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Original article

Serum interleukin-6 levels correlate with resistance to treatment of chronic hepatitis C infection with pegylated-interferon-α2b plus ribavirin

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Background: Interleukin (IL)-6, a pleiotropic cytokine, is increased in various types of chronic liver disease, including chronic hepatitis C (CHC). It was reported recently that IL-6 is associated with insulin resistance, iron metabolism and interferon resistance, which may affect the outcome of antiviral treatment. In this study, we investigated the association of serum IL-6 levels with outcomes of pegylated interferon (PEG-IFN) plus ribavirin (RBV) combination therapy.

Methods: We included 149 CHC patients and measured serum IL-6 levels at baseline and at 4, 8 and 12 weeks, and the end of treatment in 49 patients. We performed univariate and multivariate regression analyses for the association of IL-6 levels and clinical and laboratory parameters and treatment responses.

Results: Serum IL-6 levels were significantly higher in CHC patients than healthy subjects. Pretreatment IL-6 levels of male patients were inversely correlated with sustained virological response (SVR) in univariate analysis (P=0.012). In male patients with SVR, serum IL-6 levels decreased significantly at 4 weeks of treatment (P=0.029) and remained significantly lower than those of non-SVR patients after 4, 8 and 12 weeks of PEG-IFN plus RBV therapy.

Conclusions: Our results suggest that baseline levels of IL-6, as well as their decrease during treatment, are correlated to outcomes of PEG-IFN plus RBV therapy in male patients. Further analyses of IL-6 may provide new strategies for difficult-to-treat CHC patients and prevention of hepatocarcinogenesis.

Introduction

HCV is one of the main causes of chronic liver disease. Chronic hepatitis can result in hepatic fibrosis, liver cirrhosis [1] and hepatocellular carcinoma (HCC) [2,3]. The current standard of care consists of a combination of pegylated interferon (PEG-IFN)-α plus ribavirin (RBV) for 48 weeks for genotypes 1 and 4, and for 24 weeks for other genotypes [4–6]. Several host factors, such as gender, age, serum HCV RNA level, progression of liver fibrosis [7] and interleukin (IL)-28B [8,9], as well as virological factors such as virus genotype, the number of mutations in the interferon sensitivity determining region (ISDR) [10] and substitutions of amino acids 70 and 91 in the core region [11], have been useful for predicting the response to PEG-IFN/RBV therapy.

IL-6 is a pleiotropic cytokine that plays a role in the acute phase response [12]. IL-6 is released from various cells, that is, leukocytes, fibroblasts, endothelial cells and macrophages, in response to following systematic or local infection, tissue injury and inflammation [13]. As for the liver, IL-6 is produced mainly by Kupffer cells [14] and induces the production of the acute phase proteins, C-reactive protein and haptoglobin [12]. Previous studies reported that serum IL-6

levels were increased, compared to healthy subjects, in patients with some liver diseases, such as chronic viral hepatitis due to HBV [15] or HCV infection [14], alcoholic hepatitis [16], non-alcoholic steatohepatitis [17] and fulminant hepatitis [18]. It also has been reported that HCV infection induced Toll-like receptor (TLR)-4 expression and increased IL-6 secretion by human B-cells *in vitro* [19], and that serum IL-6 levels in patients of chronic hepatitis C (CHC) correlated with the histological activity index and viral load [20]. Moreover, Naugler *et al.* [21] reported that oestrogen-mediated inhibition of IL-6 production reduced the liver cancer risk in females and a similar mechanism could account for the gender bias in liver cancer in humans.

Recent studies have shown that IL-6 is associated with insulin resistance (obesity and diabetes mellitus) [22–24] and iron metabolism [25], which are probably related to drug resistance in CHC. However, to date, little has been reported on the relationships between serum IL-6 levels and treatment outcomes of PEG-IFN/RBV combination therapy. Here, we conducted a cohort study to investigate whether serum IL-6 levels influenced treatment outcomes and to clarify whether IL-6 levels may be useful predictors of response to PEG-IFN/RBV combination therapy.

Methods

Patients and methods

Between December 2004 and April 2010, a total of 720 HCV-RNA-positive patients received PEG-IFN/RBV combination therapy at Tokyo Medical and Dental University Hospital (Tokyo, Japan) and associated participating hospitals in the Ochanomizu-Liver Conference Study Group. Of these, 149 patients were included in this study. All patients had histologically or clinically proven chronic active hepatitis and they were positive for antibodies against HCV and serum HCV RNA by reverse transcription (RT)-PCR. We excluded patients positive for hepatitis B surface antigen, HIV and antinuclear antibodies. In addition, patients with malignancies, infectious disease, inflammatory bowel disease, autoimmune connective tissue disease and other forms of hepatitis known to affect serum IL-6 levels, such as primary biliary cirrhosis, alcoholic liver disease and autoimmune liver disease, were excluded. We included patients with good drug adherence, who received >80% of scheduled doses of both PEG-IFN and RBV. Patients were excluded if any of the following data were absent: ISDR, core amino 70 and 91 acids substitutions and liver biopsy. Patients who were undergoing extended treatment were also excluded from the study. As control populations, we analysed levels of IL-6 in patients with chronic HBV infection (n=9) and autoimmune liver disease (n=8).

Treatment regimens

The dose of PEG-IFN and RBV was adjusted according to body weight. We treated patients with PEG-IFN-α2b (Peg-Intron; Schering–Plough Nordic Biotech, Stockholm, Sweden) 1.2–1.5 μg/kg/week subcutaneously and oral RBV (Rebetol; Schering–Plough Nordic Biotech) at 600 mg/day (body weight <60 kg), 800 mg/day (body weight between 60 and 80 kg), or 1,000 mg/day (body weight >80 kg) according to the manufacturer's information. The duration of combination therapy was determined according to virus genotype; that is, 48 weeks treatment for genotype 1 and 24 weeks for genotype 2. The dose of PEG-IFN-α2b and RBV was reduced when necessitated by side effects, that is, anaemia, decrease of neutrophils and platelets, dermatological symptoms, appetite loss and depressive tendency. Patients visited hospital and provided blood samples every 4 weeks after the first administration. Serum IL-6 levels were measured before treatment and at 4, 8 and 12 weeks, and the last administration. Serum HCV RNA was measured by a quantitative PCR assay with a sensitivity of 100 copies/ml (National Genetics Institute, Los Angeles, CA, USA). We defined SVR as undetectable serum HCV RNA at 24 weeks after the end of treatment. This study employed an intention-to-treat analysis; patients whose treatments were discontinued because of side effects or disappearance of HCV in the serum were included.

Laboratory tests and IL-6 assay

The following factors were analysed to determine whether they were related to the IL-6 levels: age, gender, body mass index (BMI), grade of inflammation and stage of fibrosis on liver biopsy, pretreatment biochemical parameters, such as haemoglobin (Hb), platelet count (Plt), alanine transaminase (ALT) level, γ-glutamyl transpeptidase (γ-GTP), total cholesterol (T-Chol), triglyceride (TG), low-density lipoprotein (LDL) cholesterol, serum HCV RNA level, amino acid substitutions in the ISDR, substitutions of amino acids 70 and 91 in the HCV core region, and sustained virological response (SVR) rate. Furthermore, we also analysed transition of serum IL-6 during PEG-IFN/RBV therapy considering SVR patients and non-SVR patients separately. For 49 of the 149 patients, serum IL-6 levels were measured before treatment and at 4, 8 and 12 weeks, and the end point of treatment. Serum samples from 10 individuals without hepatitis C (5 males and 5 females) were used as controls. Serum samples were frozen at -70°C for later assay. IL-6 was measured by chemiluminescent enzyme immunoassay (CLEIA) using a cartridge for IL-6 measurement (Human IL-6 CLEIA Fujirebio, Fujirebio, Tokyo); the detection limit was 0.2 pg/ml and the upper limit of normal range was 4 pg/ml. CLEIA had a sensitivity of 0.11 pg/ml [26]. 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.

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/m^2; 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/m²	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, ×10⁴ 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[a]				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. [a]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.

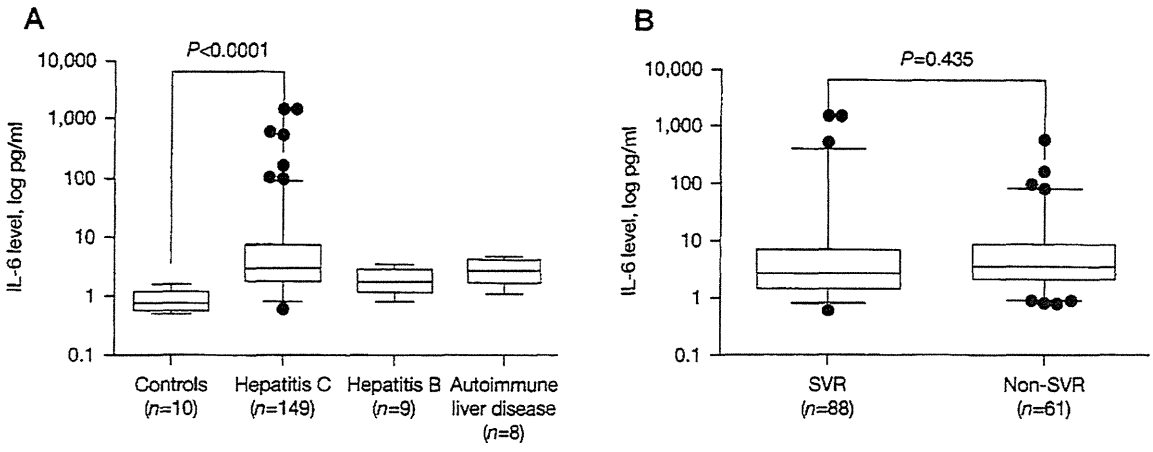
1.5 [0.8–4.8]; *P*=0.029) especially in patients with SVR (Figure 2A), whereas IL-6 did not change significantly in the first 4 weeks in female patients with SVR as well as with non-SVR (3.4 pg/ml [1.5–14.5] versus 2.3 pg/ml [1.0–91.8]; *P*=0.546; Figure 2). Among non-SVR patients, there were 7 out of 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-κβ, 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 hypertriglycaemia 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	Multivariate analysis		
			P-value	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	-	-	-	-

[a]Median (range) or *n* values are shown. [b]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; 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 genotye 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	Multivariate analysis		
			P-value	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, $\times 10^4$ 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[b]	-	-	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	-	-	-	-

[a]Median (range) or n values are shown. [b]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; 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	Multivariate analysis		
			P-value	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-chol, 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	–	–	–	–

[a]Median (range) or *n* values are shown. [b]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; 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.

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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

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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/m²	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, ×10⁴ 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-chol, 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[b]	–	–	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	–

[a]Median (range) or *n* values are shown. [b]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; 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.

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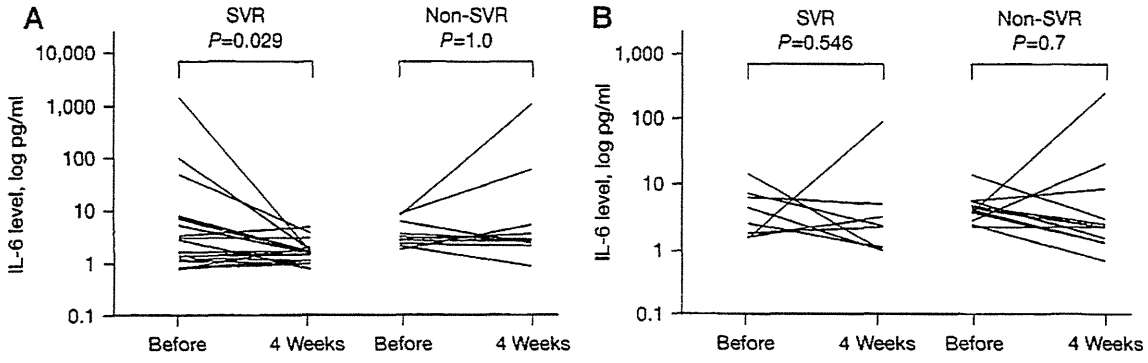
Figure 2. Serum IL-6 levels before and after 4 weeks of pegylated interferon plus ribavirin therapy



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)
		P-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)
		P-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)
		P-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)
		P-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)
		P-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)
		P-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.

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

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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

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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-λ3

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-λ3 (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-α2b (Peg-Intron, Schering-Plough Nordic Biotech, Stockholm, Sweden, at a dose of 1.2–1.5 μg/kg subcutaneously once a week) or PEG-IFN-α2a (Pegasys; Roche, Basel, Switzerland, at a dose of 180 μg 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.

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 ($\times 10^{-3}$/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.
[a]Data are shown as median (range) values.
[b]Data are expressed as mean ± SD.
[c]Data are shown as log(IU/ml)).

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 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 ($\times 10^{-3}$/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.
[a]Data are show as median (range) values.
[b]Data are expressed as mean ± SD.
[c]Data 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.

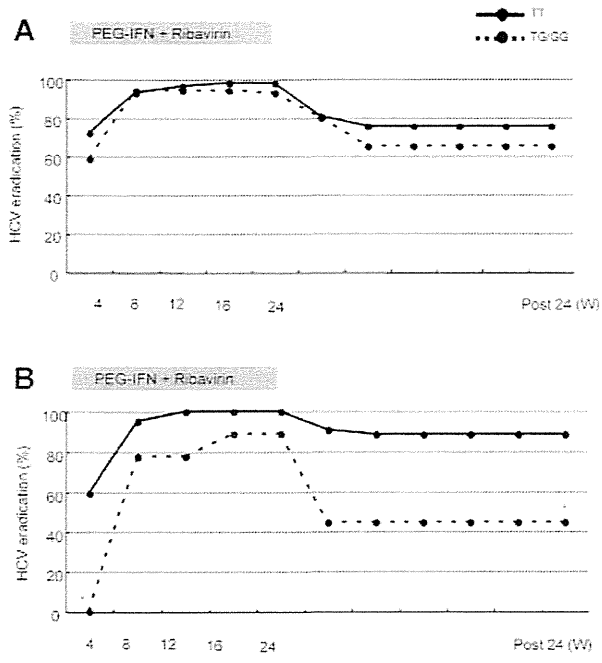

Fig. 1. Changes over time in virological response rates were confirmed in patients treated with PEG-IFN plus RBV, and the time courses were analyzed after separating the patients infected with genotypes 2a and 2b. Patients with the IL28B major (TT allele) are indicated in the figure by a continuous line 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-λ3 [O'Brien, 2009]. *IL28A* (IFN-λ2) and *IL29* (IFN-λ1) 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.

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Analysis of Interferon Signaling by Infectious Hepatitis C Virus Clones with Substitutions of Core Amino Acids 70 and 91▽§

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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 situations, 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).

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