The HCV genome is initially translated as a large polyprotein and must be processed to produce functional viral proteins. Host proteases cleave the N-terminal structural proteins, but the viral NS3-4A serine protease is essential for cleaving the nonstructural proteins. NS3-4A also interferes with the immune response by degrading immune-signaling molecules [7]. Consequently, targeting this protease using the peptidomimetic inhibitor telaprevir both interferes with viral replication and may help rescue immune signaling, leading to a rapid decrease in HCV RNA level [8, 9]. In most patients, however, viral decline after telaprevir monotherapy is short-lived, followed by viral breakthrough because of strong selection for escape mutants within several weeks. Combination therapy with IFN alone yields unsatisfactory results, and ribavirin appears to be required to avoid relapse [10]. Because telaprevir triple therapy is an extension of the current standard of care instead of an IFN-free alternative, it does not address problems associated with the cost or adverse effects of combination therapy and may limit options for retreatment; however, it is particularly promising for patients who showed at least a transient response after prior combination therapy [11]. Nonetheless, telaprevir monotherapy may provide an alternative treatment for patients unable to tolerate IFN and/or ribavirin—at least in patients with low viral loads [12]. Additional research is needed to identify factors predicting outcome of treatment and incidence of adverse effects in different populations.

A number of host factors are known to affect outcome of PEG-IFN plus ribavirin combination therapy, including age, fibrosis, obesity, hepatic steatosis, [13] low-density lipoprotein cholesterol, γ-gamma-glutamyl transpeptidase (GTP) [14], and insulin resistance [15]. A number of recent studies have also shown that common genetic variation in the IL28B locus on chromosome 19 is strongly associated with spontaneous clearance and outcome after combination therapy [16–19]. Viral factors have also been shown to predict response to combination therapy, including HCV genotype [20], baseline viral titer [13, 20], amino acid substitutions at positions 70 and 91 of the HCV core protein, and the NS5A IFN Sensitivity Determining Region (ISDR) [21, 22]. Because telaprevir directly targets the virus and often results in selection for escape mutants, it is likely that additional predictive factors affecting response to treatment will be uncovered.

Combination therapy is poorly tolerated among some patients, and ribavirin-induced anemia is a serious adverse effect of the therapy that may result in dose reduction or discontinuation. Recent studies have shown an association between genetic variation in the ITPA locus and change in hemoglobin levels during treatment [23–25]. Although it does not appear to affect outcome of therapy [23, 24] (but see [25]), patients with an anemia-susceptible genotype may require greater reductions in ribavirin dose, which is associated with poorer response to therapy [26]. Telaprevir also moderately affects hemoglobin levels, but rash is the most common side effect of telaprevir therapy [10].

In the current study, we examined 94 patients with genotype 1 who received triple therapy to identify predictors for response to treatment and to assess effects of triple therapy on hemoglobin levels.

METHODS

Patients

Ninety-four Japanese patients who participated in a phase 3 clinical trial of the triple therapy in 2010 at Hiroshima University Hospital, Sapporo Kosei Hospital, and Toranomon Hospital (16, 17, and 61 patients, respectively) were investigated. Inclusion criteria for the study included remaining positive for genotype 1 HCV RNA for >6 months; having an HCV RNA level ≥5.0 log IU/mL, as determined by the COBAS TaqMan HCV test (Roche Diagnostics KK); and being aged 20–65 years, with a body weight >40 kg and <120 kg at the time of entry into the study. Exclusion criteria included cirrhosis; results positive for hepatitis B surface antigen or antibody against HIV; previous or current hepatocellular carcinoma; possible overlapping liver diseases, such as autoimmune hepatitis, hemochromatosis, Wilson disease, alcoholic liver disease, or renal disease; or creatinine clearance ≤50 mL/min at baseline, hemoglobin level <12 g/dL, neutrophil count <1500 neutrophils/mm^3, or platelet count <100,000 platelets/mm^3 at baseline. Patient profiles are shown in Tables 1 and 2.

All patients were treated with PEG-IFN-α-2b, ribavirin, and telaprevir triple therapy. Telaprevir (750 mg; MP-424; Mitsubishi Tanabe Pharma) was administered every 8 h after meals. PEG-IFN-α-2b (Schering Plough) was injected subcutaneously at a median dose of 1.5 μg/kg per week. Ribavirin (Schering Plough) dose was adjusted by body weight (600 mg for ≤60 kg; 800 mg for >60 to ≤80 kg; and 1000 mg for >80 kg), based on guidelines by the Ministry of Health, Labor and Welfare of Japan [27], and the drug was administered orally after breakfast and dinner. Triple therapy with telaprevir was given for 12 weeks, followed by an additional 12 weeks of PEG-IFN-α-2b and ribavirin combination therapy. Triple therapy was withdrawn if hemoglobin levels were <8.5 g/dL. Ribavirin dose was reduced by 200 mg/day in patients who were receiving 600 or 800 mg/day (or by 400 mg in those receiving 1000 mg/day) when hemoglobin levels decreased to <12 g/dL and by an additional 200 mg if levels decreased to <10 g/dL. In addition, ribavirin dose was also reduced by 200 mg in patients with a hemoglobin level <13 g/dL at baseline and in those in whom the level decreased by 1 g/dL to <13 g/dL within 1 week. PEG-IFN dose was decreased to one-half when leukocyte count decreased to <1500 leukocytes/mm^3, neutrophil count decreased to <750 neutrophils/mm^3, or platelet count decreased to <80 × 10^3 platelets/mm^3; PEG-IFN was withdrawn if these factors decreased to <1000 leukocytes/mm^3, 500 neutrophils/mm^3, or 50 × 10^3 platelets/mm^3, respectively. Triple therapy was suspended temporarily when

Table 1. Patient Characteristics

	Total (n = 94)	SVR (n = 69)	Non-SVR (n = 25)
Response to previous therapy (naive/relapser/NR)	25/44/25	20/41/8	5/3/17
Age	57 (23–65)	57 (23–65)	56 (40–65)
Sex (M/F)	52/42	42/27	10/15
Height (cm)	163.6 (141.8–189.2)	164.7 (141.8–189.2)	157.7 (148.5–181.5)
Weight (kg)	61 (41–92.5)	61.7 (41–92.5)	58.8 (44.9–80.3)
rs8099917 (TT/TG/GG)	50/41/3	47/21/1	3/20/2
rs1127354 (CC/CA/AA)	75/18/1	55/13/1	20/5/0
Viral genotype (1b/others)	93/1	69/0	24/1
Core 70 (W/M/ND)	50/43/1	43/26/0	7/17/1
Core 91 (W/M/ND)	48/45/1	39/30/0	9/15/1
ISDR (0–1/≥2/ND)	82/8/4	61/5/3	3/21/1
WBC (/mm^3)	4800 (2800–8100)	4900 (2800–8100)	4660 (3000–7900)
Plt ($\times 10^4$/mm^3)	17.7 (9.1–33.8)	18 (9.9–33.8)	16 (9.1–23.9)
Hb (g/dL)	14.3 (12.3–16.6)	14.5 (12.5–16.5)	14.1 (12.3–16.6)
ALT (IU/L)	39 (12–302)	38 (12–302)	46 (17–135)
γGTP (IU/L)	36 (11–233)	33 (11–233)	53 (19–226)
Virus titer (log IU/mL)	6.7 (5.1–7.7)	6.8 (5.1–7.7)	6.7 (5.4–7.6)
Days to first ribavirin reduction	17 (2–168)	18 (2–168)	14 (7–73)
Duration of telaprevir administration (days)	85 (29–85)	85 (29–85)	84 (35–85)
Duration of peg-interferon injection (days)	162 (22–165)	162 (22–165)	162 (30–165)
Duration of ribavirin administration (days)	169 (29–169)	169 (29–169)	168 (36–169)
Effect of therapy (SVR/BT/TR/NR)	69/4/19/2	–	–

NOTE. All patients were infected with genotype 1. Counts are listed for categorical values and the median and range are reported for continuous variables. ND, not determined, data unavailable.

hemoglobin levels decreased to <8.5 g/dL. Treatment was resumed with PEG-IFN and 200 mg ribavirin if hemoglobin levels increased to ≥8.5 g/dL within 2 weeks after withdrawal. Reduction of telaprevir dose was not permitted. It was discontinued if severe adverse effects appeared, and therapy was continued with PEG-IFN and ribavirin alone. Erythropoietin was not used to elevate hemoglobin levels.

Virologic response was analyzed on an intent-to-treat basis. The successful end point of treatment was sustained virological response (SVR) for patients who showed undetectable HCV RNA for 24 weeks after cessation of treatment. In transient responders (or persons who experienced relapse), HCV RNA levels became undetectable by the end of treatment but became positive again during the follow-up period. In patients with viral breakthrough, HCV RNA became undetectable during the treatment period but then became positive again before the end of the treatment period. The remaining patients whose HCV RNA never became undetectable were nonresponders. We also defined rapid virological response (RVR) as undetectable HCV RNA at week 4 of treatment and early virological response as a >2 $\log_{10}$ decrease in HCV RNA levels by week 12 of treatment. All participants gave written informed consent to participate in the study according to the process approved by the ethical committee of each hospital and conforming to the ethical guidelines of the 1975 Declaration of Helsinki.

HCV RNA Levels

HCV RNA levels were measured using the TaqMan reverse-transcription polymerase chain reaction (PCR) test. The measurement range of this assay was 1.2–7.8 log IU/mL. Samples that exceeded the measurement range were diluted with phosphate-buffered saline and reanalyzed.

ISDR and Core Amino Acid Substitutions

Amino acid substitutions in the HCV core and ISDR regions were determined using direct sequencing of PCR products after extraction and reverse transcription of HCV RNA with use of serum samples kept frozen at −80°C. Core amino acid substitutions at positions 70 and 91 (core70 and core91, respectively) were determined according to Akuta et al [14, 28], and the number of ISDR substitutions was determined using the methods of Enomoto et al [21, 29, 30].

Single-Nucleotide Polymorphism (SNP) Genotyping

We genotyped each patient for 2 SNPs: rs8099917, an IL28B SNP previously reported to be associated with therapy outcome, and rs1127354 [31], an ITPA SNP reported to be associated with ribavirin-induced anemia [23]. Samples were genotyped using the Illumina HumanHap610-Quad Genotyping BeadChip or with the Invader or TaqMan assay, as described elsewhere [32, 33].

Table 2. Patient Characteristics Grouped by Treatment History

	Total (n = 94)	Naive (n = 25)	Relapser (n = 44)	NR (n = 25)
Age	56.5 (23–65)	54 (23–64)	57.5 (44–65)	57 (40–65)
Sex (M/F)	52/42	13/12	27/17	12/13
Height (cm)	163.5 (142–189)	163 (147–189)	167.5 (142–177)	160 (149–174)
Weight (kg)	61 (41–93)	57 (42–80)	63.5 (41–93)	59 (45–77)
rs8099917 (TT/GT/GG)	50/41/3	15/9/1	33/11/0	2/21/2
rs1127354 (CC/CA/AA)	75/18/1	18/6/1	34/10/0	23/2/0
Viral genotype (1b/others)	93/1	25/0	44/0	24/1
Core 70 (W/M/ND)	50/43/1	13/12/0	28/16/0	9/15/1
Core 91 (W/M/ND)	48/45/1	14/11/0	23/21/0	11/13/1
ISDR (0–1/≥2/ND)	82/8/4	25/0/0	38/4/2	19/4/2
WBC (/mm^3)	4800 (2800–8100)	5390 (3000–7500)	4750 (2800–8100)	4700 (3040–8000)
Plt ($\times 10^4$/mm^3)	18 (9–34)	20 (15–30)	16.5 (10–34)	16 (9–24)
Hb (g/dL)	14.3 (12.3–17)	14.1 (12.5–16.1)	14.45 (12.3–17)	14.4 (12.3–16.6)
ALT (IU/L)	38.5 (12–302)	35 (12–113)	39.5 (16–302)	45 (17–135)
γGTP (IU/L)	36 (11–233)	31 (11–141)	34 (14–233)	49 (21–226)
Virus titer (log IU/mL)	6.7 (5.1–7.7)	6.7 (5.1–7.4)	6.7 (5.4–7.6)	6.7 (5.8–7.7)
Days to first ribavirin reduction	18 (3–168)	18 (3–52)	18 (3–168)	15 (8–52)
Duration of telaprevir administration (days)	85 (29–85)	85 (29–85)	85 (32–85)	85 (35–85)
Duration of peg-interferon injection (days)	162 (22–165)	163 (22–165)	162.5 (30–165)	162 (30–165)
Duration of ribavirin administration (days)	169 (29–169)	168 (29–169)	169 (32–169)	169 (36–169)
Effect of therapy (SVR/BT/TR/NR)	69/4/19/2	20/0/5/0	41/1/2/0	8/3/12/2

NOTE. Counts are listed for categorical values and the median and range are reported for continuous variables.

Statistical Analysis

Statistical analysis was performed using PASW Statistics, version 18 (SPSS) and R, version 2.11. Categorical data were analyzed using χ^2 and Fisher's exact tests, and continuous data were analyzed using the nonparametric Mann-Whitney U test. To identify independent predictive factors, variables that were significant at the .05 level in univariate tests were considered as candidate factors for multiple logistic regression analysis. The model was reduced using AIC-based forward and/or backward stepwise selection with bootstrap validation. Odds ratios (ORs) were corrected for over-optimism with use of penalized maximum likelihood.

RESULTS

Effect of the Triple Therapy by Previous Response to PEG-IFN Plus Ribavirin Therapy

Patient profiles are shown in Tables 1 and 2. After triple therapy, 69 (73%) of 94 patients achieved SVR. Of the 25 treatment-naive patients, 20 (80%) eradicated the virus, and the remaining 5 achieved transient response. Similarly, 49 (71%) of the 69 patients who had received prior treatment achieved SVR with triple therapy. Of note, however, 41 (93%) of 44 patients who had responded transiently to previous treatment were able to eradicate the virus with use of triple therapy. Conversely, only 8 (32%) of 25 patients who had failed to respond to prior treatment were able to achieve SVR with use of triple therapy, and 2 of these patients also failed to respond to triple therapy. None of the 4 patients in whom viral breakthrough occurred were treatment naive, and 3 of the 4 were nonresponders to prior treatment.

IL28B SNP Genotypes

The genotype of IL28B SNP rs8099917 was determined for each patient. The frequency of the rs8099917 risk allele (G) was 0.25 among all patients, 0.17 among patients who achieved SVR, 0.38 among patients with viral breakthrough, and 0.5 among both transient responders and nonresponders. Patients with the rs8099917 TT genotype were significantly more likely to achieve SVR (94% vs 50%; P = 4.6E-6; Figure 1) and had significantly higher baseline viral loads (6.9 vs 6.45 log IU/mL; P = .0056; Figure 2D), compared with patients with GT or GG genotypes.

Loss of Hemoglobin During and After Triple Therapy

The triple therapy resulted in hemoglobin loss in all patients, but the pattern differed by ITPA SNP rs1127354 genotype (Figure 3). The frequency of the rs1127354 minor allele (A) was 0.11 among all patients, 0.11 among patients who achieved SVR, .13 among transient responders, and 0 in both patients with viral breakthrough and nonresponders. There was no effect of rs1127354 genotype on SVR (73% for both CC and non-CC genotypes), but ribavirin dosage reduction was required significantly earlier in patients with genotype CC than in those with non-CC genotypes (18 days vs 29 days, respectively; P = 3.2E-5; Figure 4). Although hemoglobin loss

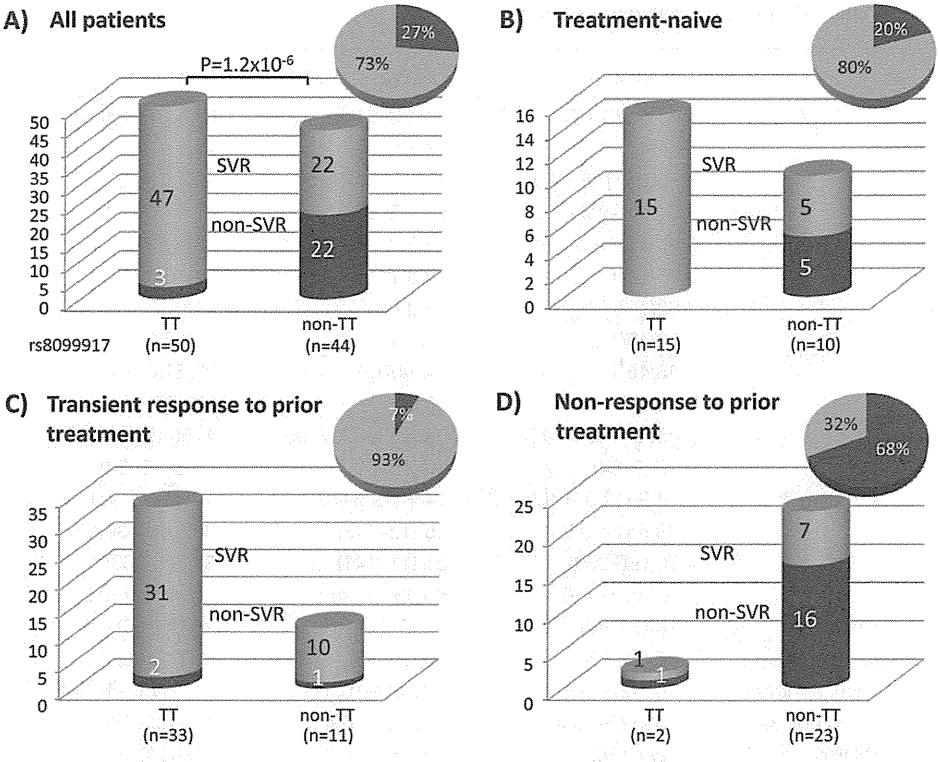

Figure 1. SVR frequency after triple therapy grouped by IL128B SNP rs8099917 genotype and by response to previous interferon (IFN) treatment. *A*, All patients. *B*, Treatment-naive patients. *C*, Previously treated patients who responded transiently to therapy. *D*, Previously treated patients who failed to respond to therapy. Inset pie charts indicate percentage of SVR *(light gray)* and non-SVR *(dark gray)* patients.

resulted in dose reduction according to the treatment protocol, no significant effects on SVR rate resulting from dose reduction were observed.

Viral Substitutions

The 43 patients (46%) with a substitution at position 70 of the HCV core protein (core70) were significantly less likely to achieve SVR than were patients with wild-type core70 (60% vs 86%; $P = .01$). There was no difference in SVR rate due to substitution at position 91 (core91; 81% vs 67%; $P = .17$) (Figure 2). There was also no difference in SVR rate due to substitutions in the NS5A ISDR region ($P = .43$). Patients with rs8099917 genotype TT were significantly more likely to be associated with wild-type core70 or core91 ($P = .006$ and $P = .031$, respectively). There was no association between rs809917 genotype and ISDR substitutions ($P = .94$).

Predictive Factors for RVR

RVR, defined as undetectable HCV RNA levels at week 4 of treatment, is a strong on-treatment predictor of SVR [34]. Previous IFN treatment, time to first ribavirin dose reduction, and baseline hemoglobin levels were each significant univariate predictors, but only hemoglobin level was a significant independent predictor of RVR under multiple logistic regression ($P = .028$; OR, 3.11).

Predictive Factors for SVR

Significant univariate predictors for SVR included clinical factors (γGTP level; rs8099917 genotype), viral factors (core70 substitutions), response to prior treatment (relapse or nonresponse), and on-treatment factors (RVR) (Table 3). Of these, nonresponse to prior treatment, rs8099917 genotype, RVR, and core70 substitutions were retained in the multivariate model, and nonresponse to prior treatment (OR, .17; $P = .01$), rs8099917 genotype (OR, .12; $P = .014$), and RVR (OR, 14.0; $P = .0064$) were identified as significant independent predictors for SVR. When only pretreatment factors were considered, nonresponse to prior treatment (OR, .14; $P = .0028$) and rs8099917 genotype (OR, .19; $P = .027$) were the only independent predictors.

DISCUSSION

This study showed that patients undergoing PEG-IFN, ribavirin, and telaprevir triple therapy for chronic hepatitis C genotype 1 infection achieve a higher SVR rate than typically expected under combination therapy alone in Japanese patients. Moreover, patients who showed transient response in previous treatment were more likely to achieve SVR after triple therapy, whereas nonresponders to prior treatment remained unlikely to eradicate the virus. Considering that telaprevir has a mode of

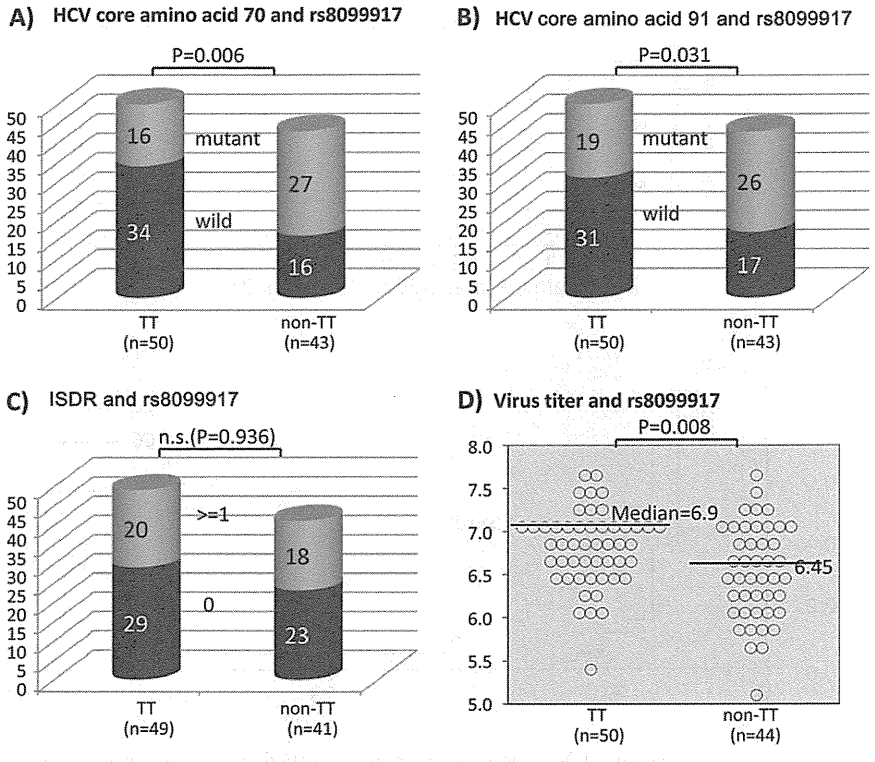

Figure 2. Viral factors and IL128B SNP rs8099917 genotype. *A*, Substitutions at HCV core amino acid 70. *B*, Substitutions at core amino acid 91. *C*, Frequency of patients with ≥2 substitutions in the NS5A interferon sensitivity determining region. *D*, Baseline viral load.

action different from that of IFN and ribavirin, [5] it is surprising that triple therapy does not better improve SVR rates among prior nonresponders, suggesting that additional unknown factors contribute to nonresponse. However, the duration of triple therapy, followed by standard of care, was limited to 24 weeks in this study; therefore, it is possible that prior nonresponders and patients who experienced relapse may benefit from a longer duration of therapy.

The most interesting result from this study is the high SVR rate among patients who previously experienced relapse, even

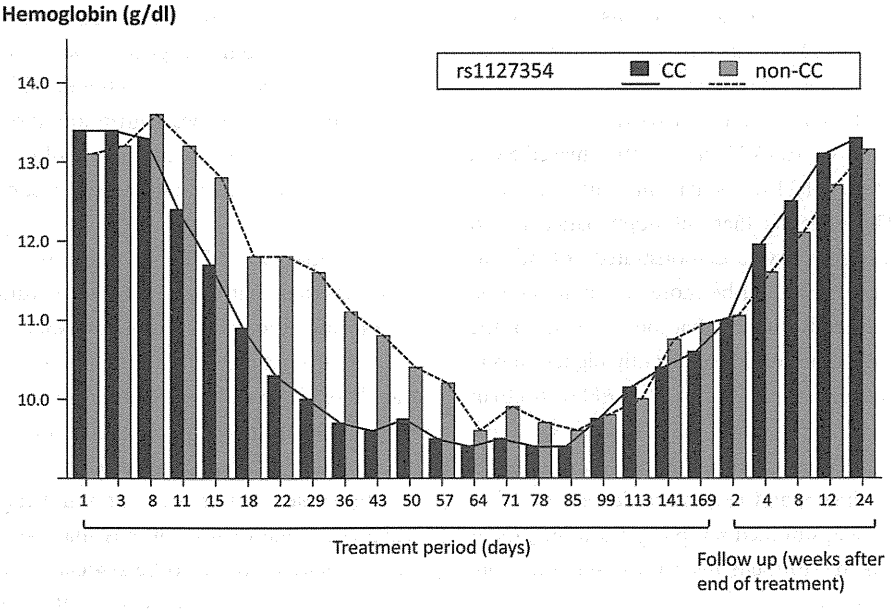

Figure 3. Change in hemoglobin level by ITPA SNP during triple therapy. Hemoglobin levels in patients grouped by ITPA SNP rs1127354 genotype (solid line represents CC; dashed line represents non-CC).

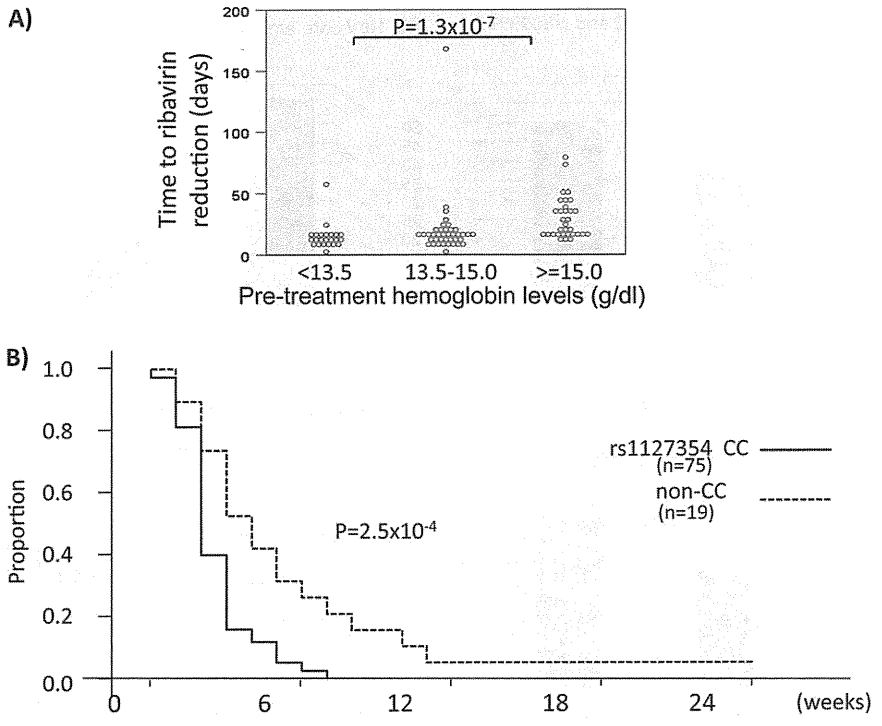

Figure 4. Ribavirin dose reduction during triple therapy. *A*, Number of days of treatment until first ribavirin dose reduction, by pretreatment hemoglobin levels. *B*, Kaplan-Meier curve for dose reduction grouped by ITPA SNP rs1127354 genotype (solid line represents CC; dashed line represents non-CC).

compared with that of naive patients. This is partly because of the higher frequency of the favorable rs8099917 TT genotype among patients who previously experienced relapse (33 [75%] of 44) than among naive patients (15 [60%] of 25), which perhaps reflects the fact that all patients who previously experienced relapse demonstrated at least a transient response to combination therapy and that this group is less likely to include as many patients with non-TT genotypes. All of the treatment-naive patients with the favorable genotype (15 [100%] of 15) achieved SVR, compared with 31 (94%) of 33 patients who previously experienced relapse; conversely, only one-half of the treatment-naive patients with unfavorable rs8099917 genotypes (5 [50%] of 10) achieved SVR, compared with only 1 (9%) of 11 of the patients who previously experienced relapse. This suggests that, although patients who previously experienced relapse have a demonstrated potential to respond to the therapy, there should be more variability among naive patients. Another consideration is that the frequency of the favorable wild-type core70 amino acid was slightly higher among patients who previously experienced relapse (28 [64%] of 44) than among naive patients (13 [52%] of 25). It should be noted, however, that the small number of patients in this study limits the conclusions that can be drawn, and results should be verified in a larger study, perhaps using stratified sampling based on patient background with regard to treatment history to establish more homogeneous patient populations.

In this and a number of other studies, variation in the IL28B locus remains the strongest predictor of SVR reported to date [16–18, 35]. It is unclear which, if any, of the reported SNPs is the primary or functional SNP, but most studies report results for rs8099917 and/or rs12979860, which are under strong linkage disequilibrium in Japanese patients and fall within the intergenic region upstream of IL28B. Although the mechanism is unknown, IL28B and the other 2 members of the IFN-λ family, IL28A and IL29, code for type III IFNs, which are similar to type I IFNs but use a highly tissue-specific receptor [36, 37]. IFN-stimulated genes appear to be initially down-regulated in patients with the favorable rs8099917 TT genotype [38], which may help to prevent desensitization and promote maximal induction of IFN-stimulated genes, although mechanistic studies are needed to understand the connection between IL28B and SVR.

In addition to IL28B polymorphisms, a number of studies have reported that amino acid substitutions in the HCV core protein and the ISDR region of NS5A independently predict treatment outcome after combination therapy [14, 22, 28, 30], and these findings have recently been extended to triple therapy[39, 40]. In this study, substitution at core70 was significant in univariate tests and was selected for inclusion in the multivariate model, but it was not significant in multiple logistic regression. One reason for this may be that core substitutions were initially reported to be associated with nonresponse [22], whereas this study focused on SVR because of the very small number of nonresponders. Terms that are significant in univariate but not multivariate tests may be correlated with each

Table 3. Predictive Factors Associated With SVR in Chronic Hepatitis C Virus Genotype 1 Patients Who Received Pegylated Interferon/Ribavirin/Telaprevir Triple Therapy

	Simple				Multiple			
Variable	*n*	OR	*P*		OR	(95% CI)	*P*	
Treatment-naive	94	1.6	.389					
Previous non-responder	94	0.1	5.5E-08	***	0.17	(.04–.66)	.010	*
Previous relapser	94	10.7	5.2E-05	***				
Age	94	0.8	.939					
Sex (male vs female)	94	1.5	.100					
BMI (kg/m2)	94	0.9	.558					
rs8099917 (TT vs GT/GG)	94	0.1	1.7E-06	***	0.12	(.02–.65)	.014	*
rs1127354 (CC vs AC/AA)	94	1.0	.980					
Core aa70 (wt vs mutant)	93	0.2	.0053	**	0.35	(.09–1.31)	.119	
Core aa91 (wt vs mutant)	93	0.5	.111					
ISDR (0–1 vs ≥2)	90	1.7	.308					
Viral load	94	1.1	.560					
ALT (IU/L)	94	0.9	.142					
gammaGTP	94	0.7	.0009	***				
Hemoglobin (g/dL)	94	1.4	.292					
WBC (/mm^3)	94	1.3	.271					
Platelets (×104/mm^3)	94	1.7	.165					
Total cholesterol (mg/dL)	94	1.7	.160					
LDL cholesterol (mg/dL)	94	2.6	.018	*				
Days to first ribavirin dose reduction	94	1.2	.129					
RVR	94	10.8	4.4E-05	***	14.00	(2.10–93.2)	.006	**
EVR	94	7992.0	.004	**				

NOTE. Results of simple and multiple logistic regression are shown. The multivariate model was constructed using stepwise selection of univariate terms significant at the .05 level. Symbols: * ($P < .05$), ** ($P < .01$), *** ($P < .001$).

other, and only the factor with the strongest effect remains significant. In this case, core70 is significantly correlated with the stronger rs8099917 genotype ($r = .31$; $P = .0027$), although other studies have shown that these terms contribute independently, especially when a larger number of patients are included [39]. Without knowing the mechanism underlying either factor, it is not possible to determine whether the underlying factors that they represent are in fact independent or whether they represent different aspects of a common unknown factor.

Although novel therapies that are not based on IFN and ribavirin are urgently needed, the pending introduction of protease inhibitors represents a pivotal addition to the treatment arsenal, especially for patients who show at least partial response to combination therapy. Because telaprevir is effective as monotherapy, even if only briefly until resistant mutations emerge, alternate combination therapies based on telaprevir and another component designed to raise the barrier to resistance may provide an adequate alternative for older patients and patients unable to tolerate IFN or ribavirin. Furthermore, identification of additional SNPs associated with anemia and other adverse effects will help reduce complications and the need for dose reductions and may lead to treatment guidelines for at-risk patients, such as administration of erythropoietin to stimulate erythropoiesis [41]. Ribavirin dose reductions were required significantly earlier in patients with ITPA SNP genotype CC, compared with patients with non-CC genotypes, which may contribute to poorer response if cumulative ribavirin administration decreases to <80% of the planned dose [26], although ribavirin dose reduction did not affect SVR rate in this study.

In conclusion, triple therapy with PEG-IFN, ribavirin, and telaprevir resulted in higher rates of SVR, compared with PEG-IFN plus ribavirin combination therapy, especially among treatment-naive patients and patients who showed transient response to prior treatment. ITPA polymorphisms predict ribavirin-induced anemia but are not associated with SVR, whereas IL28B polymorphisms and early viral kinetics remain the strongest predictors of SVR with use of triple therapy. Considering both host and viral factors, we identified 2 subgroups of patients who responded well to triple therapy: patients with the favorable rs8099917 TT genotype (47 [94%] of 50) and patients with non-TT genotypes who had wild-type core70 and core91 amino acids (7 [78%] of 9). Patients matching these conditions would benefit most from this 24-week triple therapy, whereas a longer duration of therapy should perhaps be considered for the remaining difficult-to-treat patients.

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Amino Acid Substitutions in Hepatitis C Virus Core Region Predict Hepatocarcinogenesis Following Eradication of HCV RNA by Antiviral Therapy

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Substitution of amino acid (aa) 70 and/or 91 in the core region of HCV genotype 1b (HCV-1b) is an important predictor of hepatocarcinogenesis, but its impact on the development of hepatocellular carcinoma (HCC) following eradication of HCV RNA by antiviral therapy is not clear. 1,273 patients with HCV-related chronic liver disease, with sustained virological response, defined as negative HCV RNA at 24 weeks after cessation of interferon monotherapy or interferon plus ribavirin combination therapy, were included in a follow-up study to evaluate the impact of aa substitution in the core region on hepatocarcinogenesis. Twenty six patients developed HCC during the follow-up. The cumulative rates of new HCC were 3.2%, 4.8%, and 8.6% at the end of 5, 10, and 15 years, respectively. The rates in patients infected with HCV-1b/Gln70(His70) [glutamine (histidine) at aa 70] were significantly higher than in patients infected with HCV-1b/Arg70 (arginine at aa 70) ($P = 0.007$; log-rank test) and HCV-2a/2b ($P < 0.001$; log-rank test). The rates in patients infected with HCV-1b/Arg70 were not significantly higher than in those infected with HCV-2a/2b ($P = 0.617$; log-rank test). Multivariate analysis identified HCV-1b/Gln70(His70) (HR 10.5, $P < 0.001$), advanced fibrosis (HR 9.03, $P = 0.002$), and old age (HR 3.09, $P = 0.066$) as determinants of hepatocarcinogenesis. In conclusion, aa substitution in the core region of HCV-1b at the start of antiviral therapy is an important predictor of HCC following eradication of HCV RNA. This study emphasizes the importance of detection of aa substitutions in the core region before antiviral therapy. ***J. Med. Virol. 83:1016–1022, 2011.*** © 2011 Wiley-Liss, Inc.

KEY WORDS: HCV; genotype; sustained virological response; hepatocellular carcinoma; core region; glutamine

INTRODUCTION

Infection with hepatitis C virus (HCV) is often persistent and can progress to chronic hepatitis, cirrhosis of the liver, and hepatocellular carcinoma (HCC) [Niederau et al., 1998; Kenny-Walsh, 1999]. At present, interferon (IFN), in combination with ribavirin, is the mainstay for treatment of HCV infection. In Japan, HCV genotype 1b (HCV-1b) and high viral loads account for more than 70% of HCV infections, making it difficult to treat patients with chronic hepatitis C [Tsubota et al., 2005].

Despite numerous lines of epidemiological evidence of an association between HCV infection and the development of HCC, it remains controversial whether the virus itself plays a direct role or an indirect role in the pathogenesis of HCC [Koike, 2005]. It has become evident that the HCV core region is potentially oncogenic in transgenic mice, but the clinical impact of the core region on hepatocarcinogenesis is still unclear [Moriya et al., 1998]. Previous reports indicated that amino acid (aa) substitutions at position 70 and/or 91 in the HCV core region of patients infected with HCV-1b are pretreatment predictors of poor virological response to pegylated IFN (PEG-IFN)/ribavirin combination therapy and triple therapy of

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telaprevir/PEG-IFN/ribavirin [Akuta et al., 2005, 2007a, 2010; Donlin et al., 2007], and also affect hepatocarcinogenesis [Akuta et al., 2007b; Fishman et al., 2009; Hu et al., 2009; Nakamoto et al., 2010]. These reports support the oncogenic potential of the core region from the clinical aspect. However, hepatocarcinogenesis still occurs even after eradication of HCV RNA by antiviral therapy [Ikeda et al., 2003, 2005; Tokita et al., 2005; Kobayashi et al., 2007; Hirakawa et al., 2008], though whether substitutions of aa 70 and/or 91 in the core region also affect hepatocarcinogenesis following eradication of HCV RNA await further investigation.

The present study included 1,273 patients with HCV-related chronic liver disease, with sustained virological response, defined as negative HCV RNA at 24 weeks after cessation of antiviral therapy (IFN monotherapy or IFN plus ribavirin combination therapy). The aims of this study were to evaluate the impact of aa substitutions in the core region detected at the start of antiviral therapy on hepatocarcinogenesis following eradication of HCV RNA.

PATIENTS AND METHODS

Patients

Among 4,570 consecutive patients infected with HCV, in whom antiviral therapy (IFN monotherapy or IFN plus ribavirin combination therapy) was initiated between February 1987 and June 2010 at the Toranomon Hospital, 1,273 were selected for the present study. We included patients who fulfilled the following criteria: (1) Patients positive for anti-HCV (by a third-generation enzyme immunoassay, Chiron Corp., Emerville, CA) and for HCV RNA by qualitative or quantitative analysis, before antiviral therapy. (2) Patients with sustained virological response, defined as negative HCV RNA at 24 weeks after cessation of antiviral therapy, based on HCV RNA qualitative analysis (Amplicor, Roche Diagnostics, Manheim, Germany) or by the COBAS TaqMan HCV test (Roche Diagnostics, Tokyo, Japan). (3) Patients without HCC, before and during IFN therapy. (4) Patients infected with a single genotype of HCV-1b, 2a, or 2b. (5) Patients negative for hepatitis B surface antigen (by radioimmunoassay, Dainabot, Tokyo). (6) Patients free of coinfection with the human immunodeficiency virus. (7) Lifetime cumulative alcohol intake <500 kg (mild to moderate alcohol intake). (8) Patients free of other types of hepatitis, and without hemochromatosis, Wilson disease, primary biliary cirrhosis, alcoholic liver disease, and autoimmune liver disease. (9) Each signed a consent form of the study protocol that had been approved by the human ethics review committee.

Table I summarizes the profile and laboratory data at the start of antiviral therapy of 1,273 patients with sustained virological response. They included 783 males and 490 females, aged 15–83 years (median, 53 years). The median follow-up time, from the end of antiviral therapy until the last visit, was 1.1 years (range, 0.0–18.0 years).

Laboratory Investigations

Blood samples were frozen at −80°C within 4 hr of collection and were not thawed until used for testing. HCV genotype was determined by PCR using a mixed primer set derived from nucleotide sequences of the NS5 region [Chayama et al., 1993]. HCV RNA was quantitated by branched DNA assay version 2.0 (Chiron Corp.), AMPLICOR GT HCV Monitor version 2.0 using the 10-fold dilution method (Roche Molecular Systems, Inc., Pleasanton, CA), or COBAS TaqMan HCV test (Roche Diagnostics). A high viral load was defined as branched DNA assay value of

TABLE I. Clinical Profile and Laboratory Data at the Start of Antiviral Therapy

Demographic data	
Number of patients	1,273
Sex (male/female)	783/490
Age (years)*	53 (15–83)
Body mass index (kg/m^2)*	22.7 (14.4–38.0)
Laboratory data	
Serum aspartate aminotransferase (IU/L)*	48 (11–1,386)
Serum alanine aminotransferase (IU/L)*	68 (10–2,009)
Total cholesterol (mg/dl)*	168 (79–328)
Fasting plasma glucose (mg/dl)*	93 (69–290)
HCV genotype (1b/2a/2b)*	664/433/176
Level of viremia (high viral load/low viral load)	838/415
Treatment regimen	
IFN monotherapy/IFN plus ribavirin	545/728
Histological findings	
Stage of fibrosis (F1/F2/F3/F4)	508/224/62/47
Amino acid substitutions in the HCV genotype 1b	
Core aa 70 [arginine/glutamine (histidine)]	348/127
Core aa 91 (leucine/methionine)	321/156

The enrolled patients had sustained virological response, defined as negative HCV RNA at 24 weeks after cessation of antiviral therapy.
Data are numbers and percentages of patients, except those denoted by asterisk (*), which represent the median (range) values.

≥1.0 Meq/ml, AMPLICOR GT HCV Monitor ≥100 × 10^3 IU/ml, or COBAS TaqMan HCV test ≥5.0 log IU/ml. Low viral load was defined as branched DNA assay value of <1.0 Meq/ml, AMPLICOR GT HCV Monitor <100 × 10^3 IU/ml, or COBAS TaqMan HCV test <5.0 log IU/ml. The lower limit of HCV RNA qualitative analysis (Amplicor, Roche Diagnostics, Manheim) was 100 copies/ml, and that of COBAS TaqMan HCV test was 1.2 log IU/ml. Samples with undetectable HCV RNA at 24 weeks after cessation of antiviral therapy by qualitative analysis or COBAS TaqMan HCV test were defined as HCV RNA-negative.

Detection of Amino Acid Substitutions in the Core Regions of HCV-1b

In the present study, aa substitutions in the core region of HCV-1b were analyzed by direct sequencing. HCV RNA was extracted from serum samples at the start of antiviral therapy and reverse transcribed with a random primer and MMLV reverse transcriptase (Takara Syuzo, Tokyo, Japan). Nucleic acids of the core region were amplified by nested PCR using the following primers. The first-round PCR was performed with CE1 (sense, 5′-GTC TGC GGA ACC GGT GAG TA-3′, nucleotides: 134–153) and CE2 (antisense, 5′-GAC GTG GCG TCG TAT TGT CG-3′, nucleotides: 1096–1115) primers, and the second-round PCR with CC9 (sense, 5′-ACT GCT AGC CGA GTA GTG TT-3′, nucleotides: 234–253) and CE6 (antisense, 5′-GGA GCA GTC GTT CGT GAC AT-3′, nucleotides: 934–953) primers. All samples were initially denatured at 95°C for 2 min. The 35 cycles of amplification were set as follows: denaturation for 30 sec at 95°C, annealing of primers for 30 sec at 55°C, and extension for 1 min at 72°C with an additional 7 min for extension. Then, 1 μl of the first PCR product was transferred to the second PCR reaction. Other conditions for the second PCR were the same as the first PCR, except that the second PCR primers were used instead of the first PCR primers. The amplified PCR products were purified by the QIA quick PCR purification kit (Qiagen, Tokyo, Japan) after agarose gel electrophoresis and then used for direct sequencing. Dideoxynucleotide termination sequencing was performed with the Big Dye Deoxy Terminator Cycle Sequencing kit (Perkin-Elmer, Tokyo, Japan).

Using HCV-J (accession no. D90208) as a reference [Kato et al., 1990], the sequence of 1–191 aa in the core protein of HCV-1b was determined and then compared with the consensus sequence constructed using 50 clinical samples to detect substitutions at aa 70 of arginine (Arg70) or glutamine/histidine (Gln70/His70) and aa 91 of leucine (Leu91) or methionine (Met91) [Akuta et al., 2005]. Thus, patients were classified into three HCV subgroups according to the HCV genotype and aa substitutions in the HCV-1b core region: (1) HCV-1b with Arg70, (2) HCV-1b with Gln70(His70), and (3) HCV-2a/2b.

Liver Histopathological Examination

Liver biopsy specimens were obtained percutaneously or at peritoneoscopy using a modified Vim Silverman needle with an internal diameter of 2 mm (Tohoku University style, Kakinuma Factory, Tokyo, Japan). The samples were fixed in 10% formalin and then stained with hematoxylin and eosin, Masson's trichrome, silver impregnation, and periodic acid-Schiff after diastase digestion. Each specimen submitted for examination contained ≥6 portal areas. Histopathological diagnosis was made by an experienced liver pathologist (HK) who was blinded to the clinical data. Chronic hepatitis was diagnosed based on the scoring system of Desmet et al. [1994] for histopathological assessment.

Follow-Up and Diagnosis of Hepatocellular Carcinoma

Hematological, biochemical, and virological tests were performed at least once every month until the virological response was determined. When sustained virological response was confirmed, blood tests and imaging studies (computed tomography or ultrasonography) were conducted once or twice per year in the majority of patients, except those lost to follow-up. When HCC was suspected, additional procedures, such as magnetic resonance imaging, abdominal angiography, and ultrasonography-guided tumor biopsy when necessary, were used to confirm the diagnosis.

Statistical Analysis

The cumulative rate of new cases of HCC was calculated using the Kaplan–Meier technique, and differences between the curves were tested using the log-rank test. Differences in the proportion of new cases of HCC according to groups were analyzed according to the period between the end of antiviral therapy and appearance of HCC. Stepwise Cox regression analysis was used to determine independent predictive factors that were associated with the development of HCC. The hazard ratio (HR) and 95% confidence interval (95%CI) were also calculated. Potential predictive factors associated with the development of HCC included the following variables: sex, age, body mass index, AST, ALT, total cholesterol, fasting plasma glucose, HCV genotype, level of viremia, treatment regimen, stage of fibrosis, and HCV subgroup according to HCV genotype in combination with aa substitutions in the core region. Variables that achieved statistical significance ($P < 0.05$) on univariate analysis were entered into a multivariate Cox proportional hazard model to identify significant independent factors. Statistical comparisons were performed using The Statistical Package for Social Sciences software (SPSS, Inc., Chicago, IL). All P values of less than 0.05 by the two-tailed test were considered significant.

RESULTS

Rate of New Cases of HCC in Patients With Sustained Virological Response

During the follow-up, 26 patients (2.0%) developed HCC. The median interval between the end of antiviral therapy and detection of HCC (latency to HCC) was 2.5 years (range, 0.0–15.9 years). The cumulative rates of new cases of HCC were 3.2%, 4.8%, and 8.6% at the end of 5, 10, and 15 years, respectively.

HCC Rate According to HCV Genotype and Amino Acid Substitutions in the Core Region of HCV-1b

During the follow-up, 7 (5.5%), 5 (1.4%), and 12 (2.0%) patients developed HCC in the HCV-1b with Gln70(His70), HCV-1b with Arg70, and HCV-2a/2b groups, respectively. The median latency to HCC was 1.1 years (range, 0.0–14.0 years), 3.9 (range, 0.0–15.9), and 2.8 (range, 0.0–12.9), respectively, and the cumulative rates of new cases of HCC were 10.6%, 3.6%, 3.0% at the end of 5 years; 10.6%, 6.3%, 5.2% at the end of 10 years; and 62.7%, 6.3%, 7.2% at the end of 15 years, respectively. The rates were significantly different among the three HCV subgroups ($P < 0.001$; log-rank test; Fig. 1). Especially, the rates for HCV-1b with Gln70(His70) were significantly higher than those for HCV-1b with Arg70 ($P = 0.007$; log-rank test) and HCV-2a/2b ($P < 0.001$; log-rank test). However, the rates for the HCV-1b with Arg70 group were not significantly higher than those for the HCV-2a/2b group ($P = 0.617$; log-rank test).

During the follow-up, 4 (2.6%) and 7 (2.2%) patients with HCV-1b/Met91, and HCV-1b/Leu91 developed HCC, respectively. In these two subgroups, the respective median latency to HCC was 3.4 years (range, 0.0–14.0 years) and 1.1 (range, 0.0–12.4), and the cumulative rates of new cases of HCC were 1.3%, 8.6% at the end of 5 years; 5.4%, 8.6% at the end of 10 years; and 36.9%, 14.7% at the end of 15 years. The rates for the HCV-1b/Met91 group were not significantly different from those for the HCV-1b/Leu91 group ($P = 0.908$; log-rank test).

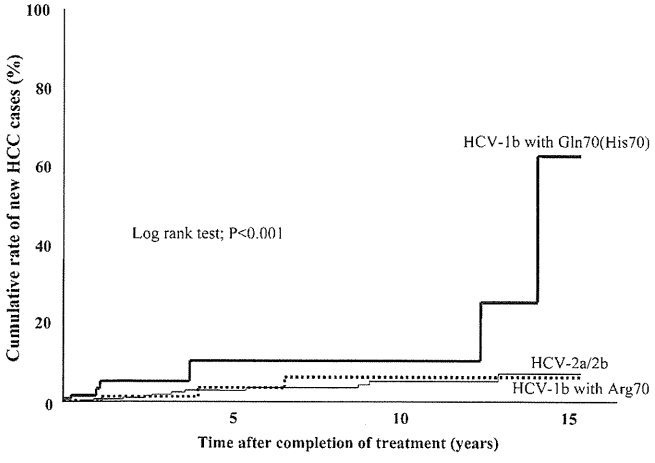

Fig. 1. Cumulative rates of new cases of HCC according to HCV genotype and amino acid substitutions in the core region of HCV-1b. The rates were significantly different among the three HCV groups ($P < 0.001$; log-rank test). Especially, the rate in patients with HCV-1b/Gln70(His70) was significantly higher than those of patients with HCV-1b/Arg70 ($P = 0.007$; log-rank test) and HCV-2a/2b ($P < 0.001$; log-rank test). Furthermore, the rate in patients with HCV-1b/Arg70 was not significantly higher than that in HCV-2a/2b ($P = 0.617$; log-rank test).

Predictive Factors Associated With the Development of HCC in Patients of Sustained Virological Response

Next, we analyzed the predictor of HCC using data of the entire group. There were significant relationships between the rate of new cases of HCC and male sex ($P = 0.003$), severe fibrosis (F3,4) ($P < 0.001$), old age (≥55 years) ($P = 0.002$), high levels of AST (≥39 IU/L) ($P = 0.023$), and HCV-1b/Gln70(His70) (log-rank test). These five factors were entered into multivariate analysis, which then identified three parameters that independently tended to or significantly influenced the development of HCC; HCV-1b/Gln70(His70) (HR 10.5, $P < 0.001$), advanced stage of fibrosis (F3,4; HR 9.03, $P = 0.002$), and old age (≥55 years; HR 3.09, $P = 0.066$; Table II).

Predictors of HCC in HCV-1b Patients With Sustained Virological Response

Finally we analyzed the data of 664 patients with HCV-1b to determine the predictors of HCC with sustained virological response. Univariate analysis identified three parameters that significantly correlated with the development of HCC: male sex ($P = 0.005$), old age ($P = 0.020$), and HCV-1b with Gln70(His70) ($P = 0.007$; log-rank test). These three factors were entered into multivariate analysis, which then identified HCV-1b with Gln70(His70) as the single parameter that significantly influenced the development of HCC (HR 8.19, $P = 0.034$).

DISCUSSION

Previous studies reported that the risk factors for hepatocarcinogenesis after elimination of HCV RNA

TABLE II. Results of Multivariate Analysis (Cox Proportional Hazard Model) for Factors Associated With Hepatocarcinogenesis in Patients With Sustained Virological Response

Factors and categories	Hazard ratio (95%CI)	*P*-Value
HCV group		
HCV-2a/2b	1	
HCV-1b with Arg70	1.15 (0.24–5.56)	0.863
HCV-1b with Gln70(His70)	10.5 (2.89–38.2)	<0.001
Fibrosis stage		
F1,2	1	
F3,4	9.03 (2.32–35.2)	0.002
Age (years)		
<55	1	
≥55	3.09 (0.93–10.3)	0.066

were severe fibrosis, male sex, and old age at the start of IFN treatment [Ikeda et al., 2003, 2005; Tokita et al., 2005; Kobayashi et al., 2007; Hirakawa et al., 2008]. In the present study, multivariate analysis identified HCV-1b with Gln70(His70), advanced fibrosis stage, and old age as determinants of HCC in patients with a sustained virological response. The present study is the first report to indicate that aa substitution in the core region at the start of antiviral therapy also influences hepatocarcinogenesis following eradication of HCV RNA. This result should be interpreted with caution since races other than the Japanese and patients infected with HCV-1a were not included. Any generalization of the results should await confirmation by studies of patients of other races and those infected with HCV-1a.

Despite numerous lines of epidemiological evidence linking HCV infection to the development of HCC, it remains controversial whether HCV itself plays a direct or indirect role in the pathogenesis of HCC [Koike, 2005]. Evidence suggests that the HCV core region is potentially oncogenic in the transgenic mice [Moriya et al., 1998], though the clinical impact of the core region on hepatocarcinogenesis remains unclear. Previous reports indicated that aa substitutions in the core region of HCV-1b are pretreatment predictors of poor virological response to antiviral therapy [Akuta et al., 2005, 2007a, 2010; Donlin et al., 2007], and also are etiological factors in HCC [Akuta et al., 2007b; Fishman et al., 2009; Hu et al., 2009; Nakamoto et al., 2010]. Importantly, the present study indicated that aa substitution in the core region at the start of antiviral therapy also affects the development of HCC even after the eradication of HCV RNA, and this is the first report to suggest the persistent oncogenic potential of the core region regardless of HCV RNA persistence. Previous reports identified the PA28γ-dependent pathway as one of the mechanisms of HCV-associated hepatocarcinogenesis. Moriishi et al. [2003, 2007] reported that knockout of the PA28γ gene induces accumulation of HCV core protein in the nuclei of hepatocytes of HCV core gene transgenic mice and disrupts the development of both hepatic steatosis and HCC. Furthermore, the HCV core protein also enhances the binding of liver X receptor α (LXRα) and retinoid X receptor α (RXRα) to the LXR-response element in the presence of PA28γ [Moriishi et al., 2007]. Thus, it seems that PA28γ plays a crucial role in the development of HCV-associated steatosis and HCC. However, these basic studies were performed under the state of HCV RNA persistence [Moriya et al., 1998; Moriishi et al., 2003, 2007; Koike, 2005], and further studies should be performed to investigate the oncogenic potential of aa substitution in the core region detected at the start of antiviral therapy on hepatocarcinogenesis following eradication of HCV RNA.

The association between HCV genotype and the risk of HCC is not clear. A study of Italian cohort indicated that the rate of HCC in patients infected with HCV-1b was significantly higher than that of patients infected with HCV-2a/2c [Bruno et al., 2007]. On the other hand, the present study of Japanese patients indicated that the rates in patients infected with HCV-1b were not significantly higher than those in those infected with HCV-2a/2b. The discrepancy between the present result and the above Italian study may be explained by differences in host factors [Montes-Cano et al., 2010], and/or differences in viral factors, such as the distribution of HCV-1b with Arg70 or Gln70(His70), and geographic diversities of HCV-1b [Nakano et al., 1999].

Previous studies showed that the 12- and 24-week regimen of telaprevir/PEG-IFN/ribavirin achieved sustained virological response rates of 35–60% and 61–69% in patients infected with HCV-1, respectively [Hézode et al., 2009; McHutchison et al., 2009; Akuta et al., 2010]. Furthermore, the PROVE3 study also showed that the 24- and 48-week regimen of triple therapy achieved sustained virological response rates of 51% and 53%, respectively, in patients infected with HCV-1 who had been unsuccessfully treated with PEG-IFN/ribavirin [McHutchison et al., 2010]. While it is anticipated that larger numbers of HCV-1 patients will achieve sustained virological response in response to telaprevir/PEG-IFN/ribavirin, a larger proportion of patients could develop HCC following eradication of HCV RNA by antiviral therapy. Hence, our study indicated that aa substitutions in the core region of HCV-1b should be detected before eradication of HCV RNA by antiviral therapy. Especially, even if patients of HCV-1b with Gln70(His70) could achieve sustained virological response, blood tests and imaging studies should be conducted at regular intervals in this high risk group for early detection and treatment of HCC.

Genetic variations near the IL28B gene are pretreatment predictors of poor virological response to the combination therapy of PEG-IFN/ribavirin and triple therapy of telaprevir/PEG-IFN/ribavirin [Ge et al., 2009; Suppiah et al., 2009; Tanaka et al., 2009; Akuta et al., 2010; Rauch et al., 2010], but their impact on hepatocarcinogenesis are unknown at this stage. In this study, 387 of 1,273 patients were evaluated for HCC according to genetic variation in rs8099917 (data not shown). A preliminary study based on a small number of patients showed that the HCC rate in genotype TT of treatment sensitive type (2.2%) was not significantly different from that in genotype non-TT of treatment resistant type (1.6%). Unfortunately, we could analyze the effect of rs8099917 on HCC following eradication of HCV RNA by antiviral therapy. Further studies of larger patient populations should be performed to investigate the relationship between genetic variations near the IL28B gene and HCC.

The limitations of the present study were that viral factors associated with hepatocarcinogenesis were incompletely investigated. Ogata et al. [2003] reported that HCV-1b strains might be associated with HCC

on the basis of the secondary structure of the amino-terminal portion of the HCV NS3 protein. Giménez-Barcons et al. [2001] reported that high amino acid variability within the NS5A of HCV might be associated with HCC in patients with HCV-1b-related cirrhosis. In the present study, the clinical impact of other regions on hepatocarcinogenesis could not be investigated, except for aa 70 and 91 in the HCV core region. The results should also be interpreted with caution since patients infected with HCV-1a were not included. Other limitations include lack of analysis of the effects of life-style related diseases (such as diabetes, insulin resistance or non-alcoholic steatohepatitis) on hepatocarcinogenesis, except for fasting plasma glucose and total cholesterol [Sumida et al., 2010a,b]. The impact of viral factors and life-style related diseases on hepatocarcinogenesis should also be investigated in future studies.

In conclusion, aa substitution in the core region of HCV-1b at the start of antiviral therapy is an important predictor of hepatocarcinogenesis following eradication of HCV RNA. This study emphasizes the importance of detection of aa substitutions in the core region before antiviral therapy.

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Amino Acid Substitution in Hepatitis C Virus Core Region and Genetic Variation Near the Interleukin 28B Gene Predict Viral Response to Telaprevir with Peginterferon and Ribavirin

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Genetic variation near the IL28B gene and substitution of amino acid (aa) 70 and 91 in the core region of hepatitis C virus (HCV) genotype 1b can predict the response to pegylated interferon (PEG-IFN)/ribavirin combination therapy, but its impact on triple therapy of telaprevir/PEG-IFN/ribavirin is not clear. The aims of this study were to investigate the predictive factors of sustained virological response to a 12-week or 24-week regimen of triple therapy in 72 of 81 Japanese adults infected with HCV genotype 1. Overall, sustained virological response and end-of-treatment response were achieved by 61% and 89%, respectively. Especially, the sustained virological response was achieved by 45% and 67% in the 12- and 24-week regimens, respectively. Multivariate analysis identified rs8099917 near the IL28B gene (genotype TT) and substitution at aa 70 (Arg70) as significant determinants of sustained virological response. Prediction of response to therapy based on a combination of these factors had high sensitivity, specificity, and positive and negative predictive values. The efficacy of triple therapy was high in the patients with genotype TT, who accomplished sustained virological response (84%), irrespective of substitution of core aa 70. In the patients having genotype non-TT, those of Arg70 gained high sustained virological response (50%), and sustained virological response (12%) was the worst in patients who possessed both genotype non-TT and Gln70(His70). *Conclusion:* This study identified genetic variation near the IL28B gene and aa substitution of the core region as predictors of sustained virological response to a triple therapy of telaprevir/PEG-IFN/ribavirin in Japanese patients infected with HCV genotype 1b. (HEPATOLOGY 2010;52:421-429)

Hepatitis C virus (HCV) usually causes chronic infection that can result in chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma (HCC).[1,2] At present, treatments based on interferon (IFN), in combination with ribavirin, are the mainstay for combating HCV infection. In Japan, HCV genotype 1b (HCV-1b) in high viral loads (>100 KIU/mL) accounts for more than 70% of HCV infections, making it difficult to treat patients with chronic hepatitis C.[3] Such background calls for efficient treatments of Japanese patients with chronic HCV infection.

Even with pegylated IFN (PEG-IFN) combined with ribavirin, a sustained virological response lasting over 24 weeks after the withdrawal of treatment is achieved in at most 50% of the patients infected with HCV-1b and high viral loads.[4,5] Recently, a new strategy was introduced in the treatment of chronic HCV infection by

Abbreviations: aa, amino acid; ALT, alanine aminotransferase; AST, aspartate aminotransferase; γGTP, gamma-glutamyl transpeptidase; HBsAg, hepatitis B surface antigen; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; IFN, interferon; NPV, negative predictive value; PEG-IFN, pegylated interferon; PPV, positive predictive value

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means of inhibiting protease in the NS3/NS4 of the HCV polyprotein. Of these, telaprevir (VX-950) was selected as a candidate agent for treatment of chronic HCV infection.[6] Later, it was found that telaprevir, when combined with PEG-IFN and ribavirin, gains a robust antiviral activity.[7,8] Specifically, HCV RNA is suppressed below the limits of detection in the blood in almost all patients infected with HCV-1 during triple therapy of telaprevir with PEG-IFN and ribavirin.[9] However, treatment-resistant patients who do not achieve sustained virological response by the triple therapy have been reported.[9-11] The underlying mechanism of the response to the treatment is still not clear.

Amino acid (aa) substitutions at position 70 and/or 91 in the HCV core region of patients infected with HCV-1b and high viral loads are pretreatment predictors of poor virological response to PEG-IFN plus ribavirin combination therapy,[12-14] and also affect clinical outcome, including hepatocarcinogenesis.[15,16] Furthermore, a recent report showed that aa substitutions in the core region can also be used before therapy to predict very early dynamics (within 48 hours) after the start of triple therapy of telaprevir with PEG-IFN and ribavirin.[17] However, it is not clear at this stage whether aa substitutions in the core region can be used before therapy to predict sustained virological response to triple therapy.

Recent reports showed that genetic variations near the IL28B gene (rs8099917, rs12979860) on chromosome 19 is a host-related factor, which encodes IFN-λ-3, are pretreatment predictors of virological response to 48-week PEG-IFN plus ribavirin combination therapy in individuals infected with HCV-1,[18-21] and also affect clinical outcome, including spontaneous clearance of HCV.[22] However, it is not clear at this stage whether genetic variation near the IL28B gene can be used before therapy to predict sustained virological response to triple therapy.

The present study included 81 patients with HCV-1b and high viral loads who received the triple therapy of telaprevir with PEG-IFN plus ribavirin. The aims of the study were to identify the pretreatment factors that could predict sustained virological response, including viral- (aa substitutions in the HCV core and NS5A regions) and host-related factors (genetic variation near the IL28B gene).

Patients and Methods

Study Population. Between May 2008 and September 2009, 81 patients infected with HCV were recruited for this study at the Department of Hepatology in Toranomon Hospital in Metropolitan Tokyo. The study protocol was in compliance with the Good Clinical Practice Guidelines and the 1975 Declaration of Helsinki and was approved by the Institutional Review Board. Each patient gave informed consent before participating in this trial. Patients were divided into two groups: 20 (25%) patients were allocated to a 12-week regimen of triple therapy (telaprevir [MP-424], PEG-IFN, and ribavirin) (the T12PR12 group), and 61 patients (75%) were assigned to a 24-week regimen of the same triple therapy for 12 weeks followed by dual therapy of PEG-IFN and ribavirin for 12 weeks (the T12PR24 group).

All of 81 patients met the following inclusion and exclusion criteria: (1) diagnosis of chronic hepatitis C. (2) HCV-1 confirmed by sequence analysis. (3) HCV RNA levels of $\geq$5.0 log IU/mL determined by the COBAS TaqMan HCV test (Roche Diagnostics, Tokyo, Japan). (4) Japanese (Mongoloid) ethnicity. (5) Age at study entry of 20-65 years. (6) Body weight $\geq$35 kg and $\leq$120 kg at the time of registration. (7) Lack of decompensated liver cirrhosis. (8) Negativity for hepatitis B surface antigen (HBsAg) in serum. (9) Negative history of HCC. (10) No previous treatment for malignancy. (11) Negative history of autoimmune hepatitis, alcohol liver disease, hemochromatosis, and chronic liver disease other than chronic hepatitis C. (12) Negative history of depression, schizophrenia or suicide attempts, hemoglobinopathies, angina pectoris, cardiac insufficiency, myocardial infarction or severe arrhythmia, uncontrollable hypertension, chronic renal dysfunction or creatinine clearance of $\leq$50 mL/minute at baseline, diabetes requiring treatment or fasting glucose level of $\geq$110 mg/dL, autoimmune disease, cerebrovascular disorders, thyroidal dysfunction uncontrollable by medical treatment, chronic pulmonary disease, allergy to medication or anaphylaxis at baseline. (13) Hemoglobin level of $\geq$12 g/dL, neutrophil count $\geq$1500/mm^3, and platelet count of $\geq$100,000/mm^3 at baseline. Pregnant or breast-feeding women or those willing to become pregnant during the study and men with a pregnant partner were excluded from the study. Furthermore, 72 of 81 patients were followed for at least 24 weeks after the completion of triple therapy. The treatment efficacy was evaluated by HCV-RNA negative at the end of treatment (end-of-treatment response) and 24 weeks after the completion of therapy (sustained virological response), based on the COBAS TaqMan HCV test (Roche Diagnostics).

Telaprevir (MP-424; Mitsubishi Tanabe Pharma, Osaka, Japan) was administered at 750 mg or 500 mg

Table 1. Profile and Laboratory Data at Commencement of Telaprevir, Peginterferon and Ribavirin Triple Therapy in Japanese Patients Infected with HCV Genotype 1

Demographic data	
Number of patients	81
Sex (M/F)	44 / 37
Age (years)*	55 (23-65)
History of blood transfusion	24 (29.6%)
Family history of liver disease	13 (16.0%)
Body mass index (kg/m^2)*	22.5 (13.2-32.4)
Laboratory data*	
HCV genotype (1a/ 1b)	1/80
Level of viremia (log IU/mL)	6.7 (5.1-7.6)
Serum aspartate aminotranlferase (IU/L)	34 (15-137)
Serum alanine aminotransferase (IU/L)	42 (12-175)
Serum albumin (g/dL)	3.9 (3.2-4,6)
Gamma-glutamyl transpeptidase (IU/L)	36 (9-229)
Leukocyte count (/mm^3)	4,800 (2,800-8,100)
Hemoglobin (g/dL)	14.3 (11.7-16.8)
Platelet count ($\times$ 10^4/mm^3)	17.1 (9.1-33.8)
Alpha-fetoprotein (μ g/L)	4 (2-39)
Total cholesterol (mg/dL)	180 (110-276)
Fasting plasma glucose (mg/dL)	92 (64-125)
Treatment	
PEG-IFNα -2b dose (μ g/kg)*	1.5 (1.3-2.0)
Ribavirin dose (mg/kg)*	11.7 (7.2-18.4)
Telaprevir dose (1,500 / 2,250 mg/day)	10/71
Treatment regimen (T12PR12 group / T12PR24 group)	20/61
Amino acid substitutions in the HCV genotype 1b	
Core aa 70 (arginine / glutamine [histidine] /ND)	47/33/1
Core aa 91 (leucine / methionine / ND)	43/37/1
ISDR of NS5A (wild-type / non wild-type / ND)	76/4/1
Genetic variation near IL28B gene	
rs8099917 genotype (TT / TG / GG / ND)	42/30/2/7
rs 12979860 genotype (CC / CT / TT /ND)	42/32/2/5
Past history of IFN therapy	
Treatment-naive / Relapsers to previous treatment / nonresponders to previous treatment	27/33/21

Data are number and percentages of patients, except those denoted by asterisk (*), which represent the median (range) values. ND, not determined.

three times a day at an 8-hour (q8) interval after the meal. PEG-IFNα-2b (PEG-Intron; Schering Plough, Kenilworth, NJ) was injected subcutaneously at a median dose 1.5 μg/kg (range: 1.3-2.0 μg/kg) once a week. Ribavirin (Rebetol; Schering Plough) was administered at 200-600 mg twice a day after breakfast and dinner (daily dose: 600-1000 mg).

PEG-IFN and ribavirin were discontinued or their doses reduced, as required, upon reduction of hemoglobin level, leukocyte count, neutrophil or platelet count, or the development of adverse events. Thus, the dose of PEG-IFN was reduced by 50% when the leukocyte count decreased below 1500/mm^3, neutrophil count below 750/mm,^{3} or platelet count below 80,000/mm^3; PEG-IFN was discontinued when these counts decreased below 1000/mm^3, 500/mm^3 or 50,000/mm,^{3} respectively. When hemoglobin decreased to <10 g/dL, the daily dose of ribavirin was reduced from 600 to 400 mg, from 800 to 600 mg and 1000 mg to 600 mg, depending on the initial dose. Ribavirin was withdrawn when hemoglobin decreased to <8.5 g/dL. However, the dose of telaprevir (MP-424) remained the same, and its administration was stopped when the discontinuation was appropriate for the development of adverse events. In those patients who discontinued telaprevir, treatment with PEG-IFNα-2b and ribavirin was also terminated.

Table 1 summarizes the profiles and laboratory data of the 81 patients at the commencement of treatment. They included 44 males and 37 females, ages 23 to 65 years (median, 55 years).

Measurement of HCV RNA. The antiviral effects of the triple therapy on HCV were assessed by measuring plasma HCV RNA levels. In this study, HCV RNA levels during treatment were evaluated at least once every month before, during, and after therapy. HCV RNA concentrations were determined using the COBAS TaqMan HCV test (Roche Diagnostics). The linear dynamic range of the assay was 1.2-7.8 log IU/mL, and the undetectable samples were defined as negative.

Detection of Amino Acid Substitutions in Core and NS5A Regions of HCV-1b. In the present study, aa substitutions of the core region and NS5A-ISDR (IFN-sensitivity determining region) of HCV-1b were analyzed by direct sequencing. HCV RNA was extracted from serum samples at the start of treatment and reverse transcribed with random primer and MMLV reverse transcriptase (Takara Syuzo, Tokyo). Nucleic acids were amplified by polymerase chain reaction (PCR) using the following primers: (1) Nucleotide sequences of the core region: The first-round PCR was performed with CE1 (sense, 5′-GTC TGC GGA ACC GGT GAG TA-3′, nucleotides: 134-153) and CE2 (antisense, 5′-GAC GTG GCG TCG TAT TGT CG-3′, nucleotides: 1096-1115) primers, and the second-round PCR with CC9 (sense, 5′-ACT GCT AGC CGA GTA GTG TT-3′, nucleotides: 234-253) and CE6 (antisense, 5′-GGA GCA GTC GTT CGT GAC AT-3′, nucleotides: 934-953) primers. (2) Nucleotide sequences of NS5A-ISDR: The first-round PCR was performed with ISDR1 (sense, 5′-ATG CCC ATG CCA GGT TCC AG-3′, nucleotides: 6662-6681) and ISDR2 (antisense, 5′-AGC TCC GCC AAG GCA GAA GA-3′, nucleotides: 7350-7369) primers, and the second-round PCR with ISDR3 (sense, 5′-ACC GGA TGT GGC AGT GCT CA-3′, nucleotides: 6824-6843) and ISDR4 (antisense, 5′-GTA ATC CGG GCG TGC CCA TA-3′, nucleotides: 7189-7208) primers. ([1,2]; nested PCR.) All samples were initially denatured at 95°C for 2 minutes. The 35 cycles of

amplification were set as follows: denaturation for 30 seconds at 95°C, annealing of primers for 30 seconds at 55°C, and extension for 1 minute at 72°C with an additional 7 minutes for extension. Then 1 μL of the first PCR product was transferred to the second PCR reaction. Other conditions for the second PCR were the same as the first PCR, except that the second PCR primers were used instead of the first PCR primers. The amplified PCR products were purified by the QIA quick PCR purification kit (Qiagen, Tokyo) after agarose gel electrophoresis and then used for direct sequencing. Dideoxynucleotide termination sequencing was performed with the Big Dye Deoxy Terminator Cycle Sequencing kit (PerkinElmer, Tokyo).

With the use of HCV-J (Access. No. D90208) as a reference,[23] the sequence of 1-191 aa in the core protein of HCV-1b was determined and then compared with the consensus sequence constructed on 81 clinical samples to detect substitutions at aa 70 of arginine (Arg70) or glutamine/histidine (Gln70/His70) and aa 91 of leucine (Leu91) or methionine (Met91).[12] The sequence of 2209-2248 aa in the NS5A of HCV-1b (ISDR) reported by Enomoto et al.[24] was determined and the numbers of aa substitutions in ISDR were defined as wildtype (0, 1) or nonwildtype ($\geq$2).

Genetic Variation Near the IL28B Gene. Samples for genome-wide association survey were genotyped using the Illumina HumanHap610-Quad Genotyping BeadChip. Genotyping data were subjected to quality control before the data analysis. Genotyping for replication and fine mapping was performed by use of the Invader assay, TaqMan assay, or direct sequencing as described.[25,26]

In this study, genetic variations near the IL28B gene (rs8099917, rs12979860), reported as the pretreatment predictors of treatment efficacy and clinical outcome,[18-22] were investigated.

Statistical Analysis. Nonparametric tests (chi-squared test and Fisher's exact probability test) were used to compare the characteristics of the groups. Univariate and multivariate logistic regression analyses were used to determine those factors that significantly contributed to sustained virological response. The odds ratios (OR) and 95% confidence intervals (95% CI) were also calculated. All P values less than 0.05 by the two-tailed test were considered significant. Variables that achieved statistical significance ($P < 0.05$) on univariate analysis were entered into multiple logistic regression analysis to identify significant independent predictive factors. Each variable was transformed into categorical data consisting of two simple ordinal numbers for univariate and multivariate analyses. The potential pretreatment factors associated with sustained virological response included the following variables: sex, age, history of blood transfusion, family history of liver disease, body mass index, aspartate aminotransferase (AST), alanine aminotransferase (ALT), albumin, gamma-glutamyl transpeptidase (γGTP), leukocyte count, hemoglobin, platelet count, HCV RNA level, alfa-fetoprotein, total cholesterol, fasting blood sugar, PEG-IFN dose/body weight, ribavirin dose/body weight, telaprevir dose/day, treatment regimen of triple therapy, past history of IFN therapy, genetic variation near the IL28B gene, and aa substitution in the core region, and NS5A-ISDR. Statistical analyses were performed using SPSS (Chicago, IL). Sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) were also calculated to determine the reliability of predictors of the response to therapy.

Results

Virological Response to Therapy. Sustained virological response was achieved by 44 of 72 (61.1%) patients. In all, 64 of 72 (88.9%) patients were considered end-of-treatment response. According to treatment regimen, sustained virological response were achieved by 45.0% (9 of 20 patients) and 67.3% (35 of 52 patients), in the T12PR12 group and the T12PR24 group, respectively. Of eight patients who could not achieve end-of-treatment response, six (75.0%) patients resulted in reelevation of viral loads regardless of HCV-RNA temporary negative, and the other two patients (25.0%) did not achieve HCV-RNA negative during treatment.

Especially in the T12PR24 group, according to the past history of treatment, sustained virological response were achieved by 76.4% (13 of 17 patients), 86.4% (19 of 22 patients), and 23.1% (3 of 13 patients), in treatment-naive, relapsers to previous treatment, and nonresponders to previous treatment, respectively.

Sustained Virological Response According to Amino Acid Substitutions in Core and NS5A Regions. According to the substitution of core aa 70, a significantly higher proportion of patients with Arg70 substitutions (74.4%) showed sustained virological response than that of patients who showed Gln70(His70) (41.4%) (Fig. 1, $P = 0.007$). In contrast, according to the substitution of core aa 91, the sustained virological response rate was not significantly different between Leu91 (65.0%) and Met91 (56.3%) (Fig. 1). Likewise, according to the numbers of aa substitutions in ISDR, the sustained virological response rate was not significantly different between wildtype