

participated in the study. The study protocol conformed to the ethical guidelines of the Declaration of Helsinki and to the relevant ethical guidelines as reflected in a *priori* approval by the ethics committees of all the participating universities and hospitals.

Statistical analyses

We used the SPSS software package (SPSS 18J; SPSS Inc., Chicago, IL, USA) and Graph Pad Prism (version 5.0; Graph Pad Software Inc., San Diego, CA, USA) for statistical analysis. The categorical variables were compared by the χ^2 test and distributions of continuous variables were compared by the unpaired Mann-Whitney U test (non-parametric test). The serum IL-6 values before treatment and at 4 weeks after treatment were analysed by the Wilcoxon signed-rank test. All *P*-values are two-tailed and those <0.05 were considered statistically significant. A multivariate logistic regression analysis with stepwise forward selection was performed with *P*-values of <0.05 as the criteria for model inclusion.

Results

Clinical characteristics of the patients

The clinical characteristics of all patients are shown in Table 1. This study enrolled 149 patients (83 male and 66 female) and the median age of patients was 57 years (males 56 years and females 58 years). The values were significantly different between males and females as follows: BMI (23.9 versus 22.0 kg/m²; *P*=0.001), Hb (15.0 versus 13.6 g/dl; *P*<0.0001), serum ALT (74 versus 50 IU/l; *P*<0.0001) and serum γ -GTP (57 versus 31 IU/l; *P*<0.0001) were higher in males than females, whereas serum T-chol was higher in females than males (155 versus 196 mg/dl; *P*<0.0001). There was no difference related to gender in age, Plt, TG, LDL cholesterol, serum HCV RNA level, substitution at amino acids 70 and 91, liver histology (grade of inflammation and stage of fibrosis) and the rate of SVR.

Serum IL-6 levels in healthy subjects and CHC patients
Pretreatment serum IL-6 levels were significantly higher in CHC compared to healthy subjects (median [IQR]: 0.75 pg/ml [0.5–1.5] versus 3.0 pg/ml [0.6–1,500]; *P*<0.0001). We also analysed serum IL-6 levels in patients with HBV infection (*n*=9) and autoimmune liver disease (*n*=8). The IL-6 levels of CHC patients tended to be higher as compared with those of chronic hepatitis B patients (median [IQR]: 1.95 pg/ml [0.8–3.5]; *P*=0.103) and also autoimmune liver disease (median [IQR]: 2.7 pg/ml [1.1–4.6]; *P*=0.442) although the serum transaminase levels of the patients were almost equivalent among each group [median 45 IU/l; *P*=0.07; Figure 1A). On the basis of the median IL-6 level of 3 pg/ml, we divided the

patients into two groups according to IL-6 levels, those with low IL-6 (≤ 3 pg/ml) and high IL-6 (>3 pg/ml).

Association of serum IL-6 level with treatment outcomes

We next determined whether pretreatment serum IL-6 levels are associated with the outcome of PEG-IFN/RBV combination therapy. Univariate analyses identified ten clinical and laboratory parameters associated with SVR and non-SVR: age, Hb, Plt, genotype, γ -GTP, LDL cholesterol, serum HCV RNA level, histological grade of inflammation, histological stage of fibrosis and ISDR substitutions (Table 2). Serum IL-6 levels did not differ significantly between SVR and non-SVR patients (median [IQR]: 2.9 pg/ml [0.6–1,500] versus 3.3 pg/ml [0.8–601]; *P*=0.435; Figure 1B). A multivariate analysis showed that stage of fibrosis, LDL cholesterol, ISDR substitutions and HCV RNA levels were independently correlated with SVR.

We next repeated the analyses separately for male and female patients. As for male patients, univariate analyses identified six clinical and laboratory parameters associated with SVR: Plt, γ -GTP, serum HCV RNA level, histological stage of fibrosis, ISDR substitutions and serum IL-6 levels (*P*=0.012; Table 3). However, in multivariate analyses, IL-6 levels did not achieve statistical significance. Analysis of female patients did not find a significant difference in serum IL-6 levels between the SVR and non-SVR groups (Table 4).

Correlation between serum IL-6 level and various host factors

Knowing that IL-6 levels were significantly associated with the treatment outcomes in male patients, we compared clinical and laboratory parameters between patient groups with low (≤ 3 pg/ml) and high (>3 pg/ml) serum IL-6 levels (Table 5). Analyses of patients overall did not find significant parameters (data not shown), while in male patients, serum IL-6 levels were positively correlated with serum TG (≤ 3 pg/ml versus >3 pg/ml: 78 mg/dl [55–329] versus 120 mg/dl [63–449]; *P*=0.029) and negatively correlated with SVR rates (79% versus 52%; *P*=0.012). Analyses of female patients showed that serum IL-6 levels were significantly associated with γ -GTP (≤ 3 pg/ml versus >3 pg/ml: 22 IU/l [12–61] versus 38 IU/l [10–131]; *P*=0.046).

On-treatment decrease of IL-6 correlates with outcomes of PEG-IFN/RBV therapy

We next analysed serum IL-6 levels before and after 4 weeks of PEG-IFN/RBV therapy and compared them between those with SVR and non-SVR separately for male and female patients. In male patients, serum IL-6 levels decreased significantly in the first 4 weeks (pretreatment versus 4 weeks: 2.7 pg/ml [0.8–1,500] versus pg/ml

Table 1. Baseline characteristics of the participating patients

Characteristic	All (n=149)	Male (n=83)	Female (n=66)	P-value
Age, years	57 (25-72)	56 (27-72)	58 (25-72)	0.221
BMI, kg/m ²	23.2 (14.9-33.2)	23.9 (18.4-29.2)	22.0 (14.9-33.2)	0.001
Grade of inflammation				0.088
A0	8	3	3	-
A1	42	19	23	-
A2	92	58	34	-
A3	5	1	4	-
Stage of fibrosis				0.065
F0	5	3	2	-
F1	79	43	36	-
F2	35	18	17	-
F3	20	16	4	-
F4	6	1	5	-
Genotype				0.094
1	119	70	49	-
2	30	13	17	-
Previous interferon therapy				0.854
No	108	61	47	-
Yes	41	22	19	-
Haemoglobin, g/dl	14.4 (10.8-16.9)	15.0 (11.9-16.9)	13.6 (10.8-15.2)	<0.0001
Platelet count, ×10 ⁴ cells/μl	16.2 (7.2-34.4)	16.3 (7.3-33.7)	16.2 (7.2-34.4)	0.412
ALT, IU/l	67 (10-408)	74 (10-408)	50 (13-369)	<0.0001
γ-GTP, IU/l	40 (10-731)	57 (13-731)	31 (10-131)	<0.0001
T-chol, mg/dl	166 (98-265)	155 (98-210)	196 (151-265)	<0.0001
TG, mg/dl	102 (55-449)	100 (55-449)	133 (62-203)	0.463
LDL cholesterol, mg/dl	80 (34-161)	77 (28-142)	86 (34-161)	0.369
Substitutions in the ISDR				0.008
≤1	118	59	59	-
≥2	31	24	7	-
Substitutions of core amino acids 70 and 91*				0.42
dW	38	20	18	-
Not dW	77	48	29	-
HCV RNA, log IU/ml	6.2 (3.7-6.9)	6.2 (3.7-6.7)	6.1 (4.3-6.9)	0.508
Virological response				0.131
SVR	88	54	34	-
Non-SVR	61	29	32	-

Median (range) or *n* values are shown. Significant difference was detected between males and females. *Double wild (dW) represents wild type at amino acids 70 and 91, whereas not dW represents single or dual substitution of amino acid 70 and 91. ALT, alanine aminotransferase; BMI, body mass index; ISDR, interferon sensitivity determining region; LDL, low-density lipoprotein; SVR, sustained virological response; T-chol, total cholesterol; TG, triglycerides; γ-GTP, γ-glutamyl transpeptidase.

1.5 [0.8-4.8]; $P=0.029$) especially in patients with SVR (Figure 2A), whereas IL-6 did not change significantly in the first 4 weeks in female patients with SVR as well as with non-SVR (3.4 pg/ml [1.5-14.5] versus 2.3 pg/ml [1.0-91.8]; $P=0.546$; Figure 2). Among non-SVR patients, there were 7 out of 23 patients whose serum IL-6 levels were increasing despite decreasing HCV RNA loads at 4 weeks of therapy. In these cases, we speculate that IL-6 predicted the treatment effect better than HCV load.

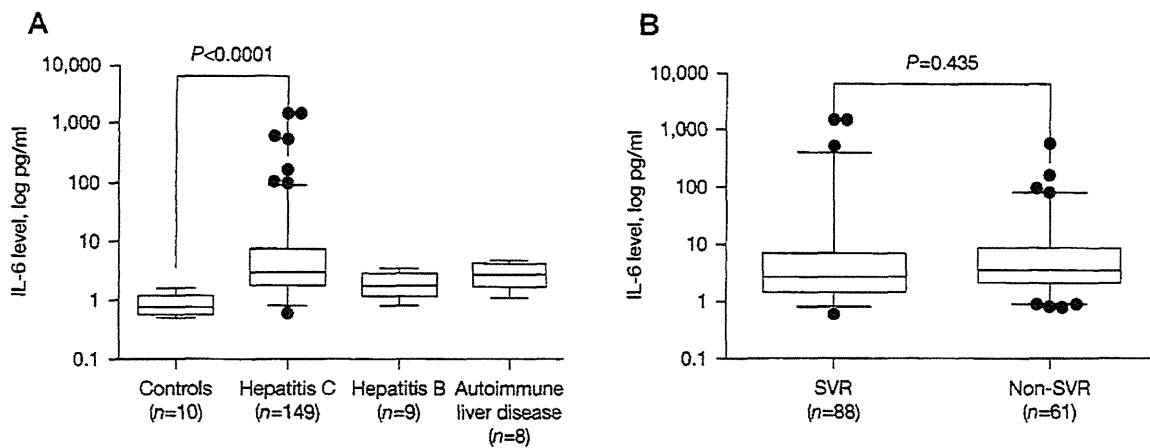
We further compared time-dependent changes of serum IL-6 levels between SVR and non-SVR patients at 8 and 12 weeks, and at the end of treatment. In male patients, on-treatment serum IL-6 levels remained significantly lower than those of non-SVR patients after

4, 8 and 12 weeks of treatment (Table 6). Among female patients, serum IL-6 levels did not differ significantly between those with SVR and non-SVR throughout the therapy.

Discussion

In this study, we confirmed that the serum IL-6 level is significantly increased in CHC patients, as previously reported [14] and demonstrated, in addition, that a baseline IL-6 level of >3 pg/ml is a good indicator of poor treatment outcomes of PEG-IFN/RBV therapy in male patients and that an early on-treatment IL-6 decrease is associated with improvement in liver

Figure 1. Pretreatment serum IL-6 levels



(A) Serum interleukin (IL)-6 levels in controls and patients with chronic hepatitis C, chronic hepatitis B and autoimmune liver disease. (B) Comparison of serum IL-6 levels according to treatment outcomes. The results are shown as box-plots. Horizontal bars indicate the median values, the outer limits of the box indicate the 25th and the 75th percentile values and whiskers above and below the boxes indicate the 5th and 95th percentile values. The values outside the ends of the whiskers are plotted individually as filled circles. Significant differences of the data were analysed using the Mann-Whitney U test. All P -values <0.05 were considered statistically significant. SVR, sustained virological response.

inflammation, decrease in HCV RNA and, finally, higher SVR, especially in male patients. Although the roles of this cytokine in HCV infection, liver inflammation and interferon responses have not been fully elucidated, IL-6, at least, could be a marker of resistance to PEG-IFN/RBV therapy for CHC patients.

It has been reported that IL-6 is produced by activation of Toll-like receptors, which constitute a major innate immune response against various pathogens, including HCV. Machida *et al.* [19] have reported that non-structural protein 5A activates the TLR-4 promoter in host cells, leading to the production of inflammatory cytokines, including interferon- β and IL-6. TLRs are transmembrane receptors that can be activated by binding of ligands [27]. Binding of ligands onto TLRs induces activation of nuclear factor- $\kappa\beta$, which leads to the production of inflammatory cytokines such as IL-6. IL-6 triggers phosphorylation of signal transducers and activators of transcription 3 (STAT3) and induces suppressor of cytokine signalling 3 (SOCS3) expression [28]. SOCS3 negatively regulates IL-6/STAT3 signalling [29]. As for HCV-infected patients, the expression of SOCS3 in the liver biopsy specimens is significantly down-regulated in rapid virological response (RVR) patients, compared to non-RVR patients, following IFN- α administration [30]. More recently, it has been reported that the SOCS3 and IL-6 expression levels in IFN-resistant cells were higher than those in IFN-sensitive cells [31]. Kupffer cells produce IL-6 followed by expression of TLR2 and TLR-4 in HCV infection

[32]. IL-6 promotes SOCS3 expression, which suppresses the JAK-STAT pathway and inhibits the formation of interferon-stimulated gene [33]. Therefore, suppression of interferon-stimulated gene through activating IL-6/SOCS3 signal results in resistance to IFN therapy. Similar to our data, these reports suggest that increased IL-6 levels were related to the resistance to IFN treatment through the cellular interferon signal attenuator, SOCS3.

Adipokines are polypeptides, secreted by adipose tissue [34], which store triglyceride in hepatocytes as well as in adipocytes. IL-6 is an adipokine and is involved in the development of metabolic syndrome. IL-6 levels are correlated with increasing visceral fat in humans [17] and intravenous administration of IL-6 increased serum triglyceride levels in rats because of an increase in hepatic triglyceride secretion [35]. This study shows that IL-6 levels are positively correlated with those of serum triglyceride in male patients, and with γ -GTP levels in female patients, and that IL-6, along with these parameters, is inversely correlated with SVR in all patients. A previous study has shown that hypertriglyceridaemia is a cause of insulin resistance, which reduces the effect of IFN therapy for CHC [36]. Although the mechanism whereby IL-6 regulates γ -GTP [37] is unclear, it has been reported that the serum IL-6 concentration is positively associated with γ -GTP. Moreover, γ -GTP is significantly correlated with the homeostatic model assessment of insulin resistance [38] and low γ -GTP is associated with a

Table 2. Comparison of clinical and laboratory characteristics of all patients based on therapeutic response

Characteristic	SVR (n=88) ^a	Non-SVR (n=61) ^a	Univariate analysis P-value	Multivariate analysis		
				OR	95% CI	P-value
Age, years	56 (25-69)	61 (30-72)	0.007	0.981	0.913-1.053	0.589
BMI, kg/m ²	23.2 (14.9-33.2)	22.7 (18.6-30.8)	0.685	-	-	-
Grade of inflammation	-	-	0.006	1.691	0.369-7.744	0.499
A0	6	0	-	-	-	-
A1	28	14	-	-	-	-
A2	53	39	-	-	-	-
A3	0	5	-	-	-	-
Stage of fibrosis	-	-	<0.0001	2.453	1.019-5.905	0.045
F0	3	2	-	-	-	-
F1	59	20	-	-	-	-
F2	19	16	-	-	-	-
F3	6	14	-	-	-	-
F4	0	6	-	-	-	-
Genotype	-	-	0.037	0.778	0.149-4.073	0.927
1	65	54	-	-	-	-
2	23	7	-	-	-	-
Haemoglobin, g/dl	14.6 (11.0-16.9)	14.0 (10.8-16.5)	0.015	0.507	0.256-1.003	0.051
Platelet count, ×10 ⁴ cells/μl	17.8 (7.3-34.4)	14.8 (7.2-27.4)	0.001	0.958	0.855-1.074	0.465
ALT, IU/l	68 (10-369)	66 (22-408)	0.728	-	-	-
γ-GTP, IU/l	37 (12-731)	52 (10-352)	0.019	0.996	0.989-1.004	0.336
T-chol, mg/dl	163 (98-265)	168 (126-226)	0.93	-	-	-
TG, mg/dl	100 (55-329)	119 (61-449)	0.11	-	-	-
LDL cholesterol, mg/dl	90 (47-161)	72 (34-132)	0.01	0.956	0.927-0.986	0.004
Substitutions in the ISDR	-	-	<0.0001	8.282	1.159-59.199	0.035
≤1	60	58	-	-	-	-
≥2	28	3	-	-	-	-
Substitutions of core amino acids 70 and 91 ^b	-	-	0.559	-	-	-
dW	22	16	-	-	-	-
Not dW	40	37	-	-	-	-
HCV RNA, log IU/ml	6.0 (3.7-6.9)	6.3 (5.1-6.7)	0.021	7.121	1.618-31.337	0.009
IL-6	-	-	0.246	-	-	-
≤3 pg/ml	48	27	-	-	-	-
>3 pg/ml	40	34	-	-	-	-

^aMedian (range) or *n* values are shown. ^bDouble wild (dW) represents wild type at amino acids 70 and 91, whereas not dW represents single or dual substitution of amino acid 70 and 91. ALT, alanine aminotransferase; BMI, body mass index; IL, interleukin; ISDR, interferon sensitivity determining region; LDL, low-density lipoprotein; SVR, sustained virological response; T-chol, total cholesterol; TG, triglycerides; γ-GTP, γ-glutamyl transpeptidase.

high rate of SVR after PEG-IFN/RBV therapy [39,40]. In this study, genotype was not a significant predictor of response to therapy. We performed analyses in subjects with good drug adherence; therefore, SVR rates were quite high in male patients with not only genotype 2 (78%; SVR/total=10/13) but also genotype 1 (63%; SVR/total=44/70).

Some reports focused on IL-6 gene polymorphism [41-43]. One study has shown that IL-6 GG or GC genotype was associated with increased serum IL-6 levels in healthy subjects [41]. Thereafter there were two reports on IL-6 genotype with CHC patients. One study has shown IL-6 GG or GC genotype to be an independent predictor of SVR to PEG-IFN/RBV combination therapy in HCV-HIV-coinfected

patients [42], whereas another study has shown that the low-producing IL-6 CC genotype was associated with HCV viral spontaneous elimination [43]. These results are not analogous to IL28B gene polymorphisms associated with response to therapy or with elimination of HCV. Unfortunately serum IL-6 levels were not analysed in these two studies [42,43]; therefore, the relationship between IL-6 polymorphisms and serum IL-6 levels associated with response to therapy or HCV clearance was obscured.

In our present study, serum IL-6 level was a predictor of response in male but not in female patients. A previous report showed that oestrogen suppressed IL-6 elevation [21] and also liver fibrosis progression [44]. In our study, female patients consisted of both

Table 3. Comparison of clinical and laboratory characteristics of male patients based on therapeutic response

Characteristic	SVR (n=54) ^a	Non-SVR (n=29) ^a	Univariate analysis P-value	Multivariate analysis		
				OR	95% CI	P-value
Age, years	55 (27-69)	58 (30-72)	0.195	-	-	-
BMI, kg/m ²	24.0 (18.4-28.4)	23.7 (19.2-29.2)	0.933	-	-	-
Grade of inflammation	-	-	0.227	-	-	-
A0	3	0	-	-	-	-
A1	14	5	-	-	-	-
A2	36	22	-	-	-	-
A3	0	1	-	-	-	-
Stage of fibrosis	-	-	0.003	3.33	1.422-7.798	0.006
F0	1	2	-	-	-	-
F1	36	7	-	-	-	-
F2	10	8	-	-	-	-
F3	6	10	-	-	-	-
F4	0	1	-	-	-	-
Genotype	-	-	0.528	-	-	-
1	44	26	-	-	-	-
2	10	3	-	-	-	-
Haemoglobin, g/dl	15.0 (11.9-16.9)	15.0 (13.0-16.5)	0.662	-	-	-
Platelet count, ×10 ⁴ cells/μl	17.9 (7.3-33.7)	16.0 (7.5-27.4)	0.048	0.976	0.847-1.124	0.733
ALT, IU/l	74 (10-339)	77 (26-408)	0.418	-	-	-
γ-GTP, IU/l	47 (13-731)	103 (21-352)	0.002	0.998	0.993-1.004	0.608
T-chol, mg/dl	160 (98-210)	150 (126-186)	0.613	-	-	-
TG, mg/dl	90 (55-329)	105 (61-449)	0.332	-	-	-
LDL cholesterol, mg/dl	81 (47-142)	72 (38-125)	0.156	-	-	-
Substitutions in the ISDR	-	-	0.001	6.717	0.987-45.708	0.052
≤1	32	27	-	-	-	-
≥2	22	2	-	-	-	-
Substitutions of core amino acids 70 and 91 ^a	-	-	1.0	-	-	-
dW	12	8	-	-	-	-
Not dW	30	18	-	-	-	-
HCV RNA, log IU/ml	5.9 (3.7-6.7)	6.4 (5.4-6.7)	0.004	3.91	0.934-16.37	0.062
IL-6	31/23	8/21	0.012	0.622	0.158-2.445	0.497
≤3 pg/ml	31	8	-	-	-	-
>3 pg/ml	23	21	-	-	-	-

^aMedian (range) or n values are shown. ^bDouble wild (dW) represents wild type at amino acids 70 and 91, whereas not dW represents single or dual substitution of amino acid 70 and 91. ALT, alanine aminotransferase; BMI, body mass index; IL, interleukin; ISDR, interferon sensitivity determining region; LDL, low-density lipoprotein; SVR, sustained virological response; T-chol, total cholesterol; TG, triglycerides; γ-GTP: γ-glutamyl transpeptidase.

premenopausal and postmenopausal patients, thus both serum IL-6 elevation and liver fibrosis progression might be unapparent. It was also reported that IL-6 positively correlates with BMI [23]. Although a significant gender difference was observed in BMI independently, IL-6 was not different between gender and was not significantly correlated to BMI in our study.

IL-6 also has been proposed to play a role in hepatocarcinogenesis and the gender disparity of HCC pathogenesis. It has been reported IL-6 levels are increased in HCC patients compared to CHC or cirrhosis patients [45,46] and may predict the development of HCC [47]. Naugler *et al.* [21] revealed that diethylnitrosamine administration increased serum IL-6 levels in male mice and treatment with an

oestrogen agonist reduced not only serum IL-6 levels but also the rate of formation of HCC. These results indicate that oestrogen regulates IL-6 production by Kupffer cells and reduces the risk of hepatocarcinogenesis in females.

In conclusion, we have shown that serum IL-6 is correlated with treatment resistance to PEG-IFN/RBV therapy, especially in male patients. Taken together with the previous reports that IL-6 is associated with insulin resistance (obesity and diabetes mellitus) and iron metabolism, which are suspected to be related to treatment outcomes of CHC and the gender bias in hepatocarcinogenesis, IL-6 is probably one of the most important targets for new treatment strategies for CHC and HCC.

Table 4. Comparison of clinical and laboratory characteristics of female patients based on therapeutic response

Characteristic	SVR (n=34) ^a	Non-SVR (n=32) ^a	Univariate analysis P-value	Multivariate analysis		
				OR	95% CI	P-value
Age, years	56 (25-68)	61 (39-72)	0.014	1.113	0.998-1.242	0.054
BMI, kg/m ²	21.9 (14.9-33.2)	22.2 (18.6-30.8)	0.544	-	-	-
Grade of inflammation	-	-	0.049	0.964	0.133-7.004	0.971
A0	3	0	-	-	-	-
A1	14	9	-	-	-	-
A2	17	17	-	-	-	-
A3	0	4	-	-	-	-
Stage of fibrosis	-	-	0.001	2.793	0.556-14.04	0.212
F0	2	0	-	-	-	-
F1	23	13	-	-	-	-
F2	9	8	-	-	-	-
F3	0	4	-	-	-	-
F4	0	5	-	-	-	-
Genotype	-	-	0.024	0.869	0.106-7.123	0.896
1	21	28	-	-	-	-
2	13	4	-	-	-	-
Haemoglobin, g/dl	13.8 (11.0-15.2)	13.4 (10.8-14.6)	0.061	-	-	-
Platelet count, ×10 ⁴ cells/μl	17.0 (9.1-34.4)	14.1 (7.2-26.2)	0.006	0.978	0.853-1.121	0.75
ALT, IU/l	43 (13-369)	55 (22-113)	0.32	-	-	-
γ-GTP, IU/l	31 (12-70)	31 (10-131)	0.221	-	-	-
T-cho, mg/dl	224 (186-265)	188 (151-226)	0.072	-	-	-
TG, mg/dl	103 (62-148)	140 (70-203)	0.242	-	-	-
LDL cholesterol, mg/dl	100 (55-161)	72 (34-132)	0.008	0.947	0.947-1.006	0.114
Substitutions in the ISDR	-	-	0.106	-	-	-
≤1	28	31	-	-	-	-
≥2	6	1	-	-	-	-
Substitutions of core amino acids 70 and 91 ^b	-	-	0.226	-	-	-
dW	10	8	-	-	-	-
Not dW	10	19	-	-	-	-
HCV RNA, log IU/ml	6.2 (4.3-6.9)	6.1 (5.1-6.7)	0.938	-	-	-
IL-6	-	-	0.47	-	-	-
≤3 pg/ml	17	19	-	-	-	-
>3 pg/ml	17	13	-	-	-	-

^aMedian (range) or n values are shown. ^bDouble wild (dW) represents wild type at amino acids 70 and 91, whereas not dW represents single or dual substitution of amino acid 70 and 91. ALT, alanine aminotransferase; BMI, body mass index; IL, interleukin; ISDR, interferon sensitivity determining region; LDL, low-density lipoprotein; SVR, sustained virological response; T-cho, total cholesterol; TG, triglycerides; γ-GTP, γ-glutamyl transpeptidase.

Acknowledgements

This study was supported by grants from the Ministry of Education, Culture, Sports, Science and Technology-Japan, the Japan Society for the Promotion of Science, Ministry of Health, Labour and Welfare-Japan, Japan Health Sciences Foundation, Miyakawa Memorial Research Foundation, Grant-in-Aid for Young Scientists and National Institute of Biomedical Innovation.

Disclosure statement

The authors declare no competing interests.

Additional file

Additional file 1: A list of the participating hospitals in the Ochanomizu-Liver Conference Study Group can be accessed via http://www.intmedpress.com/uploads/documents/AVT-10-OA-1941_Ueyama_Add_file1.pdf

References

- Williams MJ, Lang-Lenton M. Progression of initially mild hepatic fibrosis in patients with chronic hepatitis C infection. *J Viral Hepat* 2011; 18:17-22.
- Koike K. Pathogenesis of HCV-associated HCC: Dual-pass carcinogenesis through activation of oxidative stress and intracellular signaling. *Hepatol Res* 2007; 37 Suppl 2:S115-S120.

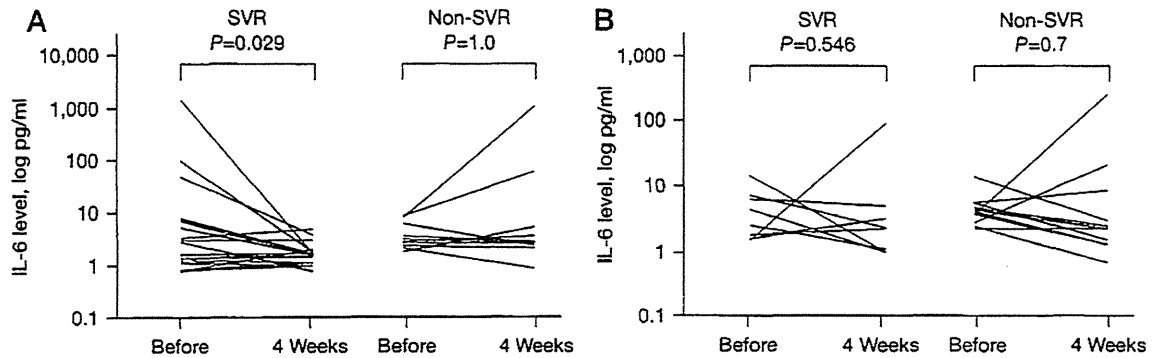
Table 5. Correlations between serum IL-6 and clinical and laboratory parameters

Characteristic	Male			Female		
	IL-6≤3 pg/ml (n=39) ^a	IL-6>3 pg/ml (n=44) ^a	P-value	IL-6≤3 pg/ml (n=36) ^a	IL-6>3 pg/ml (n=30) ^a	P-value
Age, years	56 (27-72)	56 (29-72)	0.895	59 (41-72)	56 (25-68)	0.398
BMI, kg/m ²	23.4 (19.2-29.2)	24.5 (18.4-28.9)	0.163	22.0 (14.9-30.8)	22.0 (18.5-33.2)	0.911
Grade of inflammation	-	-	0.665	-	-	0.36
A0	2	1	-	3	0	-
A1	8	11	-	12	11	-
A2	28	30	-	18	16	-
A3	0	1	-	3	1	-
Stage of fibrosis	-	-	0.393	-	-	0.496
F0	1	2	-	2	0	-
F1	24	19	-	18	18	-
F2	8	10	-	10	7	-
F3	5	11	-	2	2	-
F4	0	1	-	4	1	-
Genotype	-	-	0.239	-	-	0.549
1	35	35	-	27	22	-
2	4	9	-	9	8	-
Haemoglobin, g/dl	15.0 (13.2-16.8)	14.9 (11.9-16.9)	0.812	13.5 (12.1-15.2)	13.7 (10.8-14.8)	0.807
Platelet count, ×10 ⁴ cells/μl	16.9 (7.3-32.3)	16.2 (7.5-33.7)	0.893	15.1 (7.2-34.4)	16.9 (8.5-28.5)	0.227
ALT, IU/l	88 (32-330)	68 (10-408)	0.221	49 (19-110)	52 (13-369)	0.282
γ-GTP, IU/l	48 (15-184)	62 (13-731)	0.11	22 (12-61)	38 (10-131)	0.046
T-cho, mg/dl	163 (98-210)	148 (123-206)	0.117	201 (182-242)	188 (151-265)	0.655
TG, mg/dl	78 (55-329)	120 (63-449)	0.029	103 (67-157)	144 (62-203)	0.568
LDL cholesterol, mg/dl	78 (47-142)	74 (38-134)	0.718	89 (34-150)	82 (54-161)	0.551
Substitutions in the ISDR	-	-	0.229	-	-	0.231
≤1	25	34	-	34	25	-
≥2	14	10	-	2	5	-
Substitutions of core amino acids 70 and 91 ^a	-	-	0.29	-	-	0.769
dW	8	12	-	11	7	-
Not dW	27	21	-	16	13	-
HCV RNA, log IU/ml	6.0 (4.2-6.7)	6.3 (3.7-6.7)	0.35	6.3 (5.0-6.7)	6.1 (4.3-6.9)	0.292
Virological response	-	-	0.012	-	-	0.47
SVR	31	23	-	17	17	-
Non-SVR	8	21	-	19	13	-

^aMedian (range) or *n* values are shown. ^bDouble wild (dW) represents wild type at amino acids 70 and 91, whereas not dW represents single or dual substitution of amino acid 70 and 91. ALT, alanine aminotransferase; BMI, body mass index; IL, interleukin; ISDR, interferon sensitivity determining region; LDL, low-density lipoprotein; SVR, sustained virological response; T-cho, total cholesterol; TG, triglycerides; γ-GTP, γ-glutamyl transpeptidase.

- Saito I, Miyamura T, Ohbayashi A, *et al.* Hepatitis C virus infection is associated with the development of hepatocellular carcinoma. *Proc Natl Acad Sci U S A* 1990; 87:6547-6549.
- Fried MW, Shiffman ML, Reddy KR, *et al.* Peginterferon alfa-2a plus ribavirin for chronic hepatitis C virus infection. *N Engl J Med* 2002; 347:975-982.
- Zeuzem S, Feinman SV, Rasenack J, *et al.* Peginterferon alfa-2a in patients with chronic hepatitis C. *N Engl J Med* 2000; 343:1666-1672.
- Ascione A, De Luca M, Tartaglione MT, *et al.* Peginterferon alfa-2a plus ribavirin is more effective than peginterferon alfa-2b plus ribavirin for treating chronic hepatitis C virus infection. *Gastroenterology* 2010; 138:116-122.
- Bruno S, Shiffman ML, Roberts SK, *et al.* Efficacy and safety of peginterferon alfa-2a (40KD) plus ribavirin in hepatitis C patients with advanced fibrosis and cirrhosis. *Hepatology* 2010; 51:388-397.
- Ge D, Fellay J, Thompson AJ, *et al.* Genetic variation in IL28B predicts hepatitis C treatment-induced viral clearance. *Nature* 2009; 461:399-401.
- Tanaka Y, Nishida N, Sugiyama M, *et al.* Genome-wide association of IL28B with response to pegylated interferon-alpha and ribavirin therapy for chronic hepatitis C. *Nat Genet* 2009; 41:1105-1109.
- Enomoto N, Sakuma I, Asahina Y, *et al.* Mutations in the nonstructural protein 5A gene and response to interferon in patients with chronic hepatitis C virus 1b infection. *N Engl J Med* 1996; 334:77-82.
- Akuta N, Suzuki F, Sezaki H, *et al.* Association of amino acid substitution pattern in core protein of hepatitis C virus genotype 1b high viral load and non-virological response to interferon-ribavirin combination therapy. *Intervirology* 2005; 48:372-380.
- Heinrich PC, Castell JV, Andus T. Interleukin-6 and the acute phase response. *Biochem J* 1990; 265:621-636.
- Sehgal PB. Interleukin-6: a regulator of plasma protein gene expression in hepatic and non-hepatic tissues. *Mol Biol Med* 1990; 7:117-130.
- Oyanagi Y, Takahashi T, Matsui S, *et al.* Enhanced expression of interleukin-6 in chronic hepatitis C. *Liver* 1999; 19:464-472.

Figure 2. Serum IL-6 levels before and after 4 weeks of pegylated interferon plus ribavirin therapy



Transition of serum interleukin (IL)-6 levels during the first 4 weeks of pegylated interferon plus ribavirin therapy among (A) male and (B) female patients. Significant differences of the data were analysed using the Wilcoxon signed-rank test. All *P*-values <0.05 were considered statistically significant. SVR, sustained virological response.

Table 6. Serum IL-6 transition during pegylated-interferon plus ribavirin therapy

Sex	Variable	Treatment effect	Pretreatment	4 Weeks	8 Weeks	12 Weeks	Last treatment
Male	IL-6, pg/ml	SVR	2.7 (0.8–1,500)	1.5 (0.8–4.8)	1.7 (0.9–6.6)	1.6 (0.8–4,463)	2.0 (0.9–26.3)
		Non-SVR	3.1 (1.9–8.6)	2.8 (0.9–1,120)	2.6 (1.9–142)	4.0 (1.4–193)	1.7 (1.7–4.2)
		<i>P</i> -value	0.025	0.003	0.011	0.046	0.51
	ALT, IU/l	SVR	76 (10–339)	25 (11–200)	21 (10–219)	18 (10–265)	20 (11–177)
		Non-SVR	74 (38–203)	52 (24–201)	46 (15–187)	47 (14–215)	31 (14–121)
		<i>P</i> -value	0.966	0.012	0.037	0.026	0.127
HCV RNA, log IU/ml	SVR	5.9 (3.7–6.7)	0 (0–4.6)	0 (0–2.6)	ND	ND	
	Non-SVR	6.4 (5.4–6.7)	5.1 (3.3–5.7)	4.8 (2.7–5.6)	4.5 (3.5–4.9)	0 (0–4.8)	
	<i>P</i> -value	0.018	<0.0001	<0.0001	<0.0001	0.246	
Female	IL-6, pg/ml	SVR	3.4 (1.5–14.5)	2.3 (1.0–91.8)	1.6 (0.9–3.0)	2.0 (1.1–116)	1.8 (1.6–2.0)
		Non-SVR	4.0 (2.3–14.0)	2.2 (0.7–252)	1.8 (0.9–244)	3.6 (0.8–14.9)	2.7 (0.8–38.9)
		<i>P</i> -value	0.657	0.84	0.442	0.375	0.071
	ALT, IU/l	SVR	43 (13–96)	13 (9–29)	12 (11–30)	11 (9–28)	9 (9–11)
		Non-SVR	59 (31–113)	40 (17–87)	30 (13–130)	30 (13–112)	26 (9–64)
		<i>P</i> -value	0.152	0.003	0.001	0.005	0.014
	HCV RNA, log IU/ml	SVR	5.8 (4.3–6.4)	0 (0–4.3)	ND	ND	ND
		Non-SVR	6.1 (5.5–6.7)	5.0 (3.2–5.5)	4.6 (2.6–5.6)	3.9 (1.8–5.5)	0 (0–5.9)
		<i>P</i> -value	0.075	0.005	<0.0001	<0.0001	0.173

Median (range) values are shown. ALT, alanine aminotransferase; IL, interleukin; ND, not detectable; SVR, sustained virological response.

- Lee Y, Park US, Choi I, et al. Human interleukin 6 gene is activated by hepatitis B virus-X protein in human hepatoma cells. *Clin Cancer Res* 1998; 4:1711–1717.
- Sheron N, Bird G, Goka J, et al. Elevated plasma interleukin-6 and increased severity and mortality in alcoholic hepatitis. *Clin Exp Immunol* 1991; 84:449–453.
- van der Poorten D, Milner KL, Hui J, et al. Visceral fat: a key mediator of steatohepatitis in metabolic liver disease. *Hepatology* 2008; 48:449–457.
- Khoruts A, Stahnke L, McClain CJ, et al. Circulating tumor necrosis factor, interleukin-1 and interleukin-6 concentrations in chronic alcoholic patients. *Hepatology* 1991; 13:267–276.
- Machida K, Cheng KT, Sung VM, et al. Hepatitis C virus induces toll-like receptor 4 expression, leading to enhanced production of beta interferon and interleukin-6. *J Virol* 2006; 80:866–874.
- Malaguarnera M, DiFazio I, Laurino A, et al. Serum interleukin 6 concentrations in chronic hepatitis C patients before and after interferon-alpha treatment. *Int J Clin Pharmacol Ther* 1997; 35:385–388.
- Naugler WE, Sakurai T, Kim S, et al. Gender disparity in liver cancer due to sex differences in MyD88-dependent IL-6 production. *Science* 2007; 317:121–124.
- Pradhan AD, Manson JE, Rifai N, et al. C-reactive protein, interleukin 6, and risk of developing type 2 diabetes mellitus. *JAMA* 2001; 286:327–334.
- Moschen AR, Molnar C, Geiger S, et al. Anti-inflammatory effects of excessive weight loss: potent suppression of adipose interleukin 6 and tumour necrosis factor alpha expression. *Gut* 2010; 59:1259–1264.
- Klover PJ, Clementi AH, Mooney RA. Interleukin-6 depletion selectively improves hepatic insulin action in obesity. *Endocrinology* 2005; 146:3417–3427.

25. Pietrangelo A, Dierssen U, Valli L, *et al.* STAT3 is required for IL-6-gp130-dependent activation of hepcidin in vivo. *Gastroenterology* 2007; 132:294–300.
26. Shikano M, Sobajima H, Yoshikawa H, *et al.* Usefulness of a highly sensitive urinary and serum IL-6 assay in patients with diabetic nephropathy. *Nephron* 2000; 85:81–85.
27. Akira S, Takeda K. Toll-like receptor signalling. *Nat Rev Immunol* 2004; 4:499–511.
28. Naugler WE, Karin M. The wolf in sheep's clothing: the role of interleukin-6 in immunity, inflammation and cancer. *Trends Mol Med* 2008; 14:109–119.
29. Ogata H, Kobayashi T, Chinen T, *et al.* Deletion of the SOCS3 gene in liver parenchymal cells promotes hepatitis-induced hepatocarcinogenesis. *Gastroenterology* 2006; 131:179–193.
30. Huang Y, Feld JJ, Sapp RK, *et al.* Defective hepatic response to interferon and activation of suppressor of cytokine signaling 3 in chronic hepatitis C. *Gastroenterology* 2007; 132:733–744.
31. Suda G, Sakamoto N, Itsui Y, *et al.* IL-6-mediated intersubgenotypic variation of interferon sensitivity in hepatitis C virus genotype 2a/2b chimeric clones. *Virology* 2010; 407:80–90.
32. Szabo G, Dolganiuc A, Mandrekar P. Pattern recognition receptors: a contemporary view on liver diseases. *Hepatology* 2006; 44:287–298.
33. Gale M, Jr., Foy EM. Evasion of intracellular host defence by hepatitis C virus. *Nature* 2005; 436:939–945.
34. Marra F, Bertolani C. Adipokines in liver diseases. *Hepatology* 2009; 50:957–969.
35. Nonogaki K, Fuller GM, Fuentes NL, *et al.* Interleukin-6 stimulates hepatic triglyceride secretion in rats. *Endocrinology* 1995; 136:2143–2149.
36. Walsh MJ, Jonsson JR, Richardson MM, *et al.* Non-response to antiviral therapy is associated with obesity and increased hepatic expression of suppressor of cytokine signalling 3 (SOCS-3) in patients with chronic hepatitis C, viral genotype 1. *Gut* 2006; 55:529–535.
37. Mochizuki K, Misaki Y, Miyauchi R, *et al.* Circulating interleukin-1beta and interleukin-6 concentrations are closely associated with gamma-glutamyltranspeptidase activity in middle-aged Japanese men without obvious cardiovascular diseases. *Metabolism* 2011; 60:914–922.
38. Lonardo A, Lombardini S, Scaglioni F, *et al.* Hepatic steatosis and insulin resistance: does etiology make a difference? *J Hepatol* 2006; 44:190–196.
39. Villela-Nogueira CA, Perez RM, de Segadas Soares JA, Coelho HS. Gamma-glutamyl transferase (GGT) as an independent predictive factor of sustained virologic response in patients with hepatitis C treated with interferon-alpha and ribavirin. *J Clin Gastroenterol* 2005; 39:728–730.
40. Chen L, Borozan I, Feld J, *et al.* Hepatic gene expression discriminates responders and nonresponders in treatment of chronic hepatitis C viral infection. *Gastroenterology* 2005; 128:1437–1444.
41. Fishman D, Faulds G, Jeffery R, *et al.* The effect of novel polymorphisms in the interleukin-6 (IL-6) gene on IL-6 transcription and plasma IL-6 levels, and an association with systemic-onset juvenile chronic arthritis. *J Clin Invest* 1998; 102:1369–1376.
42. Nattermann J, Vogel M, Berg T, *et al.* Effect of the interleukin-6 C174G gene polymorphism on treatment of acute and chronic hepatitis C in human immunodeficiency virus coinfecting patients. *Hepatology* 2007; 46:1016–1025.
43. Barrett S, Collins M, Kenny C, *et al.* Polymorphisms in tumour necrosis factor-alpha, transforming growth factor-beta, interleukin-10, interleukin-6, interferon-gamma, and outcome of hepatitis C virus infection. *J Med Virol* 2003; 71:212–218.
44. Di Martino V, Lebray P, Myers RP, *et al.* Progression of liver fibrosis in women infected with hepatitis C: long-term benefit of estrogen exposure. *Hepatology* 2004; 40:1426–1433.
45. Porta C, Amici M, Quaglini S, *et al.* Circulating interleukin-6 as a tumor marker for hepatocellular carcinoma. *Ann Oncol* 2008; 19:353–358.
46. Soresi M, Giannitrapani L, D'Antona F, *et al.* Interleukin-6 and its soluble receptor in patients with liver cirrhosis and hepatocellular carcinoma. *World J Gastroenterol* 2006; 12:2563–2568.
47. Nakagawa H, Maeda S, Yoshida H, *et al.* Serum IL-6 levels and the risk for hepatocarcinogenesis in chronic hepatitis C patients: an analysis based on gender differences. *Int J Cancer* 2009; 125:2264–2269.

Accepted 22 February 2011; published online 29 July 2011

Association of IL28B Variants With Response to Pegylated-Interferon Alpha Plus Ribavirin Combination Therapy Reveals Intersubgenotypic Differences Between Genotypes 2a and 2b

Naoya Sakamoto, MD, PhD,^{1,2*} Mina Nakagawa,¹ Yasuhito Tanaka,³ Yuko Sekine-Osajima,¹ Mayumi Ueyama,¹ Masayuki Kurosaki,⁴ Nao Nishida,⁵ Akihiro Tamori,⁶ Nishimura-Sakurai Yuki,¹ Yasuhiro Itsui,^{1,7} Seishin Azuma,¹ Sei Kakinuma,^{1,2} Shuhei Hige,⁸ Yoshito Itoh,⁹ Eiji Tanaka,¹⁰ Yoichi Hiasa,¹¹ Namiki Izumi,⁴ Katsushi Tokunaga,⁵ Masashi Mizokami,¹² Mamoru Watanabe¹ and the Ochanomizu-Liver Conference Study Group

¹Department of Gastroenterology and Hepatology, Tokyo Medical and Dental University, Tokyo, Japan

²Department for Hepatitis Control, Tokyo Medical and Dental University, Tokyo, Japan

³Department of Virology & Liver Unit, Nagoya City University Graduate School of Medical Sciences, Mizuho-ku Nagoya, Japan

⁴Division of Gastroenterology and Hepatology, Musashino Red Cross Hospital, Tokyo, Japan

⁵Department of Human Genetics, Graduate School of Medicine, The University of Tokyo, Tokyo, Japan

⁶Department of Hepatology, Osaka City University Graduate School of Medicine, Osaka, Japan

⁷Department of Internal Medicine, Soka Municipal Hospital, Saitama, Japan

⁸Department of Internal Medicine, Hokkaido University Graduate School of Medicine, Sapporo, Japan

⁹Molecular Gastroenterology and Hepatology, Kyoto Prefectural University of Medicine, Kyoto, Japan

¹⁰Department of Medicine, Shinshu University School of Medicine, Matsumoto, Japan

¹¹Department of Gastroenterology and Metabolism, Ehime University Graduate School of Medicine, Ehime, Japan

¹²Research Center for Hepatitis and Immunology, International Medical Center of Japan Konodai Hospital, Ichikawa, Japan

Genetic polymorphisms of the interleukin 28B (IL28B) locus are associated closely with outcomes of pegylated-interferon (PEG-IFN) plus ribavirin (RBV) combination therapy. The aim of this study was to investigate the relationship between IL28B polymorphism and responses to therapy in patients infected with genotype 2. One hundred twenty-nine chronic hepatitis C patients infected with genotype 2, 77 patients with genotype 2a and 52 patients with genotype 2b, were analyzed. Clinical and laboratory parameters, including genetic variation near the IL28B gene (rs8099917), were assessed. Drug adherence was monitored in each patient. Univariate and multivariate statistical analyses of these parameters and clinical responses were carried out. Univariate analyses showed that a sustained virological response was correlated significantly with IL28B polymorphism, as well as age, white blood cell and neutrophil counts, adherence to RBV, and rapid virological response. Subgroup analysis revealed that patients infected with genotype 2b achieved significantly lower rapid virological response rates than those with genotype 2a. Patients with the IL28B-major allele showed higher virus clearance rates at each time point

than those with the IL28B-minor allele, and the differences were more profound in patients infected with genotype 2b than those with genotype 2a. Furthermore, both rapid and sustained virological responses were associated significantly with IL28B alleles in patients with genotype

Abbreviations: HCV, hepatitis C virus; HCC, hepatocellular carcinoma; IFN, interferon; PEG-IFN, pegylated-interferon; RBV, ribavirin; IL28B, interleukin 28B; SNPs, single nucleotide polymorphisms; BMI, body mass index; ALT, alanine transaminase; ISDR, the interferon sensitivity determining region; ITPA, inosine triphosphatase

Grant sponsor: Ministry of Education, Culture, Sports, Science and Technology-Japan; Grant sponsor: Japan Society for the Promotion of Science, Ministry of Health, Labour and Welfare-Japan; Grant sponsor: Japan Health Sciences Foundation; Grant sponsor: Miyakawa Memorial Research Foundation; Grant sponsor: National Institute of Biomedical Innovation.

Naoya Sakamoto and Mina Nakagawa contributed equally to this work.

*Correspondence to: Naoya Sakamoto, MD, PhD, Department of Gastroenterology and Hepatology, Tokyo Medical and Dental University, 1-5-45 Yushima, Bunkyo-ku, Tokyo 113-8519, Japan. E-mail: nsakamoto.gast@tmd.ac.jp

Accepted 10 January 2011

DOI 10.1002/jmv.22038

Published online in Wiley Online Library (wileyonlinelibrary.com).

2b. IL28B polymorphism was predictive of PEG-IFN plus RBV combination treatment outcomes in patients infected with genotype 2 and, especially, with genotype 2b. In conclusion, IL-28B polymorphism affects responses to PEG-IFN-based treatment in difficult-to-treat HCV patients. *J. Med. Virol.* © 2011 Wiley-Liss, Inc.

KEY WORDS: hepatitis C virus (HCV); chronic hepatitis C; genotype 2; PEG-IFN plus RBV therapy; combination therapy; IL28B; interferon- λ 3

INTRODUCTION

Hepatitis C virus (HCV) infects around 170 million people worldwide and is characterized by a high probability of developing chronic inflammation and fibrosis of the liver, leading to end-stage liver failure and hepatocellular carcinoma (HCC) [Alter, 1997; Sakamoto and Watanabe, 2009]. Since the first report in 1986, type I interferons have been the mainstay of HCV therapy [Hoofnagle, 1994]. Current standards of care consist of a combination of ribavirin (RBV) plus pegylated interferon (PEG-IFN)-alpha for 48 weeks for infection with genotypes 1 and 4, and for 24 weeks for the other genotypes [Zeuzem et al., 2000; Fried et al., 2002]. Although this treatment improved substantially sustained virological response rates, it may result also in serious adverse effects and a considerable proportion of patients require early discontinuation of treatment. Patients of African origin have even poorer treatment outcomes [Rosen and Gretch, 1999]. Given this situation, a precise assessment of the likely treatment outcomes before the initiation of treatment may improve substantially the quality of antiviral treatment.

Recently, several studies have reported that genetic polymorphisms of the IL28B locus, which encodes interferon- λ 3 (interleukin 28B), are associated with response to interferon-based treatment of chronic HCV infections with genotype 1 [Ge et al., 2009; Suppiah et al., 2009; Tanaka et al., 2009] and also spontaneous clearance of HCV [Thomas et al., 2009].

While chronic HCV infections with genotype 2 are associated with good treatment outcome, there are some refractory cases among patients infected with genotype 2, similar to genotype 1. The aims of this study were to analyze retrospectively clinical and virological factors associated with treatment response in patients with chronic HCV infection with genotype 2 who were treated with PEG-IFN plus RBV combination therapy and to clarify the relationship between IL28B polymorphism and the response to combination therapy.

PATIENTS AND METHODS

The authors analyzed retrospectively 129 patients with chronic HCV infection with genotype 2 who

received combination therapy with PEG-IFN plus RBV between December 2004 and December 2009 at 10 multicenter hospitals (liver units with hepatologists) throughout Japan. All patients had chronic active hepatitis confirmed histologically or clinically and were positive for anti-HCV antibodies and serum HCV RNA by quantitative or qualitative assays. Patients with a positive test for serum hepatitis B surface antigen, coinfection with other HCV genotypes, coinfection with human immunodeficiency virus, other causes of hepatocellular injury (such as alcoholism, autoimmune hepatitis, primary biliary cirrhosis, or a history of treatment with hepatotoxic drugs), and a need for hemodialysis were excluded.

Study Design

Each patient was treated with combination therapy with PEG-IFN- α 2b (Peg-Intron, Schering-Plough Nordic Biotech, Stockholm, Sweden, at a dose of 1.2–1.5 μ g/kg subcutaneously once a week) or PEG-IFN- α 2a (Pegasys; Roche, Basel, Switzerland, at a dose of 180 μ g subcutaneously once a week) plus RBV (Rebetol, Schering-Plough Nordic Biotech or Copegus; Roche) 600–1,000 mg daily depending on the body weight (b.w.) (b.w. <60 kg: 600 mg po daily; b.w. 60–80 kg: 800 mg po daily; b.w. >80 kg: 1,000 mg po daily; in two divided doses). The duration of the combination therapy was set at a standard 24 weeks, but treatment reduction or discontinuation was permitted by doctor's decision. The rates of PEG-IFN and RBV administration achieved were calculated as percentages of actual total dose administered of a standard total dose of 24 weeks, according to body weight before therapy. During treatment, patients were assessed as outpatients at weeks 2, 4, 6, 8, and then every 4 weeks for the duration of treatment and at every 4 weeks after the end of treatment. Biochemical and hematological testing was carried out in a central laboratory. Serum HCV RNA was measured before treatment, during treatment at 4 weekly intervals, and after therapy at 4 weekly intervals for 24 weeks, by quantitative or qualitative assays.

Patient Evaluation

The following factors were analyzed to determine whether they were related to the efficacy of combination therapy: age, gender, body mass index (BMI), previous IFN therapy, grade of inflammation and stage of fibrosis on liver biopsy, pretreatment biochemical parameters, such as white blood cells, neutrophils, hemoglobin, platelet count, alanine transaminase (ALT) level, serum HCV RNA level (log IU/ml), and single nucleotide polymorphism (SNPs) in the IL28B locus (rs8099917). Liver biopsy specimens were evaluated blindly, to determine the grade of inflammation and stage of fibrosis, by an independent interpreter who was not aware of the clinical data. Activity of inflammation was graded on a scale of 0–3: A0 shows no activity, A1 shows mild activity, A2 shows moderate activity and A3 shows severe activity. Fibrosis was staged on a scale of 0–4:

F0 shows no fibrosis, F1 shows moderate fibrosis, F2 shows moderate fibrosis with few septa, F3 shows severe fibrosis with numerous septa without cirrhosis and F4 shows cirrhosis.

Informed written consent was obtained from each patient who participated in the study. The study protocol conformed to the ethical guidelines of the Declaration of Helsinki and to the relevant ethical guidelines as reflected in a priori approval by the ethics committees of all the participating universities and hospitals.

SNP Genotyping

Human genomic DNA was extracted from whole blood of each patient. Genetic polymorphism of IL28B was determined by DigiTag2 assay by typing one tag SNP located within the IL28B locus, rs8099917 (22). Heterozygotes (T/G) or homozygotes (G/G) of the minor allele (G) were defined as having the IL28B minor allele, whereas homozygotes for the major allele (T/T) were defined as having the IL28B major allele.

Outcomes

The primary end point was a sustained biochemical and virological response. A sustained virological response was defined as serum HCV RNA undetectable at 24 weeks after the end of treatment. Secondary end points were a rapid virological response (HCV RNA undetectable in serum at week 4) and end-of-treatment virological response. In addition, tolerability (adverse events) and drug adherence were recorded and factors potentially associated with virological response explored.

Statistical Analysis

SPSS software package (SPSS 18J, SPSS, Chicago, IL) was used for statistical analysis. Discrete variables were evaluated by Fisher's exact probability test and distributions of continuous variables were analyzed by the Mann-Whitney *U*-test. Independent factors possibly affecting response to combination therapy were examined by stepwise multiple logistic-regression analysis. All *P*-values were calculated by two-tailed tests, and those of less than 0.05 were considered statistically significant.

RESULTS

Clinical Characteristics and Response to Therapy

The clinical characteristics and response rates to therapy of 129 patients are summarized in Tables I and II. Sixty-eight patients achieved a rapid virological response, whereas 44 patients remained HCV-RNA positive at week 4. Treatment reduction or cessation was permitted also to avoid side effects, and one patient stopped treatment at week 12 because he was

TABLE I. Baseline Characteristics of Participating Patients Infected With HCV Genotype 2

Total number	129
Genotype (2a/2b)	77/52
IL28B SNPs (rs8099917)	
TT/TG/GG	100/28/1
Age (years) ^a	64 (20–73)
Gender (male/female)	64/65
Body mass index (kg/m ²) ^a (N = 80)	23.7 (16.9–33.5)
Previous interferon therapy (no/yes)	102/21 (unknown 6)
Histology at biopsy (N = 96)	
Grade of inflammation	
A0/1/2/3	10/53/29/4
Stage of fibrosis	
F0/1/2/3	7/59/19/11
White blood cells (/μl) ^b (N = 94)	5,115 ± 1,630
Neutrophils (/μl) ^b (N = 94)	2,765 ± 1,131
Hemoglobin (g/dl) ^b (N = 95)	14.2 ± 1.3
Platelet count (×10 ⁻³ /μl) ^b (N = 98)	187 ± 95
ALT (IU/L) ^b (N = 95)	82 ± 78
Serum HCV-RNA level (log(IU/ml)) ^{a,c}	6.2 (3.6–7.4)
Treatment duration (>16, ≤24)	19/110

SNPs, single nucleotide polymorphisms; ALT, alanine transaminase.

^aData are shown as median (range) values.

^bData are expressed as mean ± SD.

^cData are shown as log(IU/ml).

anticipated to be a non-responder. On an intention-to-treat analysis, serum HCV-RNA levels were negative at the end of treatment in 125 of the 129 patients (97%) treated and, among them, 98 (76%) achieved a sustained virological response. The rapid virological response rate of patients infected with genotype 2b was lower significantly than that of patients infected with genotype 2a (*P* = 0.036) (Table II). The sustained virological response rate decreased with RBV drug discontinuation and dose reduction (84% and 66% with ≥80% and <80% of RBV dose, *P* = 0.021, Table III). Adherences to PEG-IFN did not influence a sustained virological response or end of treatment response significantly, while RBV adherence was associated significantly with a sustained virological response (Table III).

Factors Associated With a Sustained Virological Response

Next the host clinical and viral factors associated with a sustained virological response were analyzed. Univariate statistical analysis showed that six parameters were associated significantly with the sustained virological response rates, including age, white blood cells, neutrophils, adherence to RBV, rapid virological response and an IL28B SNP (rs8099917) (Table IV). There was no significant association of sustained virological response with gender, previous interferon therapy, stage of fibrosis, pretreatment HCV titer or adherence to PEG-IFN. Further multivariate analyses were conducted using significant factors identified by the univariate analysis (Table V). The multiple logistic-regression analysis showed that only a rapid virological response was associated with a sustained virological response (OR = 0.170, *P* = 0.019).

TABLE II. Response Rates to Therapy

Character	Number/total number (%)		
Overall			
RVR	68/112 (61)		
ETR	125/129 (97)		
SVR	98/129 (76)		
Genotype	2a	2b	P-value
RVR	46/67 (69)	22/45 (49)	0.036
ETR	74/77 (96)	51/52 (98)	NS
SVR	56/77 (73)	42/52 (81)	NS

RVR, rapid virological response; ETR, end of treatment response; SVR, sustained virological response. Bold indicated *P*-value of less than 0.05.

TABLE III. Response Rates to Treatment According to Drug Adherence

	≥ 80%	<80%	P-value
PEG-IFN adherence			
ETR	94/96 (98)	31/33 (94)	NS
SVR	75/96 (78)	23/33 (70)	NS
RBV adherence			
ETR	72/73 (99)	53/56 (95)	NS
SVR	61/73 (84)	37/56 (66)	0.021

ETR, end of treatment response; SVR, sustained virological response; PEG-IFN, pegylated interferon; RBV, ribavirin.

The rates of PEG-IFN and RBV administration achieved were calculated as percentages of actual total dose administered of a standard total dose of 24 weeks, according to body weight before therapy.

Bold indicated *P*-value of less than 0.05.

Comparison of Sustained Virological Response Rates According to IL28B SNPs

The PEG-IFN plus RBV treatment efficacy was compared after dividing the study subjects into two groups based on IL28B alleles (Table VI). Patients homozygous for the IL28B major allele (TT allele) achieved significantly higher rapid and sustained virological response

rates than those heterozygous or homozygous for the IL28B minor allele (TG/GG alleles) ($P < 0.05$). In addition, responses to PEG-IFN plus RBV treatment were analyzed after dividing the study subjects into those with genotype 2a and with genotype 2b. The rapid and sustained virological response rates tended to be higher in patients homozygous for the IL28B major allele than those heterozygous or homozygous for the

TABLE IV. Clinical and Virological Characteristics of Patients Based on Therapeutic Response

	SVR (n = 98)	Non-SVR (n = 31)	P-value
Genotype (2a/2b)		56/42	21/10
IL28B SNPs (rs8099917)			
TT/TG + GG	81/17	19/12	0.024
Age (years) ^a	56 (20–73)	61 (40–72)	0.002
Gender (male/female)	51/47	13/18	NS
Body mass index (kg/m ²) ^a	22.8 (16.9–33.5)	24.1 (20.3–27.6)	NS
Previous Interferon therapy (no/yes)	80/14	22/7	NS
Grade of inflammation (A0-1/2-3)	46/28	15/7	NS
Stage of fibrosis (F0-2/3-4)	64/10	21/1	NS
White blood cells (/μl) ^b	5,318 ± 1,617	4,489 ± 1,540	0.032
Neutrophils (/μl) ^b	2,913 ± 1,139	2,278 ± 983	0.021
Hemoglobin (g/dl) ^b	14.2 ± 1.4	14.1 ± 1.1	NS
Platelet count (×10 ⁻³ /μl) ^b	193 ± 105	171 ± 54	NS
ALT (IU/ml) ^b	79 ± 73	94 ± 92	NS
Pretreatment Serum HCV-RNA level (log(IU/ml)) ^{a,c}	6.1 (3.6–7.4)	6.3 (4.0–6.7)	NS
PEG-IFN adherence (≥ 80%/<80%)	75/23	21/10	NS
RBV adherence (≥ 80%/<80%)	61/37	12/19	0.024
RVR/non-RVR	57/24	11/20	0.001

SNPs, single nucleotide polymorphisms; ALT, alanine transaminase; RVR, rapid virological response.

^aData are show as median (range) values.

^bData are expressed as mean ± SD.

^cData are shown as log (IU/ml).

Bold indicated *P*-value of less than 0.05.

TABLE V. Multivariate Analysis for the Clinical and Virological Factors Related to Sustained Response With Peg-IFN Plus RBV Therapy in 63 Patients

Factor	Category	Odds ratio (95% CI)	P-value
Regression analysis			
RVR	RVR	1	0.019
	Non-RVR	0.170 (0.039–0.744)	
RBV adherence	≥80%	1	0.061
	<80%	0.250 (0.059–1.064)	
IL28B SNPs (rs8099917)	TT	1	0.104
	TG + GG	0.252 (0.048–1.330)	
Age		1.087 (0.976–1.211)	0.128
Neutrophils		0.999 (0.997–1.001)	0.209
White blood cells		1.000 (0.999–1.002)	0.504

CI, confidence interval; SNPs, single nucleotide polymorphisms; RVR, rapid virological response, RBV, ribavirin.

Bold indicated *P*-value of less than 0.05.

IL28B minor allele infected with both genotype 2a and 2b, and these differences were more profound in patients infected with genotype 2b than with genotype 2a. The rapid and sustained virological response rates of patients with the major IL28B allele were higher significantly than those of patients with the minor IL28B allele infected only with genotype 2b (rapid virological response: 58% and 0% with IL28B major and hetero/minor, $P = 0.002$, sustained virological response: 88% and 44% with IL28B major and hetero/minor, $P = 0.009$).

Although the rapid virological response rate of patients infected with genotype 2b was lower significantly than that of patients infected with genotype 2a, the sustained virological response rate was higher in patients infected with genotype 2b than with genotype 2a (Table II). In order to investigate that discrepancy, sustained virological response rates in patients with or without rapid virological response were analyzed according to IL28B SNPs. In patients infected with genotype 2b and a non-rapid virological response, the sustained virological response rates differed significantly between IL28B major and hetero/minor groups (sustained virological response with non-rapid virological response: 75% and 29% with IL28B major and hetero/minor, $P = 0.044$), and no one achieved a rapid

virological response among the patients infected with genotype 2b and with the IL28B hetero/minor allele. In patients infected with genotype 2a, on the contrary, there was no significant correlation of rapid and sustained virological response rates between IL28B SNPs (sustained virological response with rapid virological response: 78% and 70% with IL28B major and hetero/minor, $P = 0.630$, sustained virological response with non-rapid virological response: 57% and 43% with IL28B major and hetero/minor, $P = 0.552$).

Next, changes in virological response rates over time were investigated in patients treated with PEG-IFN plus RBV and the time course was analyzed after separating the patients infected with genotype 2a and 2b (Fig. 1). Patients with IL28B-TG and -GG showed significantly lower rates of rapid and sustained virological response, compared to patients with IL28B-TT, and greater differences were observed according to IL28B SNPs among patients infected with genotype 2b than with 2a.

Side Effects

Side effects leading to Peg-IFN plus RBV discontinuation occurred in eight patients (6.2%) and discontinuation of RBV alone occurred in four patients (3.1%).

TABLE VI. Rapid and Sustained Virological Response Rates to Treatment According to IL28B SNPs

Character	IL28B major	IL28B hetero/minor	P-value
Number/total number (%)			
Overall			
RVR	58/88 (66)	10/24 (42)	0.031
SVR	81/100 (81)	17/29 (59)	0.013
Genotype 2a			
RVR	36/50 (72)	10/17 (59)	NS
SVR	43/57 (75)	13/20 (65)	NS
Genotype 2b			
RVR	22/38 (58)	0/7 (0)	0.002
SVR	38/43 (88)	4/9 (44)	0.009

RVR, rapid virological response; ETR, end of treatment response; SVR, sustained virological response.

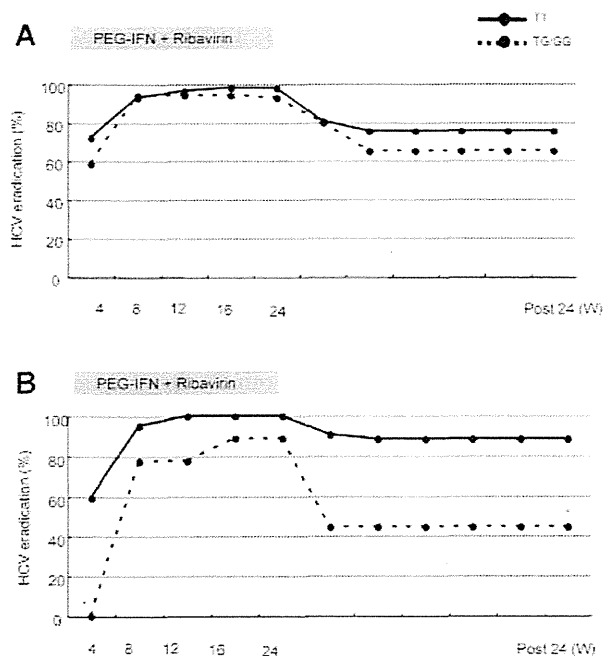


Fig. 1. Changes over time in virological response rates were confirmed in patients treated with PEG-IFN plus RBV, and the time courses were analyzed after separating the patients infected with genotypes 2a and 2b. Patients with the IL28B major (TT allele) are indicated in the figure by a continuous line and those with IL28B hetero or minor (TG or GG), by a dotted line. IL28B-TG and -GG patients showed significantly lower rates of rapid and sustained virological response, compared to IL28B-TT patients. *P*-values were two-tailed and those of less than 0.05 were considered to be statistically significant. **P* < 0.01.

Among the eight patients who withdrew from both drugs, four, including one who stopped at week 7, had achieved a sustained virological response. Among four patients who withdrew from RBV alone, three had achieved a sustained virological response. The events leading to drug withdrawal were HCC treatment ($n = 2$), general fatigue ($n = 2$), retinopathy, neuro-psychiatric event, severe dermatological symptoms suggestive of the drug-induced hypersensitivity syndrome, and arrhythmia.

DISCUSSION

Recent studies suggest that genetic variations in IL28B are strongly associated with response to therapy of chronic HCV infection with genotype 1 [Ge et al., 2009; Suppiah et al., 2009; Tanaka et al., 2009] and with spontaneous HCV clearance [Thomas et al., 2009]. In this study, univariate analyses showed that the sustained virological response was correlated significantly with IL28B polymorphism (rs8099917) as well as age, adherence to RBV and rapid virological response, and multiple logistic-regression analysis showed that only a rapid virological response was associated with a sustained virological response in all patients infected with genotype 2 (Table V). Although the IL28B

polymorphisms are not so useful for predicting the clinical outcomes of PEG-IFN plus RBV combination therapy among patients with genotype 2, compared to genotype 1, IL28B polymorphism was predictive of PEG-IFN plus RBV treatment outcomes among patients with genotype 2 and, more remarkably, among patients with genotype 2b in this study. Indeed, both rapid and sustained virological response rates according to the rs8099917 genotypes were different significantly in patients with genotype 2b but not in patients with genotype 2a. Furthermore, in the plot of virological response (Fig. 1), a stronger effect of the IL28B allele was observed in patients with genotype 2b than with genotype 2a.

It has been reported that there was no significant association between genetic variation in IL28B and response to therapy of HCV patients infected with genotype 2 or 3, indicating that the prognostic value of the risk allele for treatment response might be limited to individuals with difficult-to-treat HCV genotypes [Rauch et al., 2010]. This report lacks details of the distribution of the various genotypes. The present study agrees with a more recent report that the IL28B polymorphism was associated with a sustained virological response in patients with chronic HCV infection with genotype 2 or 3 who did not achieve a rapid virological response [Mangia et al., 2010]. In Japan, the percentage of HCV infection with genotype 1b is 70%, genotype 2a is 20% and genotype 2b is 10%, whilst other genotypes are observed only rarely. In this study, the association of IL28B polymorphism with response to therapy was analyzed in more detail, considering the subtypes 2a and 2b, and IL28B polymorphism (rs8099917) found to be linked more closely to the virological response of patients infected with genotype 2b than those with genotype 2a. A recent *in vitro* study, which constructed several chimeric virus clones between HCV-2b and HCV-JFH1 (2a), also supported subgenotypic differences between genotype 2a and 2b [Suda et al., 2010]. The authors speculated that the prognostic value of the risk allele for treatment response might be more pronounced in individuals with difficult-to-treat HCV subgenotypes, such as patients infected with genotype 2b, compared with 2a. In addition, the prevalence of the IL28B minor allele is much higher in Caucasians and African Americans than in eastern Asian populations [Thomas et al., 2009], which suggest that the effects of IL28B polymorphism could be more pronounced in non-Asian populations. In the present results, however, the sustained virological response rate of patients infected with genotype 2b was higher than that of patients with genotype 2a overall. We speculate that, among patients infected with genotype 2b, only those with the IL28B minor variant might be treatment-refractory. That possibility might be validated further by a larger cohort study with genotype 2b.

The sustained virological response rates decreased significantly with failure of adherence to RBV (Table III), which was extracted as a factor associated with sustained virological response by univariate

analysis (Table IV). Regardless of the drug adherence, end of treatment response rates of patients infected with genotype 2 were around 94–99%, but the sustained virological response rates of the patients who received a total cumulative treatment dose of RBV of <80% was reduced significantly. As reported previously, increased RBV exposure during the treatment phase was associated with an increased likelihood of a sustained virological response [McHutchison et al., 2009] and these results confirm the importance of RBV in order to prevent relapse. Furthermore, host genetic variation leading to inosine triphosphatase (ITPA) deficiency protects against hemolytic anemia in chronic hepatitis C patients receiving RBV as revealed recently [Fellay et al., 2010]. We have reported also that the *ITPA* SNP, rs1127354, is confirmed to be a useful predictor of RBV-induced anemia in Japanese patients and that the incidence of early dose reduction was significantly higher in patients with ITPA-major (CC) variant as expected and, more importantly, that a significant higher sustained virological response rate was achieved in patients with the *ITPA*-hetero/minor (CA/AA) variant with non-genotype 1 or low viral loads [Sakamoto et al., 2010].

A rapid virological response was extracted in this study as a factor associated with sustained virological response only by multivariate analysis. It has been reported recently that a rapid virological response is an important treatment predictor and that drug adherence, which is reported to affect the therapeutic efficacy in patients infected with genotype 1, had no impact on the both sustained and rapid virological responses in combination therapy for patients infected with genotype 2 [Inoue et al., 2010]. The reasons why several host factors useful for predicting the response to therapy in patients with genotype 1, such as gender, age, progression of liver fibrosis and IL28B polymorphism had no influence on the efficacy in patients with genotype 2, can be attributed to IFN-sensitive genotypes. Similarly, the other viral factors useful for predicting the response to therapy, such as viral load and amino acid substitutions in the Core and NS5A regions had no influence on treatment outcomes. In this study, patients who achieved a rapid virological response had a high sustained virological response rate, regardless of IL28B polymorphism in patients with genotype 2a but, interestingly, none of the IL28B-TG and -GG patients with genotype 2b achieved a sustained virological response (although there were nine IL28B-TG and -GG patients with genotype 2b, two could not be determined as rapid virological response because the times at which they became HCV-negative were not recorded clearly, being described as 4–8 weeks.) These results also suggest that patients with both genotype 2b and IL28B minor allele are refractory cases.

IL28B encodes a protein also known as IFN- λ 3 [O'Brien, 2009]. *IL28A* (IFN- λ 2) and *IL29* (IFN- λ 1) are found adjacent to *IL28B* on chromosome 19. These three IFN- λ cytokines, discovered in 2003 by two independent groups [Kotenko et al., 2003; Sheppard et al.,

2003] have been suggested to be involved in the suppression of replication of a number of viruses, including HCV [Robek et al., 2005; Marcello et al., 2006; Tanaka et al., 2010]. Humans have these three genes for IFN- λ , and this group of cytokines is now collectively referred to as type III IFN [Zhou et al., 2007]. IFN- λ functionally resembles type I IFN, inducing antiviral protection in vitro [Kotenko et al., 2003; Sheppard et al., 2003] as well as in vivo [Ank et al., 2006]. Type III IFN utilizes a receptor complex different from that of type I IFN, but both types of IFN induce STAT1, STAT2, and STAT3 activation by activation of a highly overlapping set of transcription factors, and the two types of IFN seem to have similar biological effects at a cellular level. Some in vitro studies have suggested that IFN- α induces expression of IFN- λ genes [Siren et al., 2005]. Other in vitro studies also suggest that IFN- λ inhibits hepatitis C virus replication through a pattern of signal transduction and regulation of interferon-stimulated genes that is distinct from IFN- α and that the anti-HCV activity of either IFN- α or IFN- λ is enhanced by a low dose of the other [Marcello et al., 2006]. A novel mechanism of the interaction between IFN- α and IFN- λ may play a key role in the suppression of HCV [O'Brien, 2009].

In conclusion, IL28B polymorphism is predictive of PEG-IFN plus RBV treatment outcomes in patients infected with genotype 2, and more remarkably with genotype 2b. These results suggest that IL-28B polymorphism affects responses to IFN-based treatment in more difficult-to-treat subpopulations of HCV patients, and that intersubgenotypic differences between genotype 2a and 2b are revealed by responses to PEG-IFN plus RBV treatment according to IL28B variants.

ACKNOWLEDGMENTS

The study is based on 10 multicenter hospitals throughout Japan, in the Kanto area (Tokyo Medical and Dental University Hospital, Musashino Red Cross Hospital, Kashiwa City Hospital, Kudanzaka Hospital, Showa General Hospital, Tsuchiura Kyodo General Hospital, Toride Kyodo General Hospital), Tokai area (Nagoya City University Hospital, Mishima Social Insurance Hospital) and Chugoku/Shikoku area (Ehime University Hospital).

REFERENCES

- Alter MJ. 1997. Epidemiology of hepatitis C. *Hepatology* 26:62S–65S.
- Ank N, West H, Bartholdy C, Eriksson K, Thomsen AR, Paludan SR. 2006. Lambda interferon (IFN-lambda), a type III IFN, is induced by viruses and IFNs and displays potent antiviral activity against select virus infections in vivo. *J Virol* 80:4501–4509.
- Fellay J, Thompson AJ, Ge DL, Gumbs CE, Urban TJ, Shianna KV, Little LD, Qiu P, Bertelsen AH, Watson M, Warner A, Muir AJ, Brass C, Albrecht J, Sulkowski M, McHutchison JG, Goldstein DB. 2010. ITPA gene variants protect against anaemia in patients treated for chronic hepatitis C. *Nature* 464:405–408.
- Fried MW, Shiffman ML, Reddy KR, Smith C, Marinos G, Goncalves FL, Haussinger D, Diago M, Carosi G, Dhumeaux D, Craxi A, Lin A, Hoffman J, Yu J. 2002. Peginterferon alfa-2a plus ribavirin for chronic hepatitis C virus infection. *N Engl J Med* 347:975–982.

- Ge D, Fellay J, Thompson AJ, Simon JS, Shianna KV, Urban TJ, Heinzen EL, Qiu P, Bertelsen AH, Muir AJ, Sulikowski M, McHutchison JG, Goldstein DB. 2009. Genetic variation in IL28B predicts hepatitis C treatment-induced viral clearance. *Nature* 461:399–401.
- Hoofnagle JH. 1994. Therapy of acute and chronic viral hepatitis. *Adv Intern Med* 39:241–275.
- Inoue Y, Hiramatsu N, Oze T, Yakushiji T, Mochizuki K, Hagiwara H, Oshita M, Mita E, Fukui H, Inada M, Tamura S, Yoshihara H, Hayashi E, Inoue A, Imai Y, Kato M, Miyagi T, Hohsui A, Ishida H, Kiso S, Kanto T, Kasahara A, Takehara T, Hayashi N. 2010. Factors affecting efficacy in patients with genotype 2 chronic hepatitis C treated by pegylated interferon alpha-2b and ribavirin: Reducing drug doses has no impact on rapid and sustained virological responses. *J Viral Hepat* 17:336–344.
- Kotenko SV, Gallagher G, Baurin VV, Lewis-Antes A, Shen M, Shah NK, Langer JA, Sheikh F, Dickensheets H, Donnelly RP. 2003. IFN-lambdas mediate antiviral protection through a distinct class II cytokine receptor complex. *Nat Immunol* 4:69–77.
- Mangia A, Thompson AJ, Santoro R, Piazzolla V, Tillmann HL, Patel K, Shianna KV, Mottola L, Petruzzellis D, Bacca D, Carretta V, Minerva N, Goldstein DB, McHutchison JG. 2010. An IL28B polymorphism determines treatment response of hepatitis C virus genotype 2 or 3 patients who do not achieve a rapid virologic response. *Gastroenterology* 139:821–827.
- Marcello T, Grakoui A, Barba-Spaeth G, Machlin ES, Kotenko SV, MacDonald MR, Rice CM. 2006. Interferons alpha and lambda inhibit hepatitis C virus replication with distinct signal transduction and gene regulation kinetics. *Gastroenterology* 131:1887–1898.
- McHutchison JG, Lawitz EJ, Shiffman ML, Muir AJ, Galler GW, McCone J, Nyberg LM, Lee WM, Ghalib RH, Schiff ER, Galati JS, Bacon BR, Davis MN, Mukhopadhyay P, Koury K, Noviello S, Pedicone LD, Brass CA, Albrecht JK, Sulikowski MS. 2009. Peginterferon alfa-2b or alfa-2a with ribavirin for treatment of hepatitis C infection. *N Engl J Med* 361:580–593.
- O'Brien TR. 2009. Interferon-alfa, interferon-lambda and hepatitis C. *Nat Genet* 41:1048–1050.
- Rauch A, Kutalik Z, Descombes P, Cai T, di Iulio J, Mueller T, Bochud M, Battagay M, Bernasconi E, Borovicka J, Colombo S, Cerny A, Dufour JF, Furrer H, Gunthard HF, Heim M, Hirschel B, Malinverni R, Moradpour D, Mullhaupt B, Witteck A, Beckmann JS, Berg T, Bergmann S, Negro F, Telenti A, Bochud PY. 2010. Genetic variation in IL28B is associated with chronic hepatitis C and treatment failure—A genome-wide association study. *Gastroenterology* 138:1240–1243.
- Robek MD, Boyd BS, Chisari FV. 2005. Lambda interferon inhibits hepatitis B and C virus replication. *J Virol* 79:3851–3854.
- Rosen HR, Gretch DR. 1999. Hepatitis C virus: Current understanding and prospects for future therapies. *Mol Med Today* 5:393–399.
- Sakamoto N, Watanabe M. 2009. New therapeutic approaches to hepatitis C virus. *J Gastroenterol* 44:643–649.
- Sakamoto N, Tanaka Y, Nakagawa M, Yatsushashi H, Nishiguchi S, Enomoto N, Azuma S, Nishimura-Sakurai Y, Kakinuma S, Nishida N, Tokunaga K, Honda M, Ito K, Mizokami M, Watanabe M. 2010. ITPA gene variant protects against anemia induced by pegylated interferon-alpha and ribavirin therapy for Japanese patients with chronic hepatitis C. *Hepatol Res* 40:1063–1071.
- Sheppard P, Kindsvogel W, Xu W, Henderson K, Schlutsmeyer S, Whitmore TE, Kuestner R, Garrigues U, Birks C, Roraback J, Ostrand C, Dong D, Shin J, Presnell S, Fox B, Haldeman B, Cooper E, Taft D, Gilbert T, Grant FJ, Tackett M, Krivan W, McKnight G, Clegg C, Foster D, Klucher KM. 2003. IL-28, IL-29 and their class II cytokine receptor IL-28R. *Nat Immunol* 4:63–68.
- Siren J, Pirhonen J, Julkunen I, Matikainen S. 2005. IFN-alpha regulates TLR-dependent gene expression of IFN-alpha, IFN-beta, IL-28, and IL-29. *J Immunol* 174:1932–1937.
- Suda G, Sakamoto N, Itsui Y, Nakagawa M, Mishima K, Onuki-Karakama Y, Yamamoto M, Funaoka Y, Watanabe T, Kiyohashi K, Nitta S, Azuma S, Kakinuma S, Tsuchiya K, Imamura M, Hiraga N, Chayama K, Watanabe M. 2010. IL-6-mediated intersubgenotypic variation of interferon sensitivity in hepatitis C virus genotype 2a/2b chimeric clones. *Virology* 407:80–90.
- Suppiah V, Moldovan M, Ahlenstiel G, Berg T, Weltman M, Abate ML, Bassendine M, Spengler U, Dore GJ, Powell E, Riordan S, Sheridan D, Smedile A, Fragomeli V, Muller T, Bahlo M, Stewart GJ, Booth DR, George J. 2009. IL28B is associated with response to chronic hepatitis C interferon-alpha and ribavirin therapy. *Nat Genet* 41:1100–1104.
- Tanaka Y, Nishida N, Sugiyama M, Kurosaki M, Matsuura K, Sakamoto N, Nakagawa M, Korenaga M, Hino K, Hige S, Ito Y, Mita E, Tanaka E, Mochida S, Murawaki Y, Honda M, Sakai A, Hiasa Y, Nishiguchi S, Koike A, Sakaida I, Imamura M, Ito K, Yano K, Masaki N, Sugauchi F, Izumi N, Tokunaga K, Mizokami M. 2009. Genome-wide association of IL28B with response to pegylated interferon-alpha and ribavirin therapy for chronic hepatitis C. *Nat Genet* 41:1105–1109.
- Tanaka Y, Nishida N, Sugiyama M, Tokunaga K, Mizokami M. 2010. Lambda-Interferons and the single nucleotide polymorphisms: A milestone to tailor-made therapy for chronic hepatitis C. *Hepatol Res* 40:449–460.
- Thomas DL, Thio CL, Martin MP, Qi Y, Ge D, O'Huigin C, Kidd J, Kidd K, Khakoo SI, Alexander G, Goedert JJ, Kirk GD, Donfield SM, Rosen HR, Tobler LH, Busch MP, McHutchison JG, Goldstein DB, Carrington M. 2009. Genetic variation in IL28B and spontaneous clearance of hepatitis C virus. *Nature* 461:798–801.
- Zeuzem S, Feinman SV, Rasenack J, Heathcote EJ, Lai MY, Gane E, O'Grady J, Reichen J, Diago M, Lin A, Hoffman J, Brunda MJ. 2000. Peginterferon alfa-2a in patients with chronic hepatitis C. *N Engl J Med* 343:1666–1672.
- Zhou Z, Hamming OJ, Ank N, Paludan SR, Nielsen AL, Hartmann R. 2007. Type III interferon (IFN) induces a type I IFN-like response in a restricted subset of cells through signaling pathways involving both the Jak-STAT pathway and the mitogen-activated protein kinases. *J Virol* 81:7749–7758.

Analysis of Interferon Signaling by Infectious Hepatitis C Virus Clones with Substitutions of Core Amino Acids 70 and 91^{†‡§}

Yusuke Funaoka,^{1†} Naoya Sakamoto,^{1,2*†} Goki Suda,¹ Yasuhiro Itsui,¹ Mina Nakagawa,^{1,2} Sei Kakinuma,¹ Takako Watanabe,¹ Kako Mishima,¹ Mayumi Ueyama,¹ Izumi Onozuka,¹ Sayuri Nitta,¹ Akiko Kitazume,¹ Kei Kiyohashi,¹ Miyako Murakawa,¹ Seishin Azuma,¹ Kiichiro Tsuchiya,¹ and Mamoru Watanabe¹

Department of Gastroenterology and Hepatology¹ and Department for Hepatitis Control,² Tokyo Medical and Dental University, Tokyo, Japan

Received 13 December 2010/Accepted 24 March 2011

Substitution of amino acids 70 and 91 in the hepatitis C virus (HCV) core region is a significant predictor of poor responses to peginterferon-plus-ribavirin therapy, while their molecular mechanisms remain unclear. Here we investigated these differences in the response to alpha interferon (IFN) by using HCV cell culture with R70Q, R70H, and L91M substitutions. IFN treatment of cells transfected or infected with the wild type or the mutant HCV clones showed that the R70Q, R70H, and L91M core mutants were significantly more resistant than the wild type. Among HCV-transfected cells, intracellular HCV RNA levels were significantly higher for the core mutants than for the wild type, while HCV RNA in culture supernatant was significantly lower for these mutants than for the wild type. IFN-induced phosphorylation of STAT1 and STAT2 and expression of the interferon-inducible genes were significantly lower for the core mutants than for the wild type, suggesting cellular unresponsiveness to IFN. The expression level of an interferon signal attenuator, SOCS3, was significantly higher for the R70Q, R70H, and L91M mutants than for the wild type. Interleukin 6 (IL-6), which upregulates SOCS3, was significantly higher for the R70Q, R70H, and L91M mutants than for the wild type, suggesting interferon resistance, possibly through IL-6-induced, SOCS3-mediated suppression of interferon signaling. Expression levels of endoplasmic reticulum (ER) stress proteins were significantly higher in cells transfected with a core mutant than in those transfected with the wild type. In conclusion, HCV R70 and L91 core mutants were resistant to interferon *in vitro*, and the resistance may be induced by IL-6-induced upregulation of SOCS3. Those mechanisms may explain clinical interferon resistance of HCV core mutants.

Hepatitis C virus (HCV) is one of the most important pathogens causing liver-related morbidity and mortality. Approximately 3% of the worldwide population is infected with HCV, which represents 170 million people, and 3 million to 4 million individuals are newly infected each year (33, 47, 62). There is no therapeutic or prophylactic vaccine available for HCV. Antiviral treatment has been shown to improve liver histology and decrease the incidence of hepatocellular carcinoma in chronic hepatitis C (CHC) (17, 64). Current therapies for CHC consist of treatment with pegylated interferon (peg-IFN), which acts both as an antiviral and as an immunoregulatory cytokine, and ribavirin (RBV), an antiviral prodrug that interferes with RNA metabolism (16, 31). However, less than 50% of patients infected with HCV genotype 1 treated in this way achieve a sustained virological response (SVR) or a cure of the infection (14, 16). Given this situation, gaining a detailed understanding of the molecular mechanisms of interferon (IFN) resistance has been a high priority in academia and industry.

The response to peg-IFN-plus-RBV treatment is affected by

several viral and host factors, including age, gender (22, 23), grade of liver fibrosis (21, 42), HCV genotype, and serum viral load (14, 59). Several viral genetic factors influence treatment outcomes, including mutations in NS5A-interferon sensitivity determining region (ISDR) (13, 38) and the core region (4, 6). Akuta et al. reported that HCV-core amino acid substitutions at positions 70 and 91 are significantly correlated with poor responses to peg-IFN-plus-RBV therapy (6) and with increased hepatocarcinogenesis (2, 3). Furthermore, it was reported recently that the core amino acid 70 and amino acid 91 substitutions are associated with a poor response to peg-IFN, RBV, and telaprevir combination therapy, respectively (1). However, the underlying molecular mechanisms of such distinct biological properties of the core 70/91 mutations are poorly understood.

In this study, we have analyzed virus infection and replication kinetics and response to interferon treatment using the HCV-JFH1 cell culture system (HCVcc) (60, 65). We constructed HCVcc expressing virus with substitutions of core amino acid 70 and amino acid 91 (R70Q, R70H, and L91M). The core mutant HCV clones were compared in terms of intracellular replication, infectious virus production, and sensitivity to alpha interferon (IFN- α). Here we have shown that the differences in sensitivity to IFN are attributable to upregulated overexpression of the cellular interferon signal attenuator SOCS3 and that this upregulation is caused by overexpression of interleukin-6 (IL-6).

* Corresponding author. Mailing address: Department of Gastroenterology and Hepatology, Tokyo Medical and Dental University, 1-5-45 Yushima, Bunkyo-ku, Tokyo 113-8519, Japan. Phone: 81 3-5803-5877. Fax: 81 3-5803-0268. E-mail: nsakamoto.gast@tmd.ac.jp.

† Y.F. and N.S. contributed equally to this work.

§ Supplemental material for this article may be found at <http://jvi.asm.org/>.

⁷ Published ahead of print on 13 April 2011.

MATERIALS AND METHODS

Reagents. Recombinant human IFN- α 2b was from Schering-Plough (Kenilworth, NJ). Beta-mercaptoethanol was from Wako (Osaka, Japan). Antibodies used were SOCS3 and SOCS1, which were from Cell Signaling (Beverly, MA), HCV core (Abcam, Cambridge, MA), NS5A (BioDesign, Saco, ME), GRP78, GADD153/CHOP (Santa Cruz Biotechnology, Santa Cruz, CA), disulfide isomerase (PDI) (Stressgen Biotechnologies, Victoria, British Columbia, Canada), and beta-actin antibody (Sigma). Secondary antibodies were peroxidase-labeled anti-mouse, anti-rabbit antibody (GE Healthcare, Connecticut), donkey anti-goat IgG-horseradish peroxidase (HRP) antibody (Santa Cruz Biotechnology, Santa Cruz, CA), and Alexa 405-labeled goat anti-mouse and Alexa 568-labeled donkey anti-rabbit IgG antibodies (Invitrogen, Carlsbad, CA).

Cells and cell culture. Huh7 cells were maintained in Dulbecco's modified minimal essential medium (DMEM) (Sigma Chemical Co, St. Louis, MO) supplemented with 2 mmol/liter L-glutamine and 10% fetal bovine serum at 37°C under 5.0% CO₂.

Sequence analyses. Nucleotide sequences were read from both strands using BigDye Terminator cycle sequencing ready reaction kits (Applied Biosystems, Foster City, CA) and an automated DNA sequencer (ABI Prism 310 genetic analyzer; Applied Biosystems).

Establishment of mutant HCV clones. In order to introduce various mutations into the core region of JFH1, plasmid pJFH1full was digested with EcoRI and BsiWI, and then the DNA fragment encompassing nucleotides 1 to 456 was subcloned into the pGEM-T Easy vector (Promega, Madison, WI). The following mutations were introduced into the DNA fragment in the subcloning vector by site-directed mutagenesis (Quick-Change II site-directed mutagenesis kit; Stratagene): R70Q, R70H, L91M, and GKPG77-80KKKK. Finally, the EcoRI-BsiWI fragments were subcloned back into the parental plasmid, pJFH1full.

In vitro RNA synthesis and transfection. Full-length HCV expression plasmids were as follows: pJFH1full, which encodes the full-length HCV-JFH1 sequence (60), pR70Q, pR70H, pL91M, and p7780K. These plasmids were linearized at their 3' ends and used as templates for HCV RNA synthesis using the RiboMax large-scale RNA production system (Promega, Madison, WI). After DNase I (RQ-1 RNase-free DNase; Promega) treatment, the transcribed HCV RNA was purified using Isogen reagent (Nippon Gene, Tokyo, Japan). For the RNA transfection, Huh7 cells were washed twice in phosphate-buffered saline (PBS), and 5×10^6 cells were suspended in Opti-MEM I (Invitrogen, Carlsbad, CA) containing 10 μ g of HCV RNA, transferred into a 4-mm electroporation cuvette, and finally subjected to an electric pulse (1,050 μ F and 270 V) using the Easy Jet system (EquiBio, Middlesex, United Kingdom). After electroporation, the cell suspension was left for 5 min at room temperature and then incubated under normal culture conditions in a 10-cm-diameter cell culture dish. Forty-eight hours after transfection, the levels of HCV replication and viral protein expression were detected by real-time PCR and Western blotting.

HCVcc infection analyses. Huh7 cells were plated on 12-well plates at a density of 1.2×10^4 cells per well. Supernatants from HCV RNA-transfected cells were inoculated onto each well at a titer of 8×10^5 copies/well (quantified by real-time reverse transcriptase PCR [RT-PCR]). Forty-eight hours after infection, various amounts of interferon were added, and the cells were harvested after 72 h of the interferon treatment (48).

RNA extraction, cDNA synthesis, and real-time RT-PCR analysis. For the detection of HCV RNA in culture supernatant, the supernatant was passed through a 0.45- μ m filter (Millex-HA, Millipore, Bedford, MA) and stored at -80°C until use. Protocols and primers for the real-time RT-PCR analysis of HCV RNA have been described previously (48). For the detection of endogenous mRNAs, total cellular RNA was isolated using an RNeasy Mini kit (Qiagen, Valencia, CA). Two micrograms of total cellular RNA was used to generate cDNA from each sample using SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA). Expression of mRNA was quantified using the TaqMan universal PCR master mix (Applied Biosystems, Foster City, CA) and the ABI 7500 real-time PCR system (Applied Biosystems, Foster City, CA).

Luciferase assays. Luciferase activities were measured using a luminometer (Lumat LB9501; Promega) using the Dual-Luciferase reporter assay system (Promega). Assays were performed in triplicate.

Western blot analysis. Western blotting was carried out as described previously (24, 53, 63). Briefly, 10 mg of total cell lysate was separated using NuPAGE 4%–12% Bis-Tris gels (Invitrogen) and blotted onto a polyvinylidene fluoride (PVDF) Western blotting membrane (Roche). The membrane was incubated with the primary antibodies followed by a peroxidase-labeled anti-IgG antibody and visualized by chemiluminescence using the ECL Western blotting analysis system (Amersham Biosciences, Buckinghamshire, United Kingdom).

Immunohistochemistry. HCV-transfected Huh7 cells were cultured on 18-mm round micro cover glasses (Matsunami, Tokyo, Japan). For detection of HCV core, lipid droplet, and endoplasmic reticulum (ER), cells were fixed with cold acetone for 15 min. The cells were incubated with the primary antibodies for 1 h at 37°C. The fluorescent secondary antibodies were Alexa 405 goat anti-mouse and 568 donkey anti-rabbit IgG antibodies (Invitrogen, Carlsbad, CA). Lipid droplets (LDs) were visualized by using Bodipy 493/503 dye (Invitrogen). Cells were mounted with Vecta Shield mounting medium and 4',6-diamidino-2-phenylindole (DAPI) (Vector Laboratories, Burlingame, CA) and visualized by using a confocal laser scanning microscope (FV10i; Olympus, Tokyo, Japan).

Calculation of 50% effective concentrations (EC₅₀). The EC₅₀ was calculated as the concentration of IFN required for 50% reduction in HCV RNA expression. We used the probit regression analysis to obtain values.

Statistical analyses. Statistical analyses were performed by using Welch's *t* test. *P* values of less than 0.05 were considered statistically significant.

RESULTS

HCV core 70/91 mutants show resistance to IFN treatment.

First, we investigated sensitivity to IFN treatment of the HCV core mutant R70Q, R70H, and L91M virus clones and compared them to the wild type. The wild type and core mutants were transfected into Huh7 cells, which were cultured in the presence of various concentrations of IFN- α for 48 h. RNA was extracted from the cells and culture supernatant, and the level of HCV RNA was quantified by real-time RT-PCR. Although the levels of supernatant HCV RNA did not differ between the wild type and core mutants (Fig. 1A), the levels of cellular HCV RNA showed that all three core mutants were significantly resistant to IFN compared to the wild type, with EC₅₀s of 5.0 IU/ml, 48 IU/ml, 32 IU/ml, and 47 IU/ml for the R70Q, R70H, L91M, and mutants and the wild type, respectively (Fig. 1B). To exclude the possible effects on interferon signaling by the input HCV RNA, we performed interferon sensitivity analyses by HCVcc infection. As shown in Fig. 1C, the interferon sensitivities of HCV core mutants and the wild type were consistent with the results of HCV RNA transfection. Similarly, according to Western blotting, the core mutants were more resistant to IFN treatment than the wild type (Fig. 1D).

Core mutants show decreased secretion of viral particles.

To determine the mechanisms underlying the resistance to interferon, we compared baseline virus expression levels in cells and culture supernatants. The three core mutants, carrying R70Q, R70H, and L91M, expressed significantly higher levels of intracellular HCV RNA than the wild type, as well as the 7780K clone. (Fig. 2A). 7780K was a negative-control clone that lacked virus particle secretion (37). On the contrary, these core mutants released significantly smaller amounts of HCV RNA into the culture supernatant than the wild type, as well as the negative-control 7780K clone. (Fig. 2B). Consistent with the HCV RNA data, Western blotting showed that cellular HCV core protein levels were higher for the core amino acid 70/91 mutants than the wild type (Fig. 2C). These results suggested that the core 70/91 mutant clones were partially defective in the secretion of infectious virus particles.

Subcellular localization of wild-type and mutant core proteins and lipid droplets. It has been reported that HCV core protein localizes on the cellular LD membrane and may mediate encapsidation of viral genomic RNA and subsequent virus assembly (35, 36). Therefore, we visualized the subcellular localization of wild-type and mutant core proteins in rela-

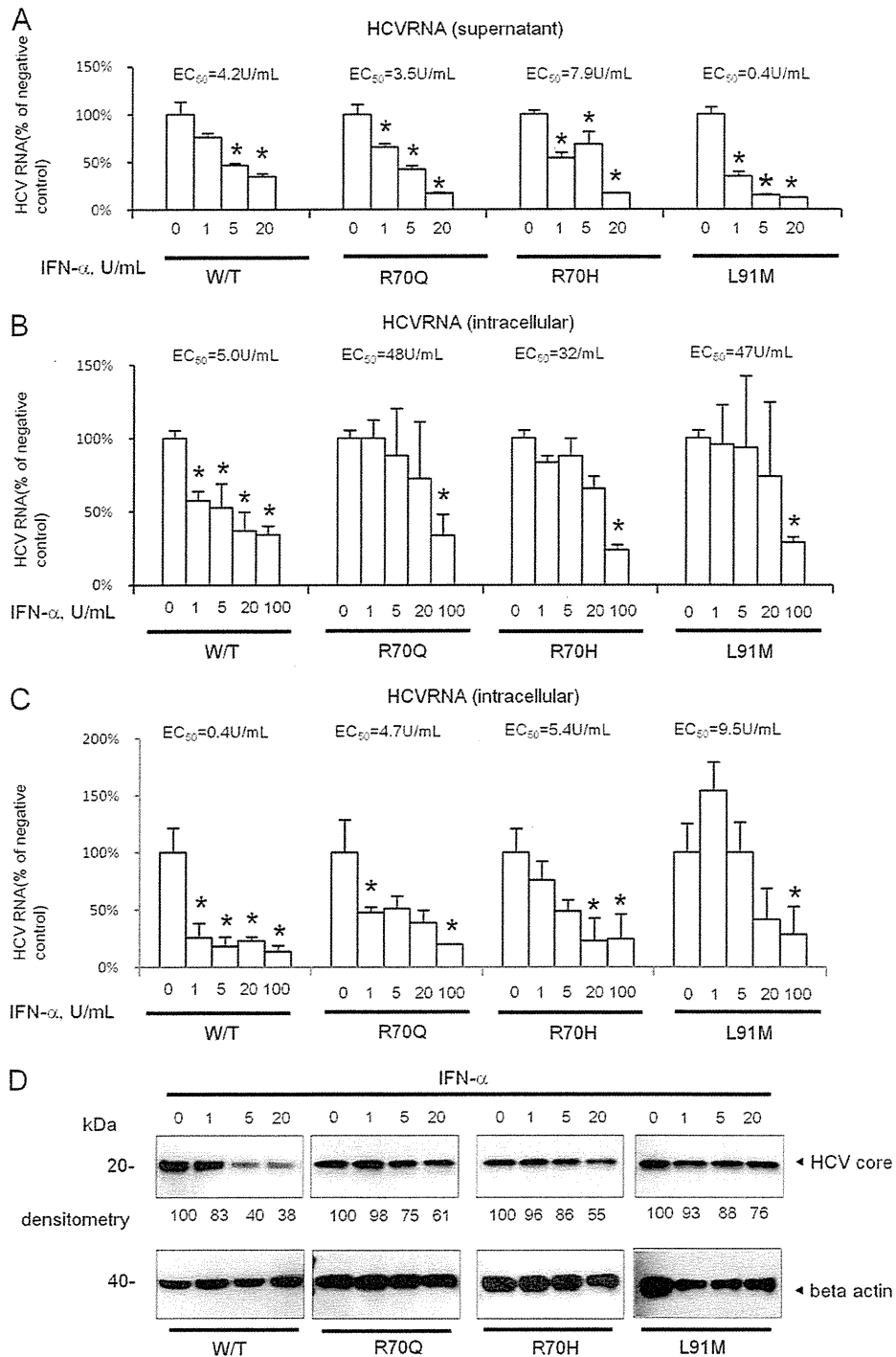


FIG. 1. Comparison of interferon sensitivity between HCV wild type and core mutant clones. The wild type and core mutants were transfected into Huh7 cells and cultured in the presence of IFN- α 2b at concentrations ranging from 0 to 100 U/ml. (A) The culture supernatant of HCV-transfected Huh7 cells was collected 72 h after transfection, and the levels of HCV core antigen in the culture supernatant were measured. The values are displayed as percentages of those for the IFN-untreated control. The experiments were repeated three times, and representative results are shown. (B) Expression of intracellular HCV RNA. Cellular RNA was harvested at 72 h posttransfection. HCV RNA was quantified by real-time RT-PCR. The values are displayed as percentages of those for the IFN-untreated control. (C) Expression of intracellular HCV RNA. Cellular RNA was harvested at 72 h postinfection. HCV RNA was quantified by real-time RT-PCR. The values are displayed as percentages of those for the IFN-untreated control. In panels A through C, asterisks indicate *P* values of less than 0.05, compared to results for the interferon-negative control. (D) Western blotting was performed to assess intracellular suppression of HCV core protein. Ten micrograms of harvested cell lysates were subjected to Western blotting using anti-HCV core antibodies. Densitometry of core protein was performed, and results are shown as percentages of the results for an IFN-negative sample.