TABLE I. Baseline Characteristics of the Study Patients (n = 274)

Age (years)	55.9 ± 11.2
Sex (female/male)	135 (49.3)/139 (50.7)
Body weight (kg)	58.0 ± 10.4
Alanine aminotransferase (IU/L)	64.5 ± 56.3
Aspartate aminotransferase (IU/L)	53.7 ± 42.2
Gamma-glutamyl transpeptidase (IU)	49.7 ± 48.5
Alkaline phosphatase (IU/L)	267.9 ± 100.6
Albumin (g/dl)	4.07 ± 0.38
Total bilirubin (mg/dl)	0.79 ± 0.30
White blood cell count (/μl)	4933 ± 1331
Hemoglobin (g/dl)	14.0 ± 1.4
Platelet count ($\times 10^3$/μl)	164 ± 50
Liver histology-activity (A0/A1/A2/A3)[a]	2 (0.7)/147 (55.3)/99 (37.2)/18 (6.8)
Liver histology-fibrosis (F0/F1/F2/F3)[a]	31 (11.6)/122 (45.9)/75 (28.2)/38 (14.3)
HCV-RNA concentration ($\log_{10}$ IU/ml)[b]	6.34 ± 0.54
Genetic polymorphisms near the *IL28B* gene (TT/TG/GG)[b]	202 (73.7)/69 (25.2)/6 (2.2)
Amino acid at HCV core 70 (wild type/mutant type/both)[c]	204 (74.4)/64 (23.4)/6 (2.2)
Response (SVR/relapse/NR)	121 (44.2)/88 (32.1)/65 (23.7)

HCV, hepatitis C virus; SVR, sustained virologic response; NR, no-response.
Percentages are shown in parentheses.
[a]Liver biopsy was not performed in eight patients.
[b]rs8099917 genetic polymorphism
[c]Before the treatment.

carried HCV with the wild-type AA at residue 70 of the HCV core region, 64 patients (23.4%) carried the mutant-type AA at residue 70, and both the wild-type AA and the mutant-type AA were identified at residue 70 in the remaining six patients (3.5%).

As a final outcome, 121 patients (44.2%) achieved a sustained virologic response, 88 patients (32.1%) relapsed, and the remaining 65 patients (23.7%) showed no-response (Fig. 1). Treatment was discontinued before 48 weeks in 11 of 65 patients who showed no-response because HCV-RNA remained detectable in serum 24 weeks after starting the therapy. The identity of the AA 70 of the core region of HCV was determined after the treatment in serum obtained at the discontinuation of the therapy in these 11 patients. Table II shows the association between the genetic polymorphisms of the rs8099917 near the *IL28B* gene, the AA substitutions of the HCV core region residue 70, and the outcome of the combination therapy. The wild-type AA was more frequently identified at residue 70 in patients with the TT genotype in comparison to those with the TG/GG genotype (82.2% vs. 52.8%, $P < 0.0001$). The rate of a sustained virologic response was significantly higher in patients with the TT genotype than those with the TG/GG genotype (107 of 202 patients, 53.0% vs. 14 of 72 patients, 19.4%, $P < 0.0001$), as well as being higher in patients carrying HCV with the wild-type AA at residue 70 of the core region than those with the mutant-type AA at this residue (101 of 204 patients, 49.5% vs. 19 of 64 patients, 29.7%, $P = 0.0083$, one patient had both the wild-type and the mutant-type AAs).

Comparison of the Amino Acid at Residue 70 of the HCV Core Region Before and After the Combination Therapy in Patients Who Showed a Relapse or No-Response

Table III shows the comparison of the AA at residue 70 of the HCV core region before and after the combination therapy in patients who showed a relapse or no-response, according to the genetic polymorphisms of the rs8099917 near the *IL28B* gene. In three of five

TABLE II. Association Between the Genetic Polymorphisms Near the *IL28B* Gene, the Amino Acid at the HCV Core Region Residue 70, and the Final Outcome of Peginterferon/Ribavirin Combination Therapy

Genetic polymorphism of rs8099917 near *IL28B* gene	Amino acid at residue 70 of the HCV core region		
	Wild type (n = 204)	Mutant type (n = 64)	Wild type + mutant type (n = 6)
TT (n = 202)	166 (92/60/14)	31 (14/9/8)	5 (1/2/2)
TG/GG (n = 72)	38 (9/9/20)	33 (5/7/21)	1 (0/1/0)

Outcomes of the combination therapy with peginterferon and ribavirin are shown in parentheses as sustained virologic response/relapse/no-response.

TABLE III. Amino Acid Substitutions of HCV Core Region Residue 70 Before and After the Combination Therapy With Peginterferon and Ribavirin in No-Responders or Relapsers

Amino acid at HCV core region residue 70	After treatment		
Before treatment	Wild type	Wild + Mutant	Mutant type
(A) Genetic polymorphisms near the *IL28B* gene (rs8099917): TT (n = 91)			
No-responders (n = 24)			
Wild type (n = 14)	13	1	0
Wild + mutant (n = 2)	0	0	2
Mutant type (n = 8)	0	0	8
Relapsers (n = 71)			
Wild type (n = 60)	60	0	0
Wild + mutant (n = 2)	0	1	1
Mutant type (n = 9)	0	0	9
(B) Genetic polymorphisms near the *IL28B* gene (rs8099917): TG/GG (n = 57)			
No-responders (n = 41)			
Wild type (n = 20)	19	1	0
Wild + mutant (n = 0)	0	0	0
Mutant type (n = 21)	0	0	21
Relapsers (n = 17)			
Wild type (n = 9)	9	0	0
Wild + mutant (n = 1)	0	1	0
Mutant type (n = 7)	0	0	7

HCV, hepatitis C virus.

patients in whom both the wild-type and mutant-type AAs had been identified at residue 70 of the HCV core region before treatment, only the mutant-type AA was identified at this residue after the treatment. All three of these patients (two no-responders and one relapser) had the TT genotype of the rs8099917. Both the wild-type and mutant-type AAs were identified at residue 70 after the treatment in two no-responders in whom only the wild-type AA had been identified before the treatment. One of them had the TT genotype at the rs 8099917 and the other patient was TG heterozygous. No change in the HCV core region residue 70 was found after the treatment in patients with the mutant-type AA at this residue before the treatment.

DISCUSSION

The present study investigated whether the combination therapy with PEG-IFN and ribavirin causes the mutation of residue 70 of the HCV core region, and whether the genetic polymorphisms of the rs8099917 locus near the *IL28B* gene influence this mutation. It is thought to be important to verify this issue, because it may be advisable to avoid the treatment of patients who have the TG/GG genotypes by the combination therapy with PEG-IFN and ribavirin so as to avoid an acquisition of the further resistance to emerging new therapies against HCV, as well as to avoid a potential enhancement of hepatocarcinogenesis.

The mutation of the AA at residue 70 was not observed before and after the treatment in all patients who had failed to achieve a sustained virologic response. The mutant-type AA was identified solely at residue 70 after the treatment in three patients who had both the wild-type and the mutant-type AAs at residue 70 before the treatment. This could be due to the selection of HCV strains with the mutant-type AA at residue 70 by the combination therapy with PEG-IFN and ribavirin, as reported previously [Kurbanov et al., 2010]. In two patients who carried only the wild-type AA before the treatment, the HCV with the mutant-type AA at residue 70 was also detected with the persistence of the wild-type AA at this residue after the treatment. The very minor HCV strain with the mutant-type AA at residue 70 which were not detected before the treatment may have been detected after the treatment due to the reduction of HCV with the wild-type AA at residue 70 by the combination therapy. Indeed, HCV with the mutant-type AA at core region residue 70 was not detectable in serum 6 months after the end of the combination therapy, suggesting that it returned to being a very minor population (data not shown). These two phenomena were observed in patients with both the TT genotype of the rs8099917, that is associated with a favorable response to the combination therapy and those with the TG/GG genotypes that is associated with an unfavorable response, without difference in the prevalence according to the genetic polymorphisms at the rs8099917 near the *IL28B* gene.

In conclusion, PEG-IFN/ribavirin combination therapy does not appear to induce the mutation of the AA at the HCV core region residue 70 regardless of the genetic polymorphism near the *IL28B* gene in Japanese patients infected with HCV genotype 1b. The combination therapy can be attempted regardless of the genetic polymorphisms near the *IL28B* gene in

treatment-naïve patients without the anxiety for the acquisition of the further resistance to the antiviral therapy. However, future studies should be undertaken to confirm the absence of the mutation at residue 70 of the HCV core region induced by the combination therapy with PEG-IFN and ribavirin. In addition, the effect of the genetic polymorphisms near the *IL28B* gene on the mutation of the AA at the HCV core region residue 70 should be investigated in the long-term observation of the natural course of chronic hepatitis C.

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Mutations in the core and NS5A region of hepatitis C virus genotype 1b and correlation with response to pegylated-interferon-alpha 2b and ribavirin combination therapy

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SUMMARY. Mutations in two regions of hepatitis C virus (HCV) have been implicated in influencing response to interferon (IFN) therapy. Substitutions in the NS5A region of HCV have been associated with response to IFN therapy, and this region has been known as the IFN sensitivity-determining region (ISDR). The mutations in the core region of HCV have also been reported to predict IFN response. The aim of this study was to investigate whether amino acid substitutions in the core region and ISDR among patients with HCV genotype 1b affect the response to IFN therapy. A total of 213 patients who completed IFN treatment were randomly selected. All patients received pegylated-IFN-alpha 2b once each week, plus oral ribavirin daily for 48 weeks. Of the 213 patients, 117 (54.9%) showed early virologic response (EVR), with HCV-negativity, at 12 weeks. Factors related to EVR on multivariate analysis were non-Gln70 and Leu91 in the core region, and ISDR mutant-type. One hundred and two (47.9%) showed a sustained virologic response (SVR). SVR occurred more frequently in patients without Gln70 (55.4%) than in those with Gln70 (21.3%) ($P < 0.0001$). SVR was achieved in 43.6% of patients with wild-type ISDR and 62.5% of patients with mutant-type ($P = 0.0227$). Of the 34 patients who simultaneously had non-Gln70 and mutant-type ISDR, 26 (76.5%) achieved SVR. Factors related to SVR on multivariate analysis were non-Gln70 and ISDR mutant-type. In conclusion, amino acid substitutions in the core region and ISDR were useful for predicting the response to IFN in patients with HCV genotype 1b.

Keywords: core region, genotype 1b, hepatitis C virus, interferon sensitivity-determining region, interferon therapy, NS5A.

INTRODUCTION

Hepatitis C virus (HCV) is a member of the Flaviviridae family and causes chronic hepatitis that can develop into potentially fatal cirrhosis and hepatocellular carcinoma [1]. It has been estimated that 170 million people are infected with HCV worldwide. Therefore, HCV infection is a major global health problem. HCV consists of four structural proteins (core, envelope 1, envelope 2 and p7) and six nonstructural proteins (NS2–NS5) [2]. HCV core protein was thought to inhibit the antiviral action of interferon (IFN) through down-regulation of transcription of IFN-induced antiviral genes [3,4]. The NS5A region includes the PKR-binding domain, which is associated with viral replication that is affected by IFN [5]. Thus, the core and NS5A regions of HCV appear to be important factors that may affect the response to IFN therapy, and mutations in the core and NS5A regions of HCV have been reported to affect response to IFN therapy [6–10]. The core region of HCV is well conserved, but substitutions of amino acid (aa) 70 and aa 91 are frequently found. Several studies reported a relation between these substitutions in the core region and IFN responsiveness [8,10]. The substitutions in the NS5A region of HCV have been closely associated with response to IFN therapy, and this region is known as the IFN sensitivity-determining region (ISDR) [6]. However, these

Abbreviations: Aa, amino acid; ALT, alanine aminotransferase; EVR, early virologic response; HCV, hepatitis C virus; IFN, interferon; ISDR, interferon sensitivity-determining region; SVR, sustained virologic response.

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relationships are little known and still controversial [10]. The aim of this study was to investigate whether amino acid substitutions in the core region and ISDR among patients with HCV genotype 1b affect the response to pegylated-IFN-alpha 2b and ribavirin combination therapy.

MATERIAL AND METHODS

A total of 891 patients with chronic hepatitis C genotype 1b and high viral load who were treated at Nagoya University Hospital and Affiliated Hospitals were enrolled; 213 patients who completed IFN treatment were randomly selected for this study. The patients' clinical characteristics are summarized in Table 1. Patients whose HCV-RNA levels were <100 KIU/mL were excluded. The core region (aa 30–110) and ISDR (aa 2209–2248) were examined by direct sequencing. All patients received subcutaneous injections of pegylated-IFN-alpha 2b (1.5 μg/kg) once each week plus oral ribavirin daily for 48 weeks. HCV-RNA in serum samples was examined at 12 weeks, at the end of IFN therapy and at 6 months after the end of treatment. Serum was stored at −80 °C for virologic examination. Early virologic response (EVR) was defined as HCV-negative at 12 weeks. Patients who were persistently negative for serum HCV-RNA and who had a normal serum alanine aminotransferase (ALT) level at 24 weeks after withdrawal of IFN treatment were considered to have sustained virologic response (SVR). Written informed consent was obtained from each patient, and the study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki.

Virologic analysis

HCV-RNA quantitative viremia load was determined by polymerase chain reaction (PCR). HCV was genotyped by direct sequencing of the 5′-untranslated region and/or E1 regions as described previously [11,12]. Genotypes were classified according to the nomenclature proposed by Simmonds *et al.* [13]. Direct sequencing of the core and NS5A-ISDR region was carried out as reported previously, but with modifications [7,14]. In brief, RNA was extracted from 140-μL serum with a commercial kit (QIAamp Viral RNA Kit; Qiagen, Valencia, CA, USA) and dissolved in 50 μL diethylpyrocarbonate-treated water. RNA (10 ng) was used for reverse transcription with oligo and random hexamer primers with a commercial kit (iScript cDNA Synthesis Kit; Bio-Rad, Hercules, CA, USA). HCV core region and NS5A-ISDR were amplified by nested PCR. In brief, each 50-μL PCR reaction contained 100 nM of each primer, 1 ng template cDNA, 5 μL GeneAmp 10 × PCR buffer, 2 μL dNTPs and 1.25 U AmpliTaq Gold (Applied Biosystems, Foster City, CA, USA). Primers for core region were sense 5′-GGGAGGTCTCGTAGACCGTGCACCATG-3′ and antisense 5′-GAGMGGKATRTACCCCATGAGRTCGGC-3′ and primers for the NS5A-ISDR were sense 5′-TGGATGGAGTGCGGTTGCACAGGTA-3′ and antisense 5′-TCTTTCTCCGTGGAGGTGGTATTG-3′. Amplification conditions consisted of 10 min at 94 °C, followed by 40 cycles of 94 °C for 10 s, 55 °C for 30 s and 72 °C for 30 s in a thermal cycler (GeneAmp PCR System 9700; Applied Biosystems). The second PCR was performed in the same reaction buffer with the first-round PCR product as template, and the following sets of primers: for the core region, sense primer 5′-AGACCGTGCACCATGAGCAC-3′ and antisense 5′-TACGCCGGGGGTCAKTRGGGCCCCA-3′; and for the NS5A-ISDR, sense 5′-CAGGTACGCTCCGGCGTGCA-3′ and antisense 5′-GGGGCCTTGGTAGGTGGCAA-3′. PCR products were separated by electrophoresis on 2% agarose gels, stained with ethidium bromide, and visualized under ultraviolet light. PCR products were then purified and sequenced with the second-round PCR primers with a dye terminator sequencing kit (BigDye Terminator v1.1 Cycle Sequencing Kit; Applied Biosystems) and an ABI 310 DNA Sequencer (Applied Biosystems). A mutation mixture was defined as viral mutants that constituted 50% or more of the total viral population.

Table 1 Clinical characteristics

Clinical characteristics	*N* =2 13
Age (years)	55.2 ± 10.6
Sex: male/female	120/93
AST(IU/L)	58.5 ± 37.7
ALT(IU/L)	66.0 ± 53.9
Platelet count (10^4/uL)	17.1 ± 5.1
HCV RNA level (KIU/mL)	1720 (100–7200)
Treatment: naive/retreatment	117/96
Body weight (kg)	55.3 ± 19.9

Data are expressed as mean ± standard deviation HCV RNA level was shown by median (range). AST, aspartate aminotransferase; ALT, alanine aminotransferase; HCV, hepatitis C virus.

Statistical analysis

Data are expressed as means ± standard deviation (SD). The paired *t*-test, the chi-square and the Fisher's exact tests were used to analyze differences in variables. A *P*-value of <0.05 was considered statistically significant. Multiple logistic regression models were used to identify factors predictive of EVR and SVR. Statview 5.0 software (SAS Institute, Inc., Cary, NC, USA) was used for all analyses.

RESULTS

Genetic heterogeneity in NS5A-ISDR and core regions of the HCV genome

The mutations in the HCV core region were measured by direct sequencing. The core region of HCV is well conserved,

Table 2 Prevalence of amino acid substitutions at 70, 75, and 91

Core 70	
Histidine	$n = 6$
Glutamine	$n = 46$
Glutamine/Histidine	$n = 1$
Arginine	$n = 160$
Core 75	
Alanine	$n = 112$
Alanine/Serine	$n = 1$
Alanine/Threonine	$n = 2$
Glutamine	$n = 1$
Serine	$n = 5$
Threonine	$n = 91$
Valine	$n = 1$
Core 91	
Leucine	$n = 162$
Methionine	$n = 51$

but substitutions of aa 70, aa 75 and aa 91 were frequently found, as previously reported. The distribution of mutations in the HCV core region at aa 70, aa 75 and aa 91 is shown in Table 2. The sequence of the HCVJ strain was defined as the consensus sequence, and the approach of counting the number of mutations to the chosen consensus sequence in ISDR was used to analyze the ISDR system. The number of NS5A-ISDR mutations was as follows: none ($n = 102$), 1 ($n = 63$), 2 ($n = 14$), 3 ($n = 8$), 4 ($n = 8$), 5 ($n = 7$), 6 ($n = 2$), 7 ($n = 4$) and 8 ($n = 5$). The relationships between substitutions of amino acids in the HCV core region and NS5A-ISDR are shown in Fig. 1. There were no significant relationships between the two regions. Thus, the HCV core region and the NS5A-ISDR were independent factors.

Virological response

Of 213 patients, 117 (54.9%) showed EVR, with HCV-negativity, at 12 weeks, and 76 became HCV-negative after 12 weeks; overall, 187 patients became HCV-negative at the end of treatment (87.8%). However, 85 patients continued to be HCV-positive after withdrawal of IFN treatment, and 102 of 213 (47.9%) patients were defined as achieving a SVR. Of 117 patients with EVR, 87 (74.4%) achieved SVR. Of 96 patients without EVR, 81 became non-SVR (84.4%). Thus, EVR was strongly associated with SVR.

Factors associated with early virologic response

The results of univariate analysis for factors predictive of EVR are shown in Table 3. The EVR rate according to amino acid substitutions of ISDR are shown in Table 4. The EVR rate of patients with more than two mutations in the ISDR (mutant-type) was 68.9%. Of 166 patients without glutamine (Gln) at aa 70 in the core region, 100 achieved EVR. The EVR rate of patients with Leu91 in the core region was 61.1%. The results of multivariate analysis for factors predictive of EVR are shown in Table 5. Factors related to EVR on multivariate analysis were non-Gln70, Leu91 and ISDR mutant-type.

Factors associated with sustained virologic response

The results of univariate analysis for factors predictive of SVR are shown in Table 6. The SVR rate according to amino acid substitutions of ISDR are shown in Table 4. SVR occurred more frequently in patients without Gln70 (55.4%) than in those with Gln70 (21.3%) (odds ratio, 0.217; 95% confidence interval (CI), 0.101–0.466; $P < 0.0001$). SVR was achieved in 43.6% of patients with wild-type ISDR and 62.5% with mutant-type ISDR (odds ratio, 0.465; 95% CI, 0.240–0.899; $P = 0.0227$). Factors related to SVR on multivariate analysis were non-Gln70 and ISDR mutant-type, as shown in Table 7.

The virological response according to amino acid substitutions in the 70 core region and ISDR

The SVR and EVR rates according to amino acid substitutions in the 70 core region and ISDR are shown in Table 8. The best response for both SVR and EVR was achieved in patients with non-Gln70 and mutant-type ISDR, and the

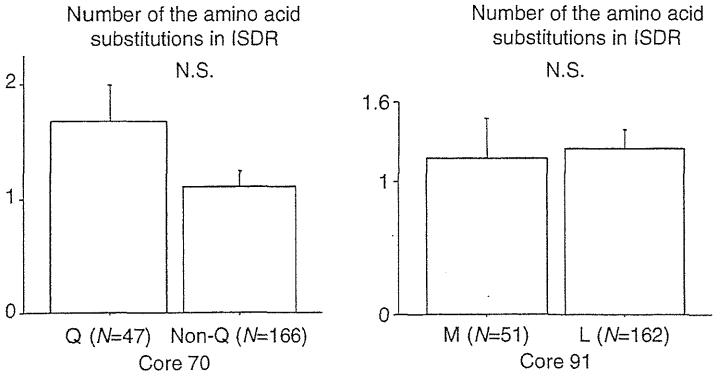

Fig. 1 The association between amino acid substitutions in core region and ISDR. ISDR, interferon sensitivity-determining region; Q, glutamine; L, leucine; M, methionine; NS, not significant.

Table 3 Univariate analysis: Factors predictive of EVR

Factors	EVR (n = 117)	Non-EVR (n = 96)	P-value
Age (years)	54.7 ± 11.3	55.9 ± 9.7	0.4511
Gender: male/female	63/54	57/39	0.7830
ALT (IU/L)	69.6 ± 64.8	61.5 ± 36.2	0.3002
AST (IU/L)	59.4 ± 40.9	57.3 ± 33.5	0.7026
PLT ($\times 10^4/mm^3$)	17.4 ± 5.1	16.9 ± 5.18	0.4955
HCV RNA level (KIU/mL)	2051.3 ± 1373.4	2006.1 ± 1462.7	0.8216
Core 70:non-Q/Q	100/17	66/30	0.0046
Core 75: A/non-A	58/59	54/42	0.3387
Core 91: L/M	99/18	63/33	0.0020
ISDR: wild/mutant	84/33	81/15	0.0327

EVR, early virologic response; AST, aspartate aminotransferase; ALT, alanine aminotransferase; PLT, platelet count; HCV, hepatitis C virus; Q, glutamine; A, alanine; L, leucine; M, methionine; ISDR, interferon sensitivity-determining region

Table 4 Amino acid substitutions of ISDR and virologic response

ISDR; number of the amino acid substitutions	0 N = 102	1 N = 63	2 N = 14	3 N = 8	4 N = 8	5 N = 7	6 N = 2	7 N = 4	8 N = 5
EVR rate (%)	51 (50.0)	33 (52.4)	10 (71.4)	4 (50.0)	7 (87.5)	4 (80.0)	0 (0)	3 (75.0)	5 (100)
SVR rate (%)	41 (40.2)	31 (49.2)	10 (71.4)	4 (50.0)	4 (50.0)	5 (71.4)	0 (0)	3 (75.0)	4 (80.0)

EVR, early virologic response; SVR, sustained virologic response.

Table 5 Multivariate analysis: Factors predictive of EVR

Factors	P-value	Risk ratio	95% CI	
Gender: male	0.3760	0.754	0.403	1.410
Age: <60 years	0.8247	0.915	0.416	2.012
AST: <60 IU/L	0.3301	1.525	0.652	3.569
ALT: <60 IU/L	0.2484	0.613	0.267	1.407
PLT: $<17 \times 10^4/mm^3$	0.0666	0.530	0.269	1.044
Core 70: nonQ	0.0242	2.406	1.121	5.165
Core 91: A	0.0022	3.409	1.557	7.463
Core 75: M	0.0683	1.863	0.954	3.635
ISDR: mutant	0.0085	0.338	0.151	0.759

EVR, early virologic response; AST, aspartate aminotransferase; ALT, alanine aminotransferase; PLT, platelet count; HCV, hepatitis C virus; ISDR, Interferon sensitivity-determining region; Q, glutamine; A, alanine; L, leucine; M; methionine.

worst response was achieved in patients with Gln70 and wild type ISDR. The SVR rates according to amino acid substitutions in the 70 core region and ISDR and EVR are shown in Table 9. The positive predictive values for SVR and non-SVR improved to 88.9% and 90.9%, respectively, when EVR was considered with the 70 core region and ISDR.

DISCUSSION

Peginterferon and ribavirin combination therapy has been standard treatment for patients with chronic hepatitis C. However, the SVR rate was almost 50% for HCV genotype 1b, which is a refractory strain. The standard doses and duration of peginterferon plus ribavirin may be suboptimal for half of the patients; patients need a new approach for eradicating HCV. Peginterferon and ribavirin therapy has been a useful treatment, but cost and adverse events have been problems. To select patients who could attain cure from HCV by current standard treatment, it is necessary to predict the response before therapy. Current guidelines for HCV treatment recommend that the selection of IFN treatment regimen depends on HCV genotypes and viral loads. Several studies have focused on sequence variation of the HCV genome and response to IFN therapy, but prediction of IFN responsiveness has been less well characterized. NS5A-ISDR heterogeneity is an important factor that may affect response to IFN, especially in Asia [6,7,9]. The ISDR interacts with PKR and regulates replication of HCV *in vitro* [5]. Mutations in the ISDR affect the interaction with PKR and may inhibit viral replication. Therefore, ISDR of not only HCV genotype 1b but also 2a and 2b could also play an important role as a predictor of IFN responsiveness in clinical research of standard IFN or Peg-IFN monotherapy [15,16]. The differences in HCV 1b subtype and race affect the utility of ISDR

Table 6 Univariate analysis: factors predictive of SVR

Factors	SVR (n = 102)	Non-SVR (n = 111)	P-value
Age (years)	53.6 ± 10.8	56.7 ± 10.2	0.0319
Gender: male/female	57/45	63/48	0.7830
ALT (IU/L)	69.6 ± 66.7	62.6 ± 38.5	0.3606
AST (IU/L)	58.8 ± 40.9	58.3 ± 34.8	0.9469
PLT ($\times 10^4/mm^3$)	17.7 ± 5.1	16.7 ± 5.0	0.1563
HCV RNA level (KIU/mL)	2111.1 ± 1504.9	1956.4 ± 1319.8	0.4386
Core 70:non-Q/Q	92/10	74/37	0.0001
Core 75: A/non-A	50/52	62/49	0.3388
Core 91: L/M	82/20	80/31	0.1984
ISDR: wild/mutant	72/30	93/18	0.0227

SVR, sustained virologic response; AST, aspartate aminotransferase; ALT, alanine aminotransferase; PLT, platelet count; HCV, hepatitis C virus; Q, glutamine; A, alanine; L, leucine; M, methionine, ISDR, Interferon sensitivity-determining region.

Table 7 Multivariate analysis: factors predictive of SVR

Factors	P-value	Risk ratio	95% CI	
Age: <60 years	0.5219	0.770	0.346	1.714
Gender: male	0.6775	1.140	0.614	2.116
AST: <60 IU/L	0.1017	0.487	0.206	1.153
ALT: <60 IU/L	0.1690	1.799	0.779	4.157
PLT: $<17 \times 10^4/mm^3$	0.4067	1.324	0.682	2.573
HCV RNA levels: <106 IU/mL	0.6409	0.841	0.405	1.743
Core70: nonQ	0.0004	0.220	0.094	0.512
Core91: M	0.5643	0.799	0.373	1.711
Core75: A	0.3993	0.757	0.396	1.446
ISDR: mutant	0.0096	2.879	1.294	6.407

SVR, sustained virologic response; AST, aspartate aminotransferase; ALT, alanine aminotransferase; PLT, platelet count, HCV, hepatitis C virus; ISDR, interferon sensitivity-determining region; Q, glutamine; A, alanine; L, leucine; M, methionine.

Table 8 The SVR and EVR rate according to amino acid substitutions in 70 core region and ISDR

Core70/ISDR	SVR (n = 102)	EVR (n = 117)
Q/wild (n = 33)	6 (18.2%)	11 (33.3%)
Q/mutant (n = 14)	4 (28.6%)	6(42.9%)
Non-Q/wild (n = 132)	66 (50.0%)	73 (55.3%)
Non-Q/mutant (n = 34)	26 (76.5%)	27 (79.4%)

SVR, sustained virologic response; EVR, early virologic response; SDR, interferon sensitivity-determining region; Q, Glutamine; ISDR, interferon sensitivity-determining region.

sequences for predicting IFN responsiveness [7,17,18]. Thus, ISDR was found to be good for predicting IFN outcome of patients in Asian countries rather than of patients in Western countries. The approach of counting the number of mutations to the HCV-J strain in the ISDR was used in the original report by Enomoto *et al.*, [6] and they classified the mutations into three groups: wild type (no mutation), intermediate (1–3 mutations) and mutant-type (more than four mutations). SVR did not occur in any of the 30 patients with wild type ISDR in the original report using standard IFN monotherapy. In the present study, 41 of 102 patients (40.2%) with the wild type ISDR (no mutation) achieved SVR because of improvement of Peg-IFN plus RBV combination therapy. We examined the association between the number of mutations and SVR with adjustment for current standard treatment. We were unable to identify a significant relation between no mutation and one mutation in ISDR and SVR. Thus, sequences of the HCV-J strain and HCV-J strain with single substitutions were defined as the wild-type, and ISDR sequences with more than two mutations were defined as the mutant-type. SVR was achieved in 43.6% of patients with wild-type ISDR and 62.5% of patients with mutant-type ISDR in this study. ISDR alone was insufficient to predict IFN responsiveness in patients who received peginterferon plus ribavirin combination therapy. We speculated that the other region would explain differences in IFN sensitivity in patients infected with wild type ISDR. HCV core, E2-PePHD and NS5A-V3 regions were reported to be associated with IFN response [8,10,19,20]. The HCV core interacts with several cell factors and modulates numerous gene expressions, including down-regulating transcription of IFN-induced antiviral genes, and it affects the inhibition of the antiviral action of IFN. Several studies indicated that the HCV core region could predict IFN responsiveness [8,10]. Therefore, the utility of substitutions of amino acids in the HCV core region combined with NS5A-ISDR sequences for predicting

Table 9 The SVR rate according to EVR amino acid substitutions in 70 core region and ISDR

Core70/ISDR	SVR of patients with EVR (n = 87)	Non SVR of patients with EVR (n = 30)	SVR of patients without EVR (n = 15)	Non SVR of patients without EVR (n = 81)
Q/wild (n = 33)	4 (40%*)	7	2	20 (90.9%**)
Q/mutant (n = 14)	3 (50%*)	3	1	7 (87.5%**)
Non-Q/wild (n = 132)	56 (76.7%*)	17	10	49 (83.1%**)
Non-Q/mutant (n = 34)	24 (88.9%*)	3	2	5 (71.4%**)

*Positive predictive value for SVR. **Positive predictive value for non-SVR. SVR, sustained virologic response; EVR, early virologic response; ISDR, interferon sensitivity-determining region; Q, glutamine.

IFN responsiveness was investigated. The non-Gln70 amino acid substitution in the HCV core region was related to SVR on univariate and multivariate analysis. SVR occurred more frequently in patients without Gln70 (50.6%) than with Gln70 (14.3%). SVR was not associated with aa 75 and aa 91 in the core region. When core 70 was considered in the analysis of ISDR, the SVR rates varied widely according to amino acid substitutions in core region 70 and ISDR. For instance, only 18.1% of patients with Gln70 and wild type ISDR achieved SVR compared with 76.4% in those with non-Gln70 and mutant-type ISDR. Despite having genotype 1b, patients with non-Gln70 and mutant-type ISDR responded to IFN as well as those with genotypes 2 and 3. Pegylated-IFN-alpha 2b and ribavirin combination therapy was suitable for treatment of Japanese patients with HCV genotype 1b, particularly those with non-Gln70 and mutant-type ISDR. Optimal duration of IFN therapy in some patients with non-Gln70 and mutant-type ISDR could be shorter than 48 weeks; and in these patients, costs and side effects could be reduced without reducing the efficacy of IFN therapy by using a shorter regimen. On the other hand, patients with Gln70 and wild type ISDR resistant to pegylated-IFN-alpha 2b and ribavirin combination therapy should receive much more powerful treatment, such as triple therapy including the new protease inhibitor, peginterferon alfa and ribavirin as their first regimen [21,22]. This is an important consideration to achieve optimal therapy and avoid unnecessary treatment. The effects of amino acid substitutions in core 70 on gene expression and core protein function were unclear, and further studies are needed to determine their mechanism. Although the effects of amino acid substitutions of the core region and ISDR were unclear, the mutation at core 70 and the ISDR system could be clinically used as a simple diagnostic tool to predict SVR in patients infected with genotype 1b. It is not easier to routinely measure the HCV sequence to determine the core 70 and ISDR sequence. Virologic response, as rapid virologic response and EVR, could be easy to measure by commercial kits in clinical practice and would be useful for prediction of achieving SVR for chronic hepatitis C patients. The present study also confirmed that EVR has been associated with SVR, but virologic response cannot be assessed before treatment. HCV sequencing analysis will become a convenient method because of progression of sequencing technology and cost reduction. In this respect, the core region and ISDR were useful predictors of virologic response. Analysis of EVR in combination with the core region and ISDR revealed that 24 of 34 patients with non-Gln70 and mutant-type ISDR and EVR achieved SVR. EVR, core region and ISDR are considered strong indicators of SVR for patients with HCV genotype 1b. Although validation of these observations in larger cohorts is required, amino acid substitutions in the core region of HCV and ISDR were useful for predicting the response to pegylated-IFN-alpha 2b and ribavirin combination therapy in patients with chronic hepatitis C genotype 1b. Combining amino acid substitutions in the core region and ISDR could improve the predictive value of SVR in patients with genotype 1b, but the efficacy is still not satisfactory. The explanation for the lack of SVR in patients with non-Gln70 and mutant-type ISDR remains unclear. The other regions of HCV or host factors are candidates for a third factor for improving the prediction of SVR [23,24].

CONCLUSION

Amino acid substitutions in the 70 core region of HCV and ISDR were useful for predicting the response to pegylated-IFN-alpha 2b and ribavirin combination therapy in patients with chronic hepatitis C genotype 1b.

Data of this study were presented in part at the 59th annual meeting of the American association for the study of liver diseases (AASLD), October 31-November 4, 2008, San Francisco, CA, USA.

DISCLOSURE

All people have nothing to disclose.

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Original Article

Impact of early elevation of serum bilirubin during treatment with pegylated interferon and ribavirin in patients with chronic hepatitis C

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Aim: Hemolytic anemia is a well-known adverse effect of interferon and ribavirin combination treatment. Herein, we analyzed the impact of early elevation of serum bilirubin level as a marker for predicting severe anemia during treatment.

Methods: We studied 245 chronic hepatitis C patients who received pegylated interferon and ribavirin combination treatment, and divided them using two different threshold levels: (i) elevation of total bilirubin of 0.5 mg/dL or more within 1 week of starting treatment; and (ii) drop of hemoglobin (Hb) by 3 g/dL or more within 4 weeks of starting treatment. We compared the dynamics in each group and then investigated independent factors for predicting a severe Hb drop (≥3 g/dL) at 4 weeks after beginning treatment and dose reduction of ribavirin.

Results: Total bilirubin levels at 1 week were significantly higher in patients with a Hb drop of 3 g/dL or more as compared to those with a drop of less than 3 g/dL ($P < 0.0001$). Hb levels at 4 weeks were significantly lower in the group of 0.5 mg/dL or more increase of total bilirubin levels than in the group with a less than 0.5 mg/dL increase ($P < 0.0001$). Therefore, elevation of total bilirubin after 1 week of treatment was shown to be an independent factor for predicting severe Hb drop (≥3 g/dL) at 4 weeks ($P < 0.0001$), and dose reduction of ribavirin during treatment ($P = 0.0321$).

Conclusion: Early elevation of serum bilirubin level was found to be a possible predictive marker of both a severe drop of Hb in the early phase of treatment and dose reduction of ribavirin.

Key words: bilirubin, hemolytic anemia, hepatitis C virus, pegylated interferon and ribavirin.

INTRODUCTION

ANEMIA IS ONE of the most common and important adverse effects of interferon (IFN) and ribavirin combination treatment that occurs in chronic hepatitis C patients.[1] Two different mechanisms of anemia are induced by such combination treatment: (i) suppression of bone marrow hematopoiesis caused by IFN;[2] and (ii) dose-dependent hemolytic anemia caused by ribavirin.[3] However, clinically, the contribution of ribavirin to anemia during this combination treatment overshadows the effect of IFN on bone marrow.[1]

Hemolytic anemia associated with ribavirin has been reported in recent studies. A median maximum decrease in hemoglobin (Hb) of 3.7 g/dL was found in patients who received pegylated interferon (PEG IFN)-α-2a plus ribavirin.[4] Another study found that 50% of patients experienced a decrease in Hb of 3 g/dL or more,[3] while a mean maximal decrease in Hb of 4.0 g/dL was reported in patients who received PEG IFN-α-2b plus ribavirin treatment.[5] Consequently, ribavirin dose modification was needed in approximately 25% of patients.[3–6]

Hemolytic anemia can induce cardiovascular morbidity, as well as substantial negative effects on cerebral function and quality of life (QOL).[1] On the other hand, a reduction in the dose of ribavirin can lower sustained viral response (SVR) rate in patients with hepatitis C, as well as both the starting dose of ribavirin and duration of treatment; thus, the cumulative dose of ribavirin is critical to achieve an optimal SVR by lowering the

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relapse rate after treatment, especially in genotype 1 infected patients.[1,7,8] So, prediction of severe anemia following ribavirin treatment is critical not only for patients' safety, but also for forming a proper dosing strategy.

It has been noted that the majority (80–85%) of bilirubin, the end product of haem, comes from Hb with only a small fraction derived from other haem-containing proteins such as cytochrome P450.[9] This report also noted that bilirubin elevation can be classified into three types: (i) pre-hepatic; (ii) hepatic; and (iii) cholestatic. Thus, it is caused by not only liver dysfunction, but also for a pre-hepatic reason: hemolysis.[9]

In this study, we found some patients with a steep elevation of serum total bilirubin level after starting PEG IFN and ribavirin combination treatment for chronic hepatitis C. The ability of early elevation of serum bilirubin to predict both a severe Hb drop in the early phase after starting combination treatment and dose reduction of ribavirin during treatment was analyzed. Furthermore, we discuss whether this marker is useful to prevent severe anemia which is one of the main causes of cessation of the treatment.

METHODS

Patients

BETWEEN 2002 AND 2009, we treated 245 patients (140 men and 105 women, mean age 52.2 ± 12.2 years) with PEG IFN-α-2b and ribavirin combination treatment at our institute. All were included and retrospectively analyzed in this study.

Patients' age and sex, initial dose of ribavirin and liver biopsy findings if available (205 patients), as well as Hb levels and serum bilirubin levels at 0, 1, 2, 4, 6, 8, 12 and 24 weeks after induction of combination treatment were analyzed.

Treatment protocol

Patients who were diagnosed with hepatitis C by both abnormalities of liver function test results and hepatitis C virus (HCV) RNA-positive findings by Cobas Amplicor HCV Monitor V2.0 test (Roche Diagnositics, Branchburg, NJ, USA) until 2007, or Cobas Taqman HCV test (Roche Diagnositics) from 2008. In some cases, liver biopsy findings based on the French Metavir score were included in this study. All patients in this study were treated with a combination of PEG IFN-α-2b (Pegintron; Schering-Plough, Kenilworth, NJ, USA) and ribavirin (Rebetol; Schering-Plough). The initial dose was determined by bodyweight (PEG IFN, 1.5 μg/kg; ribavirin, 400 mg/day for patients weighing <40 kg, 600 mg/day for those weighing 40–60 kg, 800 mg/day for those weighing 60–80 kg, 1000 mg/day for those weighing >80 kg). Dose reduction of ribavirin was done when the Hb level became less than 10 g/dL, and treatment was stopped when that became less than 8.5 g/dL.

Definition of severe Hb decline and classification of patients based on elevation of serum bilirubin

A Hb drop of 3 g/dL or more from baseline at 4 weeks after the start of combination treatment was defined as severe Hb decline in the early phase of treatment. Thus, our analyses were performed between groups divided by that threshold level. In addition, we performed analyses after dividing the patients based on a total bilirubin elevation of 0.5 mg/dL or more from the baseline after 1 week.

Dose reduction and treatment cessation by hemolytic anemia during combination treatment

In this study, 94 of 245 (38.4%) patients required a dose reduction of ribavirin, and four patients (1.6%) stopped treatment because of hemolytic anemia that developed during treatment. We performed analyses to find factors associated with dose reduction of ribavirin; however, we could not analyze factors associated with treatment cessation because of the limited number of patients.

Statistical analysis

Two-way repeated-measures ANOVA was used to compare the change of serum bilirubin level between groups divided by the extent of Hb decline as well as the change of Hb level between groups divided by the extent of serum bilirubin increase. The Mann–Whitney *U*-test was used in the comparison of values obtained at each time point. Univariate analysis was used to analyze predictive factors, with the Mann–Whitney *U*-test applied for the continuous values, and the χ^2-test applied for the categorical values. In multivariate analysis, a multiple logistic regression test was applied. $P < 0.05$ was considered to indicate statistical significance. All analyses were performed with Stat View ver. 5.0.

Ethical consideration

All data were retrospectively obtained from patients' records and the study was performed according to the Declaration of Helsinki.

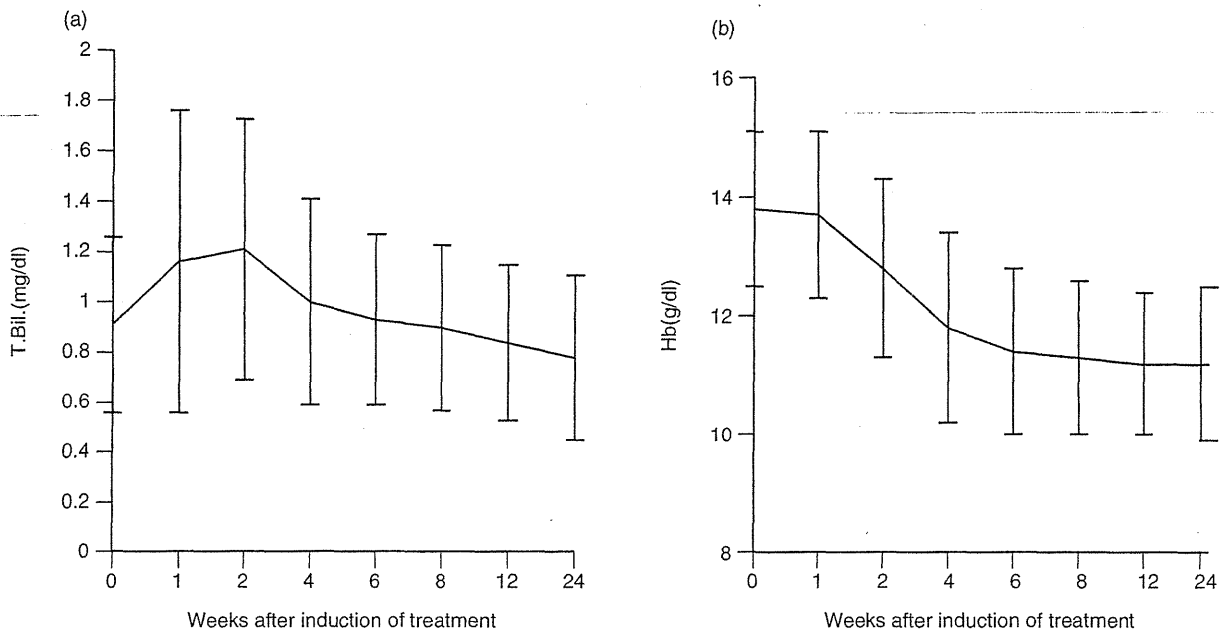

Figure 1 (a) Dynamics of serum total bilirubin (T.Bil) levels after beginning combination treatment. (b) Dynamics of hemoglobin (Hb) levels after beginning combination treatment. Each data indicate the mean values and error bars indicate the standard deviation.

RESULTS

Dynamics of Hb levels and serum bilirubin levels after induction of PEG IFN and ribavirin combination treatment

FIGURE 1(A) SHOWS the dynamics of serum total bilirubin after starting combination therapy in all of the study patients. Total bilirubin gradually increased from the baseline level until 2 weeks after starting treatment, then returned to the baseline level. Figure 1(b) shows the dynamics of Hb levels after starting treatment in all patients included in this study. Hb levels gradually declined and reached a plateau at 4–8 weeks after starting treatment.

Different dynamics of serum total bilirubin levels between patients with and without severe hemolytic anemia in the early phase of treatment

We found a steep increase of serum total bilirubin level (≥0.5 mg/dL) at 1 week after starting treatment in 62 of the 245 patients (25.3%). Furthermore, 65 (26.5%) of the patients had a severe decline in Hb (≥3 g/dL) after 4 weeks of treatment. Figure 2(a) shows the difference of dynamics of serum bilirubin levels between patients with and without severe declines of Hb. In those with a severe Hb decline, serum total bilirubin levels were increased at 1 week after beginning treatment, which was earlier than the total study population (Fig. 2a). In contrast, in patients without a steep Hb decline after 4 weeks, the change in total bilirubin level was quite similar compared to that of the total population (Fig. 2a). The difference between these groups was shown to be statistically significant by ANOVA ($P = 0.0095$). In addition, total bilirubin levels at 1 ($P < 0.0001$), 2 ($P < 0.0001$) and 4 weeks ($P = 0.0306$) after the start of treatment in the patients with a severe Hb decline were significantly higher as compared to those without a severe Hb decline.

Different dynamics of Hb levels between patients classified by the extent of elevation of serum total bilirubin

Next, we compared dynamics of Hb levels between the patients with a steep increase of serum total bilirubin (≥0.5 mg/dL) and those without steep increase (<0.5 mg/dL) at 1 week after starting treatment. Figure 2(b) shows the comparison of dynamics by ANOVA, which were not statistically significant ($P = 0.1539$).

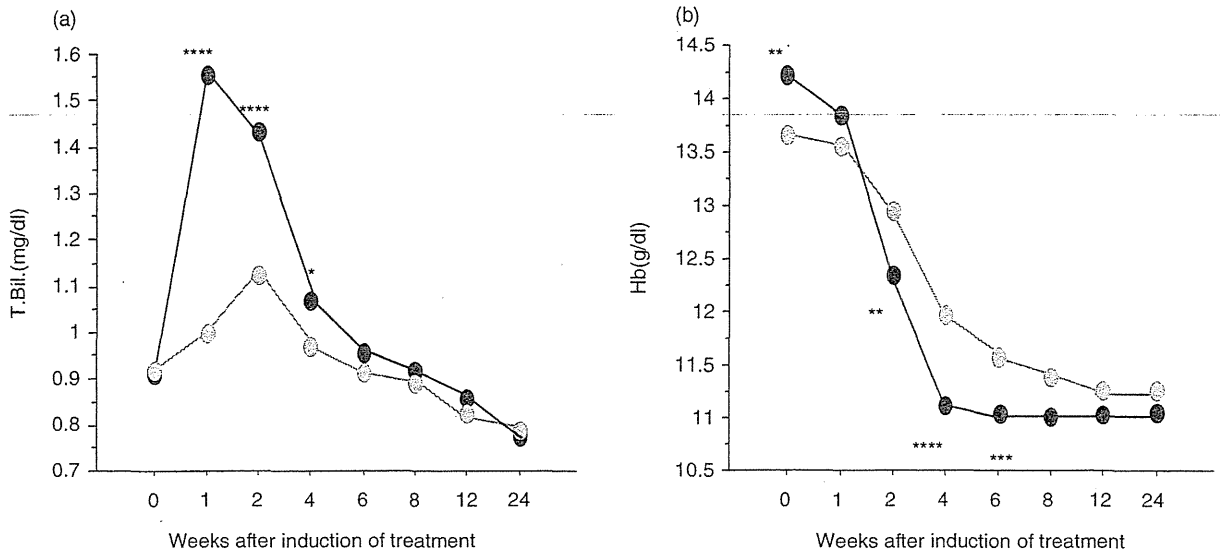

Figure 2 (a) Comparison of the dynamics of serum bilirubin levels between patients with severe hemolytic anemia (Hb decline ≥3 g/dL) to those without severe hemolytic anemia at 4 weeks after start of treatment. Two-way repeated-measures ANOVA showed a significant difference of dynamics between these two groups ($P = 0.0095$). Black lines indicate serum bilirubin levels in the group with severe hemolytic anemia, and gray lines indicate those levels in the group without severe hemolytic anemia. $^{*}P < 0.05$, $^{****}P < 0.0001$ for comparisons of each values at each time point. (b) Comparison of the dynamics of Hb levels between patients group divided by the extent of elevation of serum total bilirubin (≥0.5 vs <0.5 mg/dL at 1 week after starting treatment).Two-way repeated-measures ANOVA did not reveal a statistically significant difference between the two groups ($P = 0.1539$). Black lines indicate Hb levels in the group with elevation of serum bilirubin of ≥0.5 mg/dL, and gray lines indicate these levels in the group with elevation of serum bilirubin of <0.5 mg/dL. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{****}P < 0.0001$ for comparison of each values at each time point. Hb, hemoglobin; T.Bil, total bilirubin.

As for why it did not reach statistical significance, baseline Hb levels were higher in the group with a steep increase of serum total bilirubin ($P = 0.0043$), though that trend was reversed at 2 weeks after the start of treatment ($P = 0.0030$). Furthermore, the extent of Hb decline was higher in the group with that steep increase.

Factors associated with severe Hb drop in the early phase

Next, we investigated the predictive factors associated with a severe Hb decline after beginning treatment. By univariate analysis, male sex ($P = 0.0201$), higher initial dose of ribavirin ($P = 0.0029$), higher baseline Hb level ($P < 0.0001$) and higher elevation of serum total bilirubin after 1 week ($P < 0.0001$) were shown to be significant predictive factors (Table 1). By multivariate analysis, though higher patient age (odds ratio [OR] = 1.055, 95% confidence interval [CI] = 1.020–1.090, $P = 0.0090$), higher initial dose of ribavirin (OR = 1.266, 95% CI = 1.015–1.580, $P = 0.0361$) and higher baseline Hb level (OR = 1.765, 95% CI = 1.202–2.592, $P = 0.0038$) were selected as the significant independent factors associated with a steep decline in Hb after treatment, though a higher elevation of serum bilirubin at 1 week after starting treatment was found to be the strongest factor than the other three (OR = 9.448, 95% CI = 3.727–23.965, $P < 0.0001$) (Table 2).

Factors associated with dose reduction of ribavirin during combination treatment

To evaluate the clinical relevance of a steep increase of serum bilirubin level, we investigated the factors associated with reduction of ribavirin dose during the combination treatment. By univariate analysis, higher patient age ($P < 0.0001$), female sex ($P = 0.0207$), higher initial dose of ribavirin ($P = 0.0003$), more advanced fibrosis ($P < 0.0001$) and lower baseline Hb levels ($P = 0.0007$) were considered as significant factors associated with dose reduction of ribavirin during treatment. In contrast, a higher elevation of serum bilirubin after 1 week was not considered a statistically significant factor though it showed a tendency for significance

Table 1 Univariate analysis of factors associated with severe hemoglobin decline (Hb decline ≥3 g/dL in 4 weeks after starting treatment) after induction of combination treatment in this study

	Yes ($n = 65$)	No ($n = 180$)	*P*-value
Age, range (median), years	29–70 (56)	18–74 (53)	0.1200
Sex, *n* (M/F)	45/20	95/85	0.0201*
Initial dose of ribavirin, range (median), mg/kg bodyweight	5.4–15.8 (12.2)	2.4–16.2 (11.1)	0.0029**
Histological grading, *n*, A1/2/3	26/28/2	84/63/2	0.3114
Histological staging, *n*, F0/1/2/3/4	10/22/15/8/1	34/60/38/16/1	0.8340
Baseline Hb, range (median), g/dL	12.2–18.1 (14.6)	11.0–16.0 (13.6)	<0.0001****
Baseline Hb, range (median), g/dL	0.4–2.0 (0.9)	0.3–2.5 (0.8)	0.2711
Elevation of total bilirubin at 1 week from baseline, range (median), mg/dL	−0.6–2.7 (0.6)	−1.0–2.2 (0.1)	<0.0001****

*$P < 0.05$; **$P < 0.01$; ****$P < 0.0001$.

($P = 0.0684$) (Table 3). By multivariate analysis, higher elevation of serum bilirubin at 1 week was a significant independent factor ($P = 0.0321$), as were higher age ($P = 0.0007$), higher initial dose of ribavirin ($P < 0.0001$) and lower baseline Hb level ($P = 0.0162$) (Table 4).

DISCUSSION

HEMOLYTIC ANEMIA THAT occurs after beginning PEG IFN and ribavirin combination treatment for patients with chronic hepatitis C is a critical adverse effect that causes dose modification in 20–25% of treated patients,[1,4,5] of whom more than half must discontinue treatment.[5]

In this report, we first evaluated the extent of hemolytic anemia after beginning combination treatment in chronic hepatitis C patients. The mean Hb decrease from baseline for all patients was approximately 2.5 g/dL and reached the bottom and plateau at 4 weeks after treatment, which was similar to previous reports. We also focused on the dynamics of total bilirubin, and found that the mean elevation was 0.3 mg/dL at 2 weeks after beginning treatment (Fig 1a,b).

Table 2 Multivariate analysis of factors associated with severe hemoglobin decline after induction of combination treatment in this study

Factors	Odds ratio (95% confidence interval)	*P*-value
Age (reference)	1	
1 year old higher	1.055 (1.020–1.090)	0.0090**
Sex (reference)	1	
Male sex	1.093 (0.398–2.999)	0.8629
Initial dose of ribavirin (reference)	1	
1 mg/kg bodyweight higher	1.266 (1.015–1.580)	0.0361*
Histological grading (reference)	1	
1 point higher	1.091 (0.438–2.715)	0.8515
Histological staging (reference)	1	
1 point higher	1.088 (0.655–1.806)	0.7449
Baseline Hb (reference)	1	
1 g/dL higher	1.765 (1.202–2.592)	0.0038**
Baseline total bilirubin (reference)	1	
1 mg/dL higher	2.245 (0.701–7.189)	0.1731
Elevation of total bilirubin at 1 week from baseline (reference)	1	
1 mg/dL higher	9.448 (3.727–23.965)	<0.0001****

*$P < 0.05$; **$P < 0.01$; ****$P < 0.0001$.

Table 3 Univariate analysis of factors associated with dose reduction of ribavirin after induction of combination treatment in this study

Dose reduction of Ribavirin	Yes ($n = 94$)	No ($n = 151$)	*P*-value
Age, range (median), years	22–71 (59)	18–74 (51)	<0.0001****
Sex, *n* (M/F)	45/49	95/56	0.0207*
Initial dose of ribavirin, range (median) mg/kg bodyweight	7.0–16.2 (12.0)	2.4–16.2 (11.1)	0.0003***
Histological grading, *n*, A1/2/3	38/42/1	72/49/1	0.2052
Histological staging, *n*, F0/1/2/3/4	13/14/30/14/0	31/58/23/10/2	<0.0001****
Baseline Hb, range (median), g/dL	11.1–16.1 (13.3)	11.0–18.1 (14.2)	0.0007***
Baseline total bilirubin, range (median), mg/dL	0.4–1.8 (0.8)	0.3–2.5 (0.8)	0.9497
Elevation of total bilirubin at 1 week from baseline, range (median), mg/dL	−0.8–2.7 (0.3)	−1.0–2.2 (0.0)	0.0684†

*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$; †$P < 0.1$.
Hb, hemoglobin.

In addition, serum bilirubin levels showed a steep and rapid elevation (≥0.5 mg/dL) at 1 week after the start of treatment in 25.3% of our patients, and seemed to occur more frequently in those with severe hemolytic anemia. This was the clue that motivated us to begin this study.

Various studies have been conducted regarding serum bilirubin levels after starting PEG IFN and ribavirin combination treatment. In one report, serum bilirubin levels were significantly increased in HCV/HIV co-infected patients who were treated with atazanavir, a protease inhibitor of HIV following the start of PEG IFN and ribavirin combination treatment.[10] However, to the best of our knowledge, there are no studies focused on serum bilirubin elevation after beginning combination treatment in standard chronic hepatitis C patients.

The main pre-hepatic cause of serum bilirubin elevation is hemolysis. Thus, we speculated that the extent of serum bilirubin elevation is correlated with hemolytic anemia following the start of PEG IFN and ribavirin combination treatment. As expected, the elevation of serum bilirubin at 1 week after starting treatment in patients with a severe Hb decline at 4 weeks after starting treatment was greater than that in patients without a severe Hb decline after 4 weeks. Hepatic dysfunction

Table 4 Multivariate analysis of factors associated with dose reduction of ribavirin after induction of combination treatment

Factors	Odds ratio (95% confidence interval)	*P*-value
Age (reference)	1	
1 year old higher	1.056 (1.023–1.090)	0.0007***
Sex (reference)	1	
Male	1.144 (0.484–2.704)	0.7598
Initial dose of ribavirin (reference)	1	
1 mg/kg bodyweight higher	1.539 (1.249–1.897)	<0.0001****
Histological grading (reference)	1	
1 point higher	0.808 (0.373–1.750)	0.5886
Histological staging (reference)	1	
1 point higher	1.395 (0.904–2.153)	0.1324
Baseline Hb (reference)	1	
1 g/dL higher	0.667 (0.480–0.928)	0.0162*
Baseline total bilirubin (reference)	1	
1 mg/dL higher	1.975 (0.732–5.327)	0.1786
Elevation of total bilirubin at 1 week from baseline (reference)	1	
1 mg/dL higher)	2.143 (1.061–3.806)	0.0321*

*$P < 0.05$; ***$P < 0.001$; ****$P < 0.0001$.
Hb, hemoglobin.

might be another cause of serum bilirubin elevation, but there were no changes in liver functions in patients in this study (data not shown).

We also found that the extent of elevation of serum bilirubin could be a predictive marker for severe hemolytic anemia after beginning PEG IFN and ribavirin combination treatment, and that after 1 week of treatment was the strongest independent factor for predicting a severe Hb drop in multivariate analysis. In recent reports, an early decrease of Hb (≥2 g/dL) at 2 weeks after beginning treatment was useful for predicting the treatment discontinuation,[11,12] as were a decrease in Hb to less than 10 g/dL,[13] and ribavirin apparent clearance (CL/F), which reflects ribavirin concentration at 4 weeks after the start of combination therapy.[14–16]

Among these markers, elevation of serum bilirubin after 1 week of treatment is new and at least as useful as the others; it is easier to employ than CL/F because a calculation formula is not needed, and can predict severe hemolytic anemia earlier than Hb decline at 2 weeks after beginning treatment.

Another interesting finding is that higher baseline Hb level was another independent factor of severe decline in Hb after starting treatment as were initial ribavirin dose and age, both of which are not surprising and have been shown in several reports.[3,5,13] Similar studies found that patients with a higher baseline Hb and who were maintained on the initial dose of ribavirin showed a trend toward larger Hb decline, while they also noted that the endogenous erythropoietin response is blunted in this population.[17,18]

A severe Hb decline by ribavirin administration due to hemolysis can cause anemia, which has a negative impact not only on the QOL of the patients, but also on cardiovascular comorbidity and cerebral function; thus, dose modification is needed in some cases.[1,19] However, dose modification has been shown to reduce the efficacy of PEG IFN and ribavirin combination treatment.[19] To investigate the factors associated with dose reduction of ribavirin during such combination treatment, the extent of serum bilirubin elevation was again found to be a significant independent factor. Thus, the extent of early elevation of serum bilirubin may be an important marker to predict dose reduction of ribavirin early after the start of treatment, which would be useful not only for patients' safety but also for predicting treatment response.

In summary, significant proportions of patients have been shown to have a steep elevation of serum bilirubin level at 1 week after beginning PEG IFN and ribavirin combination treatment for chronic hepatitis C. We found that this steep elevation was significantly correlated with both a severe decline in Hb and dose reduction of ribavirin, and consider that it may be an easier and earlier predictive marker compared with those previously reported, including CL/F and Hb decline at 2 weeks after starting treatment

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Association between HCV amino acid substitutions and outcome of peginterferon and ribavirin combination therapy in HCV genotype 1b and high viral load

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Key words
amino acid substitution, chronic hepatitis C, hepatitis C virus, peginterferon and ribavirin therapy, resistance to interferon.

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Abstract

Background and Aim: We prospectively compared the sensitivity to interferon (IFN) and the efficacy of antiviral combination therapy with peginterferon (PEG-IFN) and ribavirin for chronic hepatitis C virus (HCV) genotype 1b infection according to the amino acid sequences of the HCV core, E1, and NS5A regions reported to be associated with the outcome of antiviral therapy.

Methods: A total of 107 patients with HCV genotype 1b were investigated. All patients received combination therapy with PEG-IFN alpha-2b and ribavirin. Amino acids 70 and 91 (core), 139 (E1), and 2209–2248 (NS5A) of HCV were analyzed by direct nucleotide sequencing.

Results: The reduction in HCV RNA concentration at 24 h after a single administration of conventional IFN-alpha and after the start of combination therapy was significantly less marked, and rates of complete early virologic response, end-of-treatment response, and sustained virologic response (SVR) were significantly lower (all $P < 0.0001$) in patients with glutamine at amino acid 70 ($n = 29$) than in those with arginine at that position ($n = 70$). We found no differences associated with the other amino acid positions. Amino acid 70 was an independent factor for the responses to the therapy in multivariate analysis.

Conclusion: The identity of amino acid 70 of the HCV core region affected the sensitivity to IFN; patients with glutamine at amino acid 70 of HCV showed resistance to IFN. Consequently, it strongly affected the outcome of combination therapy with PEG-IFN and ribavirin in Japanese patients with HCV genotype 1b.

Introduction

The current standard antiviral therapy for patients with chronic hepatitis C is combination therapy with peginterferon (PEG-IFN) and ribavirin.[1] The rate of sustained virologic response (SVR), which indicates the eradication of the hepatitis C virus (HCV) is around 50% in patients infected with HCV genotype 1, which is more resistant to this therapy than genotypes 2 or 3; the recommended duration of the PEG-IFN/ribavirin treatment period differs between patients with HCV genotype 1 and those with genotype 2 or 3.[1,2]

Many studies have been performed to elucidate the viral factor determining the sensitivity or resistance to the IFN-based antiviral therapy, especially for HCV genotype 1. Several amino acid substitutions have been reported to be associated with the efficacy of IFN-based antiviral therapy in patients infected with HCV genotype 1b.[3–9] In the late 1990s, Enomoto *et al.* reported that mutations in the amino acids at positions 2209–2248 of the NS5A region of HCV were closely associated with the efficacy of IFN monotherapy;[3] however, these results proved controversial.[10–22] Very recently, a few studies have reported an association between other amino acid substitutions and the rate of SVR by the PEG-IFN/ribavirin therapy in patients with HCV genotype 1b.[7,8] However, the influence of these amino acid substitutions on the sensitivity to IFN or the outcome of PEG-IFN/ribavirin combination therapy has not been fully established.

In the present study, the authors investigated the association of four HCV amino acid substitutions (70 and 91 of the core region, 139 of the E1 region, and 2209–2248 of the NS5A region), with IFN sensitivity. Its association with the combination therapy PEG-IFN and ribavirin was also investigated in Japanese patients chronically infected with HCV genotype 1b, and having high pretreatment HCV RNA concentration.

Patients and methods

Patients

A total of 148 patients with chronic hepatitis C and without cirrhosis received antiviral combination therapy with PEG-IFN and ribavirin at the Ogaki Municipal Hospital, Ogaki, Japan, between July 2005 and June 2007. Among them, 109 patients had been infected with HCV genotype 1b and had pretreatment HCV RNA concentration > 100×10^3 IU/mL, as assessed by quantitative polymerase chain reaction (PCR) assay (Amplicor HCV Monitor Test, version 2.0; Roche Molecular Systems, Pleasanton, CA, USA). Of those 109 patients, 107 patients were enrolled in the study (two patients declined to enroll). The clinical characteristics of study patients are listed in Table 1. The patient group was comprised of 52 males (48.6%) and 55 females (51.4%), with a mean age of 58.9 ± 9.0 years. Twenty-two patients (20.6%) had previously received blood transfusion. Although 32 patients (29.9%) had a history of previous antiviral therapy by monotherapy with conventional IFN or combination therapy with conventional IFN and ribavirin, no patients had a history of the combination therapy with PEG-IFN and ribavirin. The average pretreatment HCV RNA concentration was $1760 \pm 1139 \times 10^3$ IU/mL. In 102 patients who underwent pretreatment liver biopsy, the grade of liver fibrosis according to the METAVIR score[23] was F0 in 5 patients (4.9%), F1 in 61 patients (59.8%), F2 in 24 patients (23.5%), and F3 in 12 patients (11.8%), respectively. No patients had co-infection with hepatitis B virus or human immunodeficiency virus. No patients were alcohol abusers or intravenous drug users.

Table 1 Clinical characteristics of study patients ($n = 107$)

Age (years)	58.9 ± 9.0
Sex (female/male)	55 (51.4)/52 (48.6)
Body weight (kg)	59.1 ± 10.2
History of interferon therapy (naive/retreatment)	75 (70.1)/32 (29.9)
History of transfusion (−/+)	85 (79.4)/22 (20.6)
Alanine aminotransferase (IU/L)	65.8 ± 64.9
Aspartate aminotransferase (IU/L)	55.9 ± 44.3
Gamma-glutamyl transpeptidase (IU)	53.7 ± 53.6
Alkaline phosphatase (IU/L)	265.2 ± 86.4
Albumin (g/dL)	4.14 ± 0.35
Total bilirubin (mg/dL)	0.69 ± 0.28
White blood cell count (/μL)	5201 ± 1197
Hemoglobin (g/dL)	14.0 ± 1.4
Platelet count ($\times 10^3$/μL)	166 ± 51
Liver histology-activity (A0/A1/A2/A3)†	2 (2.0)/55 (53.9)/36 (35.3)/9 (8.8)
Liver histology-fibrosis (F0/F1/F2/F3)†	5 (4.9)/61 (59.8)/24 (23.5)/12 (11.8)
HCV RNA concentration ($\times 10^3$ IU/mL)	1760 ± 1139
Reduction of peginterferon dose	29 (27.1)
Reduction of ribavirin dose	49 (45.8)
Response (SVR/relapse/NR)	39 (36.5)/38 (35.5)/30 (28.0)

Percentages are shown in parentheses.
†Liver biopsy was not performed in five patients.
HCV, hepatitis C virus; NR, no response; SVR, sustained virologic response.

Single administration test of conventional interferon alpha to evaluate sensitivity to interferon

All patients were underwent a single administration test of conventional IFN alpha more than 2 weeks before the start of the combination therapy to evaluate the sensitivity of HCV to IFN in each patient. They received intramuscular administration of 6 mega-units of standard IFN alpha-2b (Intron A; Schering-Plough, Tokyo, Japan). The concentration of HCV RNA was measured before and 24 h after the single administration test and the reduction of serum HCV RNA was calculated.

Combination therapy with peginterferon and ribavirin

For combination therapy with PEG-IFN and ribavirin, all patients were given PEG-IFN alpha-2b (Pegintron, Schering-Plough) weekly and ribavirin (Rebetol, Schering-Plough) daily according to the manufacturer's recommendations. The dose of PEG-IFN and ribavirin were adjusted by patient body weight. Patients weighing ≤ 45 kg were given 60 μg of PEG-IFN alpha-2b once a week, those weighing > 45 kg and ≤60 kg were given 80 μg, those weighing > 60 kg and ≤75 kg were given 100 μg, those weighing > 75 kg and ≤90 kg were given 120 μg, and those weighing > 90 kg were given 150 μg. Patients weighing ≤ 60 kg were given 600 mg of ribavirin per day, those weighing > 60 kg and ≤80 kg were given 800 mg of ribavirin per day, and those weighing > 80 kg were given 1000 mg of ribavirin per day. All patients were scheduled to undergo 48 weeks of treatment; longer durations were not considered in this study. Serum HCV RNA concentration was measured every 4 weeks on an outpatient basis. The presence of HCV RNA in the serum was measured by the qualitative Amplicor Monitor HCV RNA assay (AMPLICOR Hepatitis C Virus (HCV) Test, version 2.0, Roche Molecular Systems; detection limit, 50 IU/mL) to confirm the undetectability of serum HCV RNA, when it was unquantifiable (under the detection limit) by the quantitative Amplicor Monitor assay (detection limit, 615 IU/mL). Patients were classified into categories as follows: rapid virologic response (RVR) was defined as undetectable serum HCV RNA at 4 weeks from the start of the combination therapy. Complete early virologic response (cEVR) was defined as undetectable serum HCV RNA within 12 weeks of the start of the therapy. End-of-treatment response (ETR) was defined as undetectable serum HCV RNA at the end of the treatment period (i.e. 48 weeks after the start of the therapy). Sustained virologic response (SVR) was defined as undetectable serum HCV RNA at 24 weeks after the end of therapy. Relapse was defined as positive serum HCV RNA during the period between the end of treatment and 24 weeks thereafter, following ETR. Null-response (NR) was defined as positive serum HCV RNA throughout the treatment period and thereafter.