia. In the fed state, glucose secretion from hepatocytes may be suppressed due to low level cell surface expression of GLUT2, as GLUT2 is a bidirectional transporter.

Hepatitis C virus infection induces mitochondria damage and ROS production, leading to JNK activation. HCV NS4A protein is involved in mitochondrial damage (Nomura-Takigawa et al., 2006). HCV NS5A protein is involved in ROS production (Dionisio et al., 2009; Wang et al., 2009; Deng et al., 2011). HCV-induced ROS production causes JNK activation, which results in the decreased phosphorylation and nuclear accumulation of FoxO1 by an unidentified mechanism. Nuclear accumulation of FoxO1 up-regulates gene expression of PEPCK and G6Pase, leading eventually to increased glucose production by gluconeogenesis (Deng et al., 2011).

These two pathways, HCV-induced down-regulation of GLUT2 expression and up-regulation of gluconeogenesis, may contribute to development of type 2 diabetes in HCV-infected patients at least to some extent. HCV-induced down-regulation of GLUT2 expression and up-regulation of gluconeogenesis may result in high concentration of glucose in HCV-infected hepatocytes. As suggested in a recent study, low glucose concentration in the hepatocytes inhibits HCV replication (Nakashima et al., 2011). Therefore, high glucose levels in the hepatocytes may confer an advantage in efficient replication of HCV.

Our understanding of HCV-induced glucose metabolic disorders will require much more work to fully unfold this pathway. Further investigation including the mechanism of HCV-induced GLUT2 downregualtion, JNK-mediated decreased phosphorylation of FoxO1, and the possible effect(s) of the dysregulation of hepatic gluconeogenesis on the HCV life cycle and host cells are currently under way.

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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