evidence of hepatic decompensation. Patients were also excluded if they had other severe or unstable conditions or evidence of organ dysfunction in excess of that consistent with the age of the patient, were unable to tolerate oral medication or had conditions that could affect the absorption of study drug, or were exposed to any investigational drug within 4 weeks of study participation or had any previous exposure to inhibitors of NS5A or NS3 protease. Laboratory findings that excluded participation were the following: alanine aminotransferase (ALT) >5× the upper limit of normal (ULN); total bilirubin ≥2 mg/dL; direct bilirubin >1.5× ULN; international normalized ratio (INR) ≥1.7; albumin ≤3.5 g/dL; hemoglobin <9.0 g/dL; white blood cells <1,500/mm^3; absolute neutrophil count <750/mm^3; platelets <50,000/mm^3; or creatinine >1.8× ULN.

Prohibited concomitant medications included inducers or inhibitors of cytochrome P450/3A4, non-study medications with anti-HCV activity, any prescription medication or herbal product not prescribed for a specific condition, liver-protection drugs, proton pump inhibitors, and erythropoiesis-stimulating agents. H_2 receptor antagonists were permitted, but administered ≥10 hours before or ≥2 hours after daclatasvir; other acid-modifying agents had to be taken ≥2 hours before or after daclatasvir.

Study Drug Dosing. All patients received oral combination therapy with daclatasvir and asunaprevir from the beginning of the study. Daclatasvir was dosed as two 30-mg tablets once-daily. Asunaprevir was initially dosed as three 200-mg tablets twice-daily; subsequently, the dose of asunaprevir was reduced to 200 mg twice-daily after reports of hepatic enzyme elevations in a clinical study of asunaprevir and Peg-IFN/RBV.[24]

Treatment was continued to week 24 for patients with HCV RNA below the assay lower limit of quantitation (LLQ; 15 IU/mL) on or after week 2; treatment was discontinued for patients with <2 $\log_{10}$ IU/mL decrease of HCV RNA from baseline or on or after week 2. For patients with viral rebound on or after week 2, or HCV RNA above LLQ on or after week 4, treatment was discontinued or weight-based Peg-IFN-RBV therapy was added for up to 48 additional weeks at the investigator's discretion, based on expected tolerance of Peg-IFN-RBV. Viral rebound was defined as an increase ≥1 $\log_{10}$ IU/mL from nadir at more than one time point or HCV RNA ≥15 IU/mL after declining to below that level.

Safety and Efficacy Assessments. Assessments, including HCV RNA, physical examination, vital signs, adverse events, laboratory tests, and review of concomitant medications, were conducted at screening, on study days 1 (baseline) through 7 and days 9, 11, and 14, at weeks 3, 4, 6, 8, 10, 12, 16, 20, and 24, and at post-treatment weeks 4, 8, 12, and 24. Twelve-lead electrocardiograms were recorded at all visits, except those at weeks 3 and 6. Additional pretreatment assessments included HCV genotype and host interleukin-28B (*IL28B*) genotype.

Serum HCV RNA levels were determined at a central laboratory using the Roche COBAS TaqMan HCV Auto assay (LLQ = 15 IU/mL; Roche Diagnostics KK, Tokyo, Japan). HCV genotype and subtype were determined at the central laboratory by polymerase chain reaction (PCR) amplification and sequencing. *IL28B* genotype was determined by PCR amplification and sequencing of the rs12979860 single-nucleotide polymorphism (SNP).

Outcome Measures. The primary efficacy endpoint was the proportion of patients with undetectable HCV RNA at 12 weeks post-treatment (SVR_{12}). Secondary endpoints included the proportions of patients with rapid virologic response (RVR; defined as undetectable HCV RNA at week 4), extended RVR (eRVR; undetectable HCV RNA at weeks 4 and 12), complete early virologic response (cEVR; undetectable HCV RNA at week 12), end-of-treatment response (EOTR; undetectable HCV RNA at week 24), and SVR at 24 weeks post-treatment (SVR_{24}).

The possible presence of HCV-resistance polymorphisms was analyzed using stored specimens. Resistance testing was performed on all samples at baseline and on samples indicative of virologic failure, defined as either (1) <2 $\log_{10}$ HCV RNA decrease from baseline at week 2, (2) virologic rebound (HCV RNA detectable after previously undetectable or ≥1 $\log_{10}$ increase from nadir), or (3) detectable HCV RNA at weeks 4 or 12 or at the end of therapy. Resistance analysis methodology included isolation of HCV RNA, PCR amplification, and population sequencing of HCV NS3 protease and NS5A domains.

Statistical Analysis. Categorical variables were summarized using counts and percents; continuous variables were summarized with univariate statistics.

Results

Patient Characteristics and Disposition. Twelve patients were screened; 2 patients failed to meet entry criteria (for HCC and elevated direct bilirubin, respectively), and 10 were enrolled and treated. Enrolled patients were generally older (median, 62 years); 6

Treatment of chronic HCV infection with pegylated interferon alpha (Peg-IFN-α) and ribavirin (RBV) elicits a sustained virologic response (SVR) in 40%-50% of treatment-naïve patients with genotype 1 infections; SVR rates in this population increase to 66% or 75% when boceprevir or telaprevir, respectively, is added to the regimen.[8-12] Response rates are influenced by viral load and genotype and by patient demographics, disease history, and genetics.[10] Peg-IFN/RBV retreatment of patients with previous nonresponse to Peg-IFN/RBV is frequently unsuccessful, with SVR rates of only 6%-9%.[13,14] Null responders are the subset of nonresponders who have responded most poorly to Peg-IFN/RBV, and their urgent need for more potent therapies has prompted the evaluation of regimens containing direct-acting antivirals (DAAs). SVR rates of 27% (genotype 1a) and 37% (genotype 1b) were achieved in null responders with a regimen combining telaprevir with Peg-IFN/RBV in a study of nonresponders.[15] These results suggest that DAA-containing regimens can benefit this population, but greater antiviral potency is needed to increase response rates further.

Combinations of two DAAs may overcome IFN nonresponsiveness in null responders by increasing antiviral activity and reducing the risk of developing resistance-associated variants.[16] In HCV-infected human hepatocyte chimeric mice, dual DAA treatment eradicated HCV without resistance, whereas resistance emerged rapidly with single DAA treatment.[17] In a clinical study that included null responders, marked antiviral effects were observed after 13 days of dual DAA treatment, supporting the evaluation of longer term dual DAA therapy reported in this study.[18] Daclatasvir (BMS-790052) is a first-in-class, highly selective nonstructural protein 5A (NS5A) replication complex inhibitor with picomolar potency and broad genotypic coverage; asunaprevir (BMS-650032) is a nonstructural protein 3 (NS3) protease inhibitor active against HCV genotypes 1a and 1b.[19,20] Daclatasvir and asunaprevir are associated with different resistance-associated variants, consistent with their different molecular targets, and showed no meaningful pharmacokinetic interactions in healthy volunteers.[20-22]

In a 24-week study of null responders in the United States, daclatasvir and asunaprevir demonstrated potent antiviral effects, both as a dual DAA regimen and in a quadruple regimen that included Peg-IFN/RBV.[23] Overall, 36% of dual-therapy recipients achieved SVR, including both of the 2 patients with genotype 1b infection. However, patients with genotype 1a experienced frequent viral breakthrough with the dual regimen and only 2 of 9 achieved SVR, suggesting subtype-associated differences in resistance barrier and response. We present the results of an open-label trial evaluating dual therapy with daclatasvir and asunaprevir in Japanese patients with chronic HCV genotype 1b infection and previous null response to Peg-IFN/RBV.

Patients and Methods

Study Design. This open-label, phase IIa study (clinicaltrials.gov identifier NCT01051414) evaluated the antiviral activity and safety of daclatasvir combined with asunaprevir in patients with HCV genotype 1 infection and previous null response to treatment with Peg-IFN/RBV, defined as <2 $\log_{10}$ reduction of HCV RNA after 12 weeks of therapy. This sentinel cohort provided safety data for review by an independent study safety committee before the enrollment of additional cohorts that will be described in a subsequent report. Written informed consent was obtained from all patients. The study was approved by institutional review boards at each site and was conducted in compliance with the Declaration of Helsinki, Good Clinical Practice Guidelines, and local regulatory requirements.

Patients. Patients eligible for enrollment in the sentinel cohort included men and women 20-75 years in age (women of childbearing potential were required to use adequate contraception) with chronic HCV genotype 1 infection for at least 6 months (all enrolled patients were genotype 1b because of the high prevalence of this subtype in Japan) and HCV RNA $\geq 10^5$ IU/mL. Eligible patients met criteria defining null responders and had no evidence of cirrhosis documented by laparoscopy, imaging, or liver biopsy within 2 years.

Patients were excluded if they had a history of HCC, coinfection with hepatitis B virus or human immunodeficiency virus, other chronic liver disease, or

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View this article online at wileyonlinelibrary.com.

DOI 10.1002/hep.24724

Potential conflict of interest: Nothing to report.

This article first published online ahead of print on 30 January 2012. The following corrections have since been made: "BMS-790052" has been replaced throughout with "daclatasvir"; "BMS-50032" has been replaced throughout with "asunaprevir". The article has been updated online and in print.

Table 1. Baseline Demographic and Disease Characteristics

Parameter	Value
N	10
Age, median years (range)	62 (52-70)
Male sex, n (%)	4 (40)
Japanese race, n (%)	10 (100)
Host *IL28B* genotype,* n (%)	
CC	2 (20)
CT	8 (80)
HCV genotype 1b, n (%)	10 (100)
HCV RNA, mean $\log_{10}$ IU/mL (SD)	6.8 (0.61)
ALT, mean U/L (SD)	60.6 (32.9)
Platelets $\times 10^9$ cells/mL, median (min, max)	150.5 (84.0, 166.0)
Total bilirubin, median mg/dL (min, max)	0.8 (0.6, 1.2)
Albumin, median g/dL (min, max)	3.9 (3.1, 4.2)
INR, median (min, max)	1.0 (1.0, 1.1)

*SNP rs12979860.

Abbreviation: *IL28B*, interleukin-28B; HCV, hepatitis C virus; SD, standard deviation; ALT, alanine aminotransferase; min, minimum; max, maximum; INR, international normalized ratio; SNP, single-nucleotide polymorphism.

were female and all were Japanese (Table 1). All enrolled patients were infected with genotype 1b, reflecting the predominance of this subtype in Japan, although the study protocol did not exclude patients with HCV genotype 1a.[6] Two patients were *IL28B* genotype CC (SNP rs12979860) and 8 were CT. Nine patients completed 24 weeks of therapy; 1 patient discontinued at week 2 because of a grade 4 total bilirubin elevation (see below). Among the 9 patients treated for 24 weeks, asunaprevir was dosed at 600 mg twice-daily for 12-21 weeks before the dose was reduced to 200 mg twice-daily (Fig. 1).

Virologic Response. Serum HCV RNA levels decreased rapidly in all patients (Fig. 2); mean reductions from baseline were 4.4 $\log_{10}$ IU/mL at week 1, 5.3 $\log_{10}$ IU/mL at week 2, and 5.8 $\log_{10}$ IU/mL from week 4 through the end of treatment. At week 4, HCV RNA was undetectable (RVR) in 4 of 10 (40%) patients and below the assay LLQ in 9 of 10 (90%; Fig. 3). No patients qualified for discontinuation or addition of pegIFN/RBV. At week 8, HCV RNA was undetectable in 9 of 10 patients (all who remained on treatment) and remained undetectable through the end of treatment and follow-up. SVR_{12}, the primary endpoint, and SVR_{24} were achieved by 90% of patients, including all 9 who completed 24 weeks of therapy. The patient who discontinued treatment at week 2 had low-level HCV RNA at discontinuation (1.8 $\log_{10}$ IU/mL), but HCV RNA was undetectable at follow-up visits 2, 3, 4, 13, and 24 weeks after discontinuation.

Viral Breakthrough and Relapse. There was no viral breakthrough during treatment or relapse of HCV RNA post-treatment. Analysis of baseline samples revealed variants reported to confer minimal to low

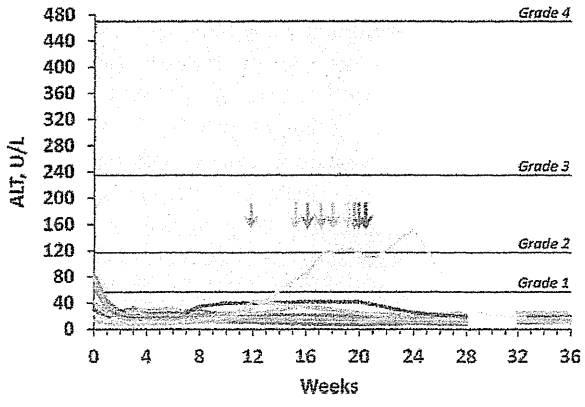

Fig. 1. ALT levels: individual patients. Serum ALT levels for the 9 patients who completed 24 weeks of treatment; the patient who discontinued at week 2 is not presented. Shaded area indicates the treatment period; arrows indicate the points at which the dose of asunaprevir was reduced from 600 to 200 mg twice-daily. Arrow and line colors are the same for each patient.

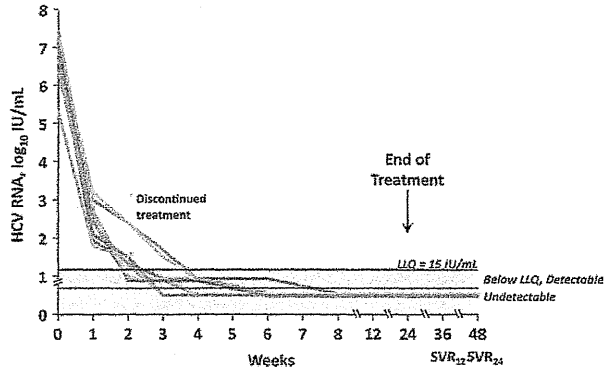

Fig. 2. HCV RNA levels: individual patients. Individual patient plasma HCV RNA levels during 24 weeks of treatment and through 24 weeks post-treatment (week 48) are shown. LLQ = 15 IU/mL.

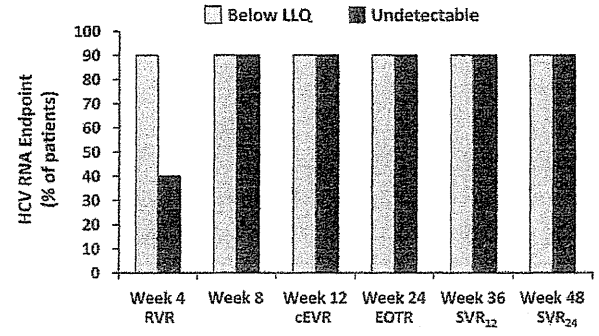

Fig. 3. HCV RNA endpoints. Categorical HCV RNA endpoints are indicated for the 10 study patients. One patient discontinued at week 2 and was counted as a treatment failure at the time points shown. However, HCV RNA was undetectable in this patient at 2, 3, 13, and 24 weeks post-treatment.

Table 2. On-Treatment Adverse Events Occurring in ≥2 Patients

Event	Patients, n (%)
Diarrhea	7 (70)
Headache	4 (40)
ALT increased	3 (30)
AST increased	3 (30)
Lymphopenia	2 (20)
Abdominal discomfort	2 (20)
Malaise	2 (20)
Pyrexia	2 (20)
Nasopharyngitis	2 (20)
Lipase increased	2 (20)
Back pain	2 (20)

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase.

levels of resistance to daclatasvir.[22] NS5A substitutions L28M and L31M were detected in 1 patient each, and Y93H was detected in 2 other patients. NS3 protease substitutions reported to confer resistance to telaprevir, boceprevir, and TMC-435 were detected[25]; T54S was identified in 1 patient, and Q80L was identified in 3. In 1 patient, both NS3 protease substitutions (T54S and Q80L) and an NS5A substitution (Y93H) were detected. There was no consistent association between detection of these variants and virologic outcomes.

Safety. The most frequently reported adverse events were diarrhea and headache, all of which were mild (grade 1) (Table 2). The patient who discontinued (see below) experienced multiple grade 3 or 4 adverse events and laboratory abnormalities on treatment. In the other 9 patients, there were no grade 3 or 4 transaminase elevations or other grade 3 or 4 events, no clinically relevant changes in electrocardiogram parameters, and no lymphopenia of any severity. Two transient grade 1 ALT elevations were reported, and 1 grade 2 elevation that began at week 16 and persisted until the end of treatment, after which it normalized within 2 weeks (Fig. 1). There were no notable differences in ALT before and after asunaprevir dose reduction.

There were two serious adverse events. A 54-year-old male was hospitalized with grade 3 pyrexia and persistent diarrhea 11 days after initiating study treatment. Loxoprofen was initiated, and body temperature normalized and diarrhea improved after 4 days. The patient remained on study treatment. The second event concerned a 60-year-old woman with a history of ulcerative colitis who discontinued study treatment after 2 weeks because of a grade 4 bilirubin elevation with multiple complicating features. Five days before discontinuation, she presented with infectious gastroenteritis and was treated with cefotiam and was subsequently hospitalized with fever, vomiting, and diarrhea. Meropenem, human serum albumin, and furosemide were initiated. At discontinuation of study drugs, laboratory findings included total bilirubin of 7.7 mg/dL and grade 3 lymphopenia and serum phosphorus reduction; transaminases and alkaline phosphatase were within normal ranges. In the week after discontinuation, white cell and eosinophil counts became elevated; total bilirubin improved and transaminases remained normal. Two weeks after discontinuation, grade 4 ALT and aspartate-aminotransferase elevations and a grade 3 lipase elevation were reported. Six weeks after discontinuation, bilirubin and transaminase elevations were resolved and lipase improved to within 2× ULN.

Discussion

This study assessed combination oral DAA therapy in a difficult-to-treat population with multiple adverse prognostic features, including HCV genotype 1b infection, primarily *IL28B* CT genotype, generally older age, and null response to previous Peg-IFN/RBV therapy.[10,13,14] These patients represent a group with a significant need for new therapeutic options.

A DAA-only therapeutic strategy may be particularly appropriate for null responders, who have previously shown only marginal response to Peg-IFN/RBV.[13,14] The combination of two highly potent DAAs cleared detectable virus rapidly in this study; HCV RNA was undetectable by week 8 in all 9 patients treated for 24 weeks. This outcome compares favorably with those observed when null responders received a combination of Peg-IFN/RBV and a single NS3 protease inhibitor, telaprevir or TMC435.[15,26] In these studies, HCV RNA remained detectable in 36% to approximately 50% of patients after 12 weeks.

HCV RNA remained undetectable 12 (SVR_{12}) and 24 weeks (SVR_{24}) post-treatment in all patients who completed treatment. This contrasts with the poor results obtained with Peg-IFN/RBV retreatment and the reported 37% SVR rate of genotype 1b null responders who received Peg-IFN/RBV and telaprevir.[10,13-15] Additional follow-up of patients from this study will assess whether SVR_{24} is predictive of long-lasting viral clearance with this dual DAA therapy, as it is with Peg-IFN/RBV. It is interesting that HCV RNA was persistently undetectable post-treatment in the patient who discontinued after only 2 weeks of treatment. With early discontinuation data from only this single case, at present, the result must be considered an anomaly. The factors that contributed to viral

clearance are uncertain, although the patient's *IL28B* CC genotype suggests increased sensitivity to endogenous interferon[27]; the possible influence of concurrent acute gastroenteritis or other complicating factors is unknown. However, coupled with the attainment of SVR_{12} in all other patients, this outcome suggests that required duration of therapy, which is currently predicated on data from Peg-IFN-based regimens, may need reassessment for DAA-only regimens, and, possibly, that certain patient populations can be treated for very short durations.

The high SVR rate is consistent with limited data from a related U.S.-based study, in which 2 of 2 null responders with HCV genotype 1b and who were treated with daclatasvir and asunaprevir achieved SVR_{24}.[23] However, only 2 of 9 patients with genotype 1a achieved SVR_{24} with the dual DAA regimen, compared with 9 of 10 patients who received both DAAs and Peg-IFN/RBV. These differences suggest that viral genotype can influence responses to DAA regimens that do not include Peg-IFN/RBV, and outcomes can be optimized with individualized therapy that considers viral genotype, among other factors. Because of the high SVR rate, the potential influence of other baseline and on-treatment parameters could not be assessed, other than to observe that unfavorable predictors of Peg-IFN/RBV response, such as older age and *IL28B* CT genotype,[27,28] had no measureable impact on outcomes.

There was no viral breakthrough on treatment. In view of the rapid emergence of resistance in some studies of short-term DAA monotherapy,[29,30] these findings support the concept that dual DAA therapy reduces the risk of viral breakthrough, in addition to increasing antiviral activity. Resistance analyses revealed that before treatment, some patients carried NS5A and NS3 polymorphisms predicted to reduce sensitivity to daclatasvir and some HCV protease inhibitors, respectively.[22,25] There was no clear relationship between the presence of these polymorphisms and minor interpatient differences in the rate of early virologic response; however, further study in larger patient cohorts will help determine whether baseline polymorphisms can influence virologic response with this regimen.

The adverse event profile of the dual DAA regimen compares favorably with the more frequent and severe events reported with Peg-IFN/RBV, although patient numbers in this study were limited. The mild diarrhea experienced by several patients has been reported previously with asunaprevir and is common with other drugs of this class.[15,18,24] Though a role for daclatasvir and/or asunaprevir in the two serious adverse events could not be ruled out and the investigator considered these events drug related, multiple confounding factors existed. The case of pyrexia was consistent with a viral infection and resolved with treatment. In the case of hyperbilirubinemia that led to discontinuation, the time course of laboratory abnormalities and related events suggests a link to the use of cefotiam and meropenem for treatment of infectious gastroenteritis. Both of these agents have been associated with vomiting, diarrhea, and hyperbilirubinemia.[31,32]

The asunaprevir dose was reduced during treatment because of transaminase elevations observed with 600 mg twice-daily in a concurrent study.[24] In this sentinel cohort, viral suppression was maintained in all patients after dose reduction, and no grade 3 or 4 transaminase elevations occurred during treatment at either dose of asunaprevir. One patient experienced grade 2 transaminase elevations that began at week 16 and persisted during treatment, despite asunaprevir dose reduction at week 19. Although these elevations were not severe, their rapid normalization post-treatment suggests a possible relationship to study treatment. None of the 9 patients treated for 24 weeks experienced transaminase elevations post-treatment. Although grade 4 transaminase elevations occurred 2 weeks post-treatment in the patient who discontinued, the timing of these events and multiple other complications suggest that they were not related directly to study treatment.

In conclusion, the combination of daclatasvir and asunaprevir achieved a high rate of SVR_{24} in patients with HCV genotype 1b infections and previous null response to Peg-IFN/RBV. These results support the concept that HCV infection can be cured with two DAAs without Peg-IFN/RBV, even in difficult-to-treat populations that lack robust IFN responsiveness. Further research will assess the benefits of DAA combinations in larger, more diverse patient populations.

Acknowledgment: The authors thank the patients and their families, research staff at all participating sites, and Bristol-Myers Squibb Research and Development colleagues.

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Original Paper

Intervirology

Intervirology 2012;55:231–241
DOI: 10.1159/000328327

Received: September 22, 2010
Accepted after revision: March 28, 2011
Published online: July 5, 2011

Amino Acid Substitution in HCV Core/NS5A Region and Genetic Variation Near *IL28B* Gene Affect Treatment Efficacy to Interferon plus Ribavirin Combination Therapy

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Key Words
Hepatitis C virus · Interferon · Ribavirin · Core region · NS5A region · ISDR · IRRDR · *IL28B*

Abstract
Objective: To evaluate predictive factors of treatment efficacy to interferon (IFN)/ribavirin in patients infected with HCV genotype 1b (HCV-1b). ***Methods:*** This study investigated pretreatment predictors, including viral- (aa substitutions in core aa 70/91 and NS5A-ISDR/IRRDR) and host-related factors (genetic variation near *IL28B* gene), to 48-week IFN/ribavirin in 490 Japanese adults infected with HCV-1b. ***Results:*** The proportion of patients who showed end-of-treatment response (ETR), sustained virological response (SVR), and SVR after ETR was 76, 54, and 76%, respectively. There was a significant positive correlation between the number of aa substitutions in ISDR and those in IRRDR. Concerning the substitution of core aa 91, the number of aa substitutions in ISDR/IRRDR of patients with Leu91 was significantly higher than that of patients with Met91. Furthermore, levels of viremia were influenced by aa substitutions in core aa 91 and ISDR/IRRDR. By multivariate analysis, rs8099917 genotype was an important predictor of ETR and SVR. With regard to viral factors, core aa 70/91 was an important predictor of ETR, and SVR after ETR. ISDR was an important predictor of SVR, and SVR after ETR. ***Conclusion:*** aa substitution in core/NS5A region and genetic variation near *IL28B* were important predictors of treatment efficacy to IFN/ribavirin.

Introduction

Treatment of chronic hepatitis C virus (HCV) infection with interferon (IFN) combined with ribavirin carries potential serious side effects and is costly, especially when used long enough to achieve a high sustained virological response (SVR) in patients infected with HCV genotype 1b (HCV-1b) and high viral loads. For these rea-

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sons, those patients who do not achieve SVR need to be identified, so as to free them of unnecessary side effects and reduce costs, preferably before the start of the combination therapy.

Viral- and host-related factors are useful as predictors of treatment efficacy to 48-week IFN/ribavirin combination therapy. With regard to viral factors, amino acid (aa) substitutions at position 70 and/or 91 in the core region of HCV-1b are pretreatment predictors of virological response to combination therapy [1–4], and also affect clinical outcome, including hepatocarcinogenesis [5, 6]. Furthermore, the NS5A region of HCV-1b, including IFN-sensitivity-determining region (ISDR) [7, 8] and IFN/ribavirin resistance-determining region (IRRDR) [9, 10], are also useful as pretreatment predictors of virological response to combination therapy [11, 12]. With regard to host factors, genetic variations near *IL28B* gene (rs8099917, rs12979860) on chromosome 19, which encodes IFN-λ-3, are pretreatment predictors of virological response to combination therapy in individuals infected with HCV-1 [13–16], and also affect clinical outcome, including spontaneous clearance of HCV [17]. A recent report identified genetic variation near *IL28B* gene and aa substitution of the core region as predictors of SVR to triple therapy of telaprevir/pegylated (PEG)-IFN/ribavirin in Japanese patients infected with HCV-1b [18]. However, to our knowledge, there are no previous reports of IFN/ribavirin combination therapy based on multivariate analysis to investigate pretreatment predictors, including all of aa substitutions in core aa 70/91 and NS5A-ISDR/IRRDR, and genetic variation near *IL28B* gene.

The aim of the present study was to investigate predictive factors of treatment efficacy, including viral- (aa substitutions in core aa 70/91 and NS5A-ISDR/IRRDR) and host-related factors (genetic variation near *IL28B* gene), to 48-week IFN/ribavirin in Japanese adults infected with HCV-1b.

Patients and Methods

Study Population

A total of 1,249 HCV-1b-infected Japanese adult patients were consecutively recruited into the study protocol of combination therapy with IFN (PEG-IFNα-2b or IFNα-2b) plus ribavirin between December 2001 and January 2009 at Toranomon Hospital, Tokyo, Japan. Among these, 490 patients, who could complete a total of 48 weeks of combination therapy, were enrolled in this retrospective study, and fulfilled the following criteria: (1) negativity for hepatitis B surface antigen (HBsAg) in serum; (2) HCV-1b only confirmed by sequence analysis; (3) HCV-RNA levels of ≥5.0 log IU/ml determined by the COBAS TaqMan HCV test (Roche Diagnostics, Tokyo, Japan) within the preceding 2 months of enrolment; (4) no hepatocellular carcinoma; (5) body weight >40 kg; (6) lack of coinfection with human immunodeficiency virus; (7) no previous treatment with antiviral or immunosuppressive agents within the preceding 3 months of enrolment; (8) none was an alcoholic; lifetime cumulative alcohol intake was <500 kg; (9) none had other forms of liver diseases, such as hemochromatosis, Wilson disease, primary biliary cirrhosis, alcoholic liver disease, or autoimmune liver disease, and (10) none of the females was pregnant or breastfeeding.

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 their informed consent before participating in this trial.

The treatment efficacy was evaluated in terms of HCV-RNA negativity at the end of treatment (end-of-treatment response (ETR)) and 24 weeks after the completion of therapy (SVR), based on the COBAS TaqMan HCV test (Roche Diagnostics). SVR in patients who achieved ETR was defined as SVR after ETR. ETR, SVR, and SVR after ETR could be evaluated in 487 (99%), 448 (91%), and 321 (66%) of 490 patients, respectively.

422 (86%) patients received PEG-IFNα-2b at a median dose of 1.4 μg/kg (range 0.7–1.9) subcutaneously each week plus oral ribavirin at a median dose of 11.1 mg/kg (range 3.7–15.1) daily for 48 weeks. The remaining 68 (14%) patients received 6 million units of IFNα-2b intramuscularly each day for 48 weeks (daily for the initial 2 weeks, followed by three times per week for 46 weeks), and oral ribavirin at a median dose of 11.3 mg/kg (range 6.8–13.4) daily for 48 weeks.

Table 1 summarizes the profiles and laboratory data of the 490 patients at the commencement of treatment. They included 310 males and 180 females aged 20–75 years (median 54).

Measurement of HCV RNA

The antiviral effects of treatment on HCV were assessed by measuring plasma HCV-RNA levels. In this study, HCV-RNA levels 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 aa Substitutions in Core, and NS5A Regions of HCV-1b

With the use of HCV-J (accession No. D90208) as a reference [19], the sequence of 1–191 aa in the core protein of HCV-1b was determined and then compared with the consensus sequence constructed on the previous study to detect substitutions at aa 70 of arginine (Arg70) or glutamine/histidine (Gln70/His70) and aa 91 of leucine (Leu91) or methionine (Met91) [1]. The sequence of 2,209–2,248 aa in the NS5A of HCV-1b (ISDR) reported by Enomoto et al. [7, 8] was determined, and the number of aa substitutions in ISDR was defined as wild-type (WT) (0, 1) or non-wild-type (non-WT) (≥2) in comparison with HCV-J. Furthermore, the sequence of 2,334–2,379 aa in the NS5A of HCV-1b (IRRDR) reported by El-Shamy et al. [9, 10] was determined and then compared with the consensus sequence constructed on the previous study. In the present study, aa substitutions of the core region and NS5A-ISDR/IRRDR of HCV-1b were analyzed by direct sequencing [10, 18].

Genetic Variation near 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 Invader assay, TaqMan assay, or direct sequencing as described previously [20, 21].

In this study, genetic variations near *IL28B* gene (rs8099917), reported as the pretreatment predictors of treatment efficacy in Japanese patients [14, 18], were investigated.

Statistical Analysis

Non-parametric tests (Mann-Whitney U test, χ^2 test and Fisher's exact probability test) were used to compare the characteristics of the groups. Correlation analysis was evaluated by the Spearman rank correlation test. Uni- and multivariate logistic regression analyses were used to determine those factors that significantly contributed to ETR, SVR, and SVR after ETR. The odds ratios (OR) and 95% confidence intervals (95% CI) were also calculated. All p values <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 uni- and multivariate analyses. Potential predictive factors associated with ETR, SVR, and SVR after ETR included the following variables: sex, age, history of blood transfusion, familial history of liver disease, body mass index, aspartate aminotransferase (AST), alanine aminotransferase (ALT), albumin, γ-glutamyl transpeptidase (GGT), leukocyte count, hemoglobin, platelet count, level of viremia, α-fetoprotein, total cholesterol, high-density lipoprotein cholesterol, low-density lipoprotein cholesterol, triglycerides, uric acid, ribavirin dose/body weight, genetic variation near *IL28B* gene, and aa substitution in the core region, and NS5A-ISDR/IRRDR. Statistical analyses were performed using SPSS software (SPSS Inc., Chicago, Ill., USA).

Results

Response to Therapy

ETR was achieved by 372 of 487 (76%) patients, SVR by 244 of 448 (54%), and SVR after ETR by 244 of 321 (76%).

Number of aa Substitutions in NS5A-ISDR and NS5A-IRRDR

As a whole, 0, 1, and ≥2 aa substitutions in ISDR were found in 56% (227 of 406), 23% (95 of 406), and 21% (84 of 406) of patients, respectively. Thus, the percentage of patients with ≤1 aa substitution in ISDR (WT) was 79% (322 of 406). Furthermore, ≤3, 4–5, and ≥6 aa substitutions in IRRDR were found in 36% (73 of 200), 34% (67 of 200), and 30% (60 of 200) of patients, respectively (fig. 1).

Table 1. Patient profile and laboratory data at commencement of the 48-week combination therapy of IFN + ribavirin in 490 patients infected with HCV-1b

Demographic data	
Number of patients	490
Male/female	310/180
Age, years	54 (20–75)
History of blood transfusion	169 (34%)
Family history of liver disease	96 (20%)
Body mass index, kg/m^2	22.6 (15.7–34.7)
Laboratory data	
Level of viremia, log IU/ml	6.4 (2.2–7.7)
Serum AST, IU/l	50 (16–296)
Serum ALT, IU/l	67 (12–836)
Serum albumin, g/dl	3.9 (3.1–4.7)
GGT, IU/l	44 (10–592)
Leukocyte count, n/mm^3	4,700 (1,200–10,900)
Hemoglobin, g/dl	14.4 (10.6–18.1)
Platelet count, × 10^4/mm^3	16.7 (6.4–37.5)
α-Fetoprotein, μg/l	5 (1–459)
Total cholesterol, mg/dl	170 (96–284)
High-density lipoprotein cholesterol, mg/dl	46 (13–95)
Low-density lipoprotein cholesterol, mg/dl	100 (32–190)
Triglycerides, mg/dl	90 (33–416)
Uric acid, mg/dl	5.5 (2.3–9.4)
Treatment	
PEG-IFNα-2b/IFNα-2b	422/68
Ribavirin dose, mg/kg	11.2 (3.7–15.1)
aa substitutions in the HCV-1b	
Core aa 70, arginine/glutamine (histidine)	266/151
Core aa 91, leucine/methionine	246/169
ISDR of NS5A, 0/1/≥2	227/95/84
IRRDR of NS5A, ≤3/4–5/≥6	73/67/60
Genetic variation near IL28B *gene*	
rs8099917 genotype, TT/TG/GG	150/65/4

Data represent number of patients with percentages in parentheses, or median (range) values.

The correlation between ISDR and IRRDR was analyzed. There was a significant positive correlation between the number of aa substitutions in ISDR and those in IRRDR ($r = 0.308$, $p < 0.001$) (fig. 2).

aa Substitutions in the Core Region and NS5A-ISDR/IRRDR

Concerning the substitution of core aa 70, the number of aa substitutions in ISDR of 256 patients with Arg70 (median 0) was not significantly different from that of 146 patients with Gln70 (His70) (median 0) (fig. 3a). Fur-

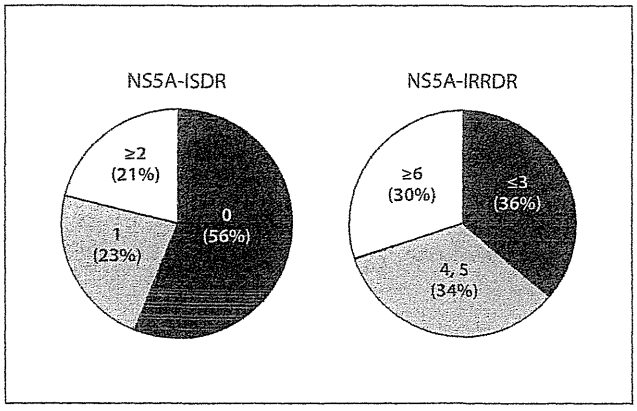

Fig. 1. The number of aa substitutions in NS5A-ISDR and NS5A-IRRDR. The percentage of patients with ≤1 aa substitution in ISDR (WT) was 79%.

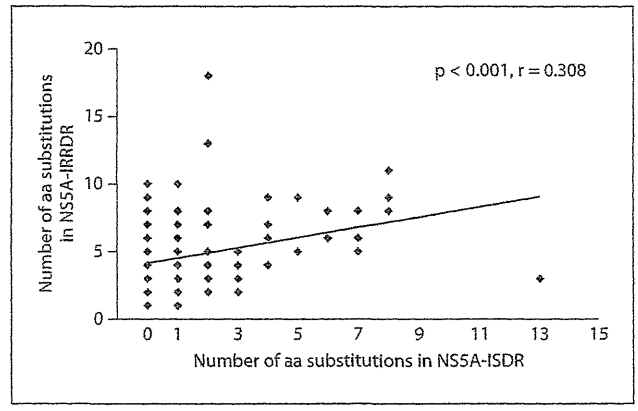

Fig. 2. Correlation between NS5A-ISDR and NS5A-IRRDR. There was a significant positive correlation between the number of aa substitutions in ISDR and that in IRRDR ($r = 0.308$, $p < 0.001$).

thermore, the number of aa substitutions in IRRDR of 123 patients with Arg70 (median 5) was also not significantly different from that of 77 patients with Gln70 (His70) (median 4) (fig. 3b).

Concerning the substitution of core aa 91, the number of aa substitutions in ISDR of 240 patients with Leu91 (median 1) was significantly higher than that of 161 patients with Met91 (median 0) ($p < 0.001$) (fig. 3c). Furthermore, the number of aa substitutions in IRRDR of 111 patients with Leu91 (median 5) was significantly higher than that of 89 patients with Met91 (median 3) ($p < 0.001$) (fig. 3d).

Viremia Level and aa Substitutions in Core Region/ISDR/IRRDR

Concerning the number of substitutions in ISDR, viremia levels of 321 patients with WT (median 6.5) were significantly higher than those of 84 patients with non-WT (median 5.7) ($p < 0.001$) (fig 4a).

Concerning the number of substitutions in IRRDR, viremia levels of 140 patients with ≤5 substitutions (median 6.4) were significantly higher than those of 60 patients with ≥6 (median 6.1) ($p = 0.027$) (fig. 4b).

Concerning the substitution of core aa 70, viremia levels of 265 patients with Arg70 (median 6.4) were not significantly different from those of 151 patients with Gln70 (His70) (median 6.3) (fig. 4c).

Concerning the substitution of core aa 91, viremia levels of 169 patients with Met91 (median 6.5) were significantly higher than those of 245 patients with Leu91 (median 6.2) ($p = 0.028$) (fig. 4d).

Thus, levels of viremia were influenced by aa substitutions in core aa 91 and ISDR/IRRDR.

Treatment Response according to the Number of aa Substitutions in IRRDR

Concerning the number of aa substitutions in IRRDR, a significantly higher proportion of patients with ≥4 aa substitutions (58%) showed SVR compared to patients with ≤3 (42%) ($p = 0.039$). In contrast, the SVR rate was not significantly different between patients with ≤4 (49%) and those with ≥5 (57%) aa substitutions. Likewise, the SVR rate was not significantly different between patients with ≤5 (51%) and those with ≥6 (55%) aa substitutions (fig. 5a).

The ETR rate was not significantly different between patients with ≤3 (74%) and those with ≥4 (82%) aa substitutions, nor between patients with ≤4 (76%) and those with ≥5 (83%). Likewise, the ETR rate was not significantly different between those with ≤5 (79%) and those with ≥6 (80%) aa substitutions (fig. 5b).

The SVR rate after ETR was not significantly different between patients with ≤3 (61%) and those with ≥4 (74%) aa substitutions, nor between patients with ≤4 (67%) and those with ≥5 (72%). Likewise, they were not significantly different between patients with ≤5 (67%) and those with ≥6 (75%) aa substitutions (fig. 5c).

Thus, it was useful as predictor of SVR to categorize into two groups of ≤4 and ≥5 aa substitutions by univariate analysis. However, the ETR and SVR after ETR rates were not significantly different according to the number of aa substitutions in IRRDR.

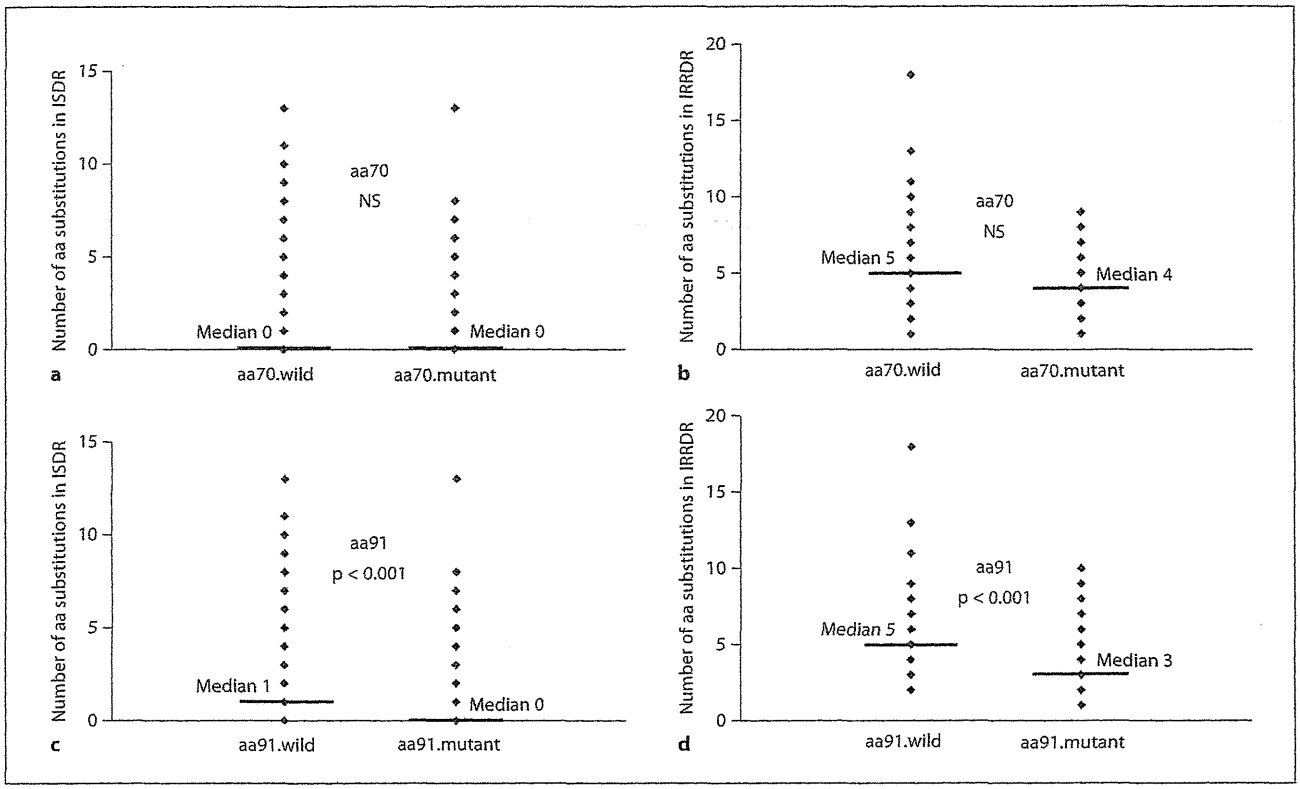

Fig. 3. aa substitutions in the core region and NS5A-ISDR/IRRDR. **a**, **b** Concerning the substitution of core aa 70, the number of aa substitutions in ISDR/IRRDR of patients with Arg70 was not significantly different from that of patients with Gln70 (His70). **c**, **d** Concerning the substitution of core aa 91, the number of aa substitutions in ISDR/IRRDR of patients with Leu91 was significantly higher than that of patients with Met91 ($p < 0.001$).

Predictors of SVR as Determined by Uni- and Multivariate Analyses

Univariate analysis identified 15 parameters that correlate with SVR: gender (male sex; $p < 0.001$), age (<55 years; $p < 0.001$), ribavirin dose (≥11.0 mg/kg; $p = 0.006$), AST (<58 IU/l; $p = 0.039$), leukocyte count (≥4,500/mm^3; $p = 0.043$), hemoglobin (≥14.0 g/dl; $p = 0.001$), platelet count (≥15.0 × 10^4/mm^3; $p < 0.001$), GGT (<50 IU/l; $p = 0.028$), uric acid (≥5.5 mg/dl; $p = 0.005$), level of viremia (<6.0 log IU/ml; $p < 0.001$), α-fetoprotein (<10 μg/l; $p < 0.001$), genetic variation in rs8099917 (genotype TT; $p < 0.001$), substitution of aa 70 (Arg70; $p < 0.001$), the number of aa substitutions in ISDR (non-WT; $p < 0.001$) and IRRDR (≥4; $p = 0.039$). Figure 6 shows the SVR rate according to aa substitution in the core/NS5A region and genetic variation near *IL28B* by univariate analysis.

Multivariate analysis that included the above variables identified 3 parameters that independently influenced SVR: genetic variation in rs8099917 (genotype TT; $p < 0.001$), gender (male sex; $p < 0.001$), and the number of aa substitutions in ISDR (non-WT; $p = 0.027$) (table 2).

Predictors of ETR as Determined by Uni- and Multivariate Analyses

Univariate analysis identified 14 parameters that correlated with ETR: gender (male sex; $p = 0.001$), age (<55 years; $p = 0.004$), AST (<39 IU/l; $p = 0.027$), hemoglobin (≥14.0 g/dl; $p = 0.035$), platelet count (≥15.0 × 10^4/mm^3; $p < 0.001$), albumin (≥3.9 g/dl; $p = 0.014$), GGT (<50 IU/l; $p < 0.001$), uric acid (≥5.5 mg/dl; $p = 0.003$), level of viremia (<6.0 log IU/ml; $p = 0.001$), low-density lipoprotein cholesterol (≥85 mg/dl; $p = 0.004$), α-fetoprotein (<10 μg/l; $p < 0.001$), genetic variation in rs8099917 (genotype TT; $p < 0.001$), substitution of aa 70 (Arg70; $p < 0.001$), and the number of aa substitutions in ISDR (non-WT; $p = 0.021$). Figure 7 shows the ETR rate according to aa

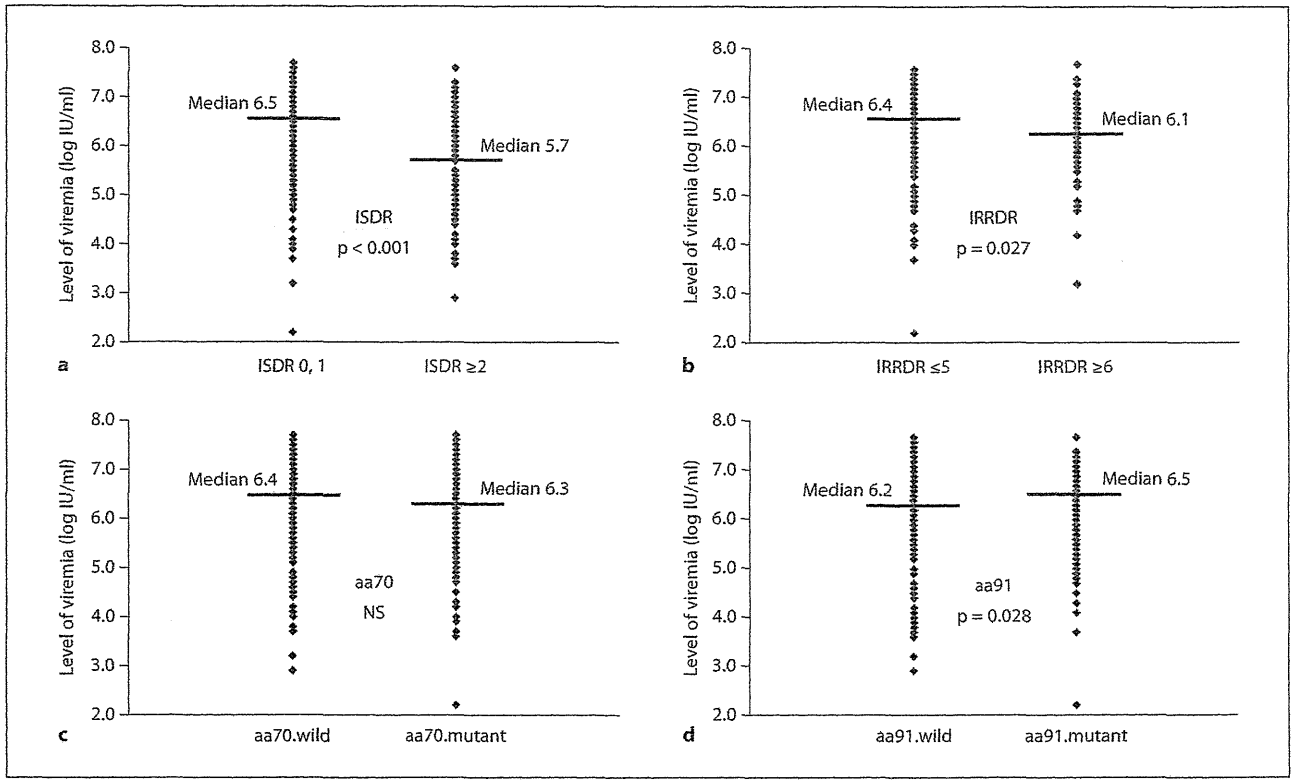

Fig. 4. Viremia level and aa substitutions in core region/ISDR/IRRDR. **a** Concerning the number of substitutions in ISDR, viremia levels of patients with WT were significantly higher than those of patients with non-WT ($p < 0.001$). **b** Concerning the number of substitutions in IRRDR, viremia levels of patients with ≤5 aa substitutions were significantly higher levels than those of patients with ≥6 ($p = 0.027$). **c** Concerning the substitution of core aa 70, viremia levels of patients with Arg70 were not significantly different from those of patients with Gln70 (His70). **d** Concerning the substitution of core aa 91, viremia levels of patients with Met91 were significantly higher than those of patients with Leu91 ($p = 0.028$). Thus, levels of viremia might be influenced by aa substitutions in core aa 91 and ISDR/IRRDR.

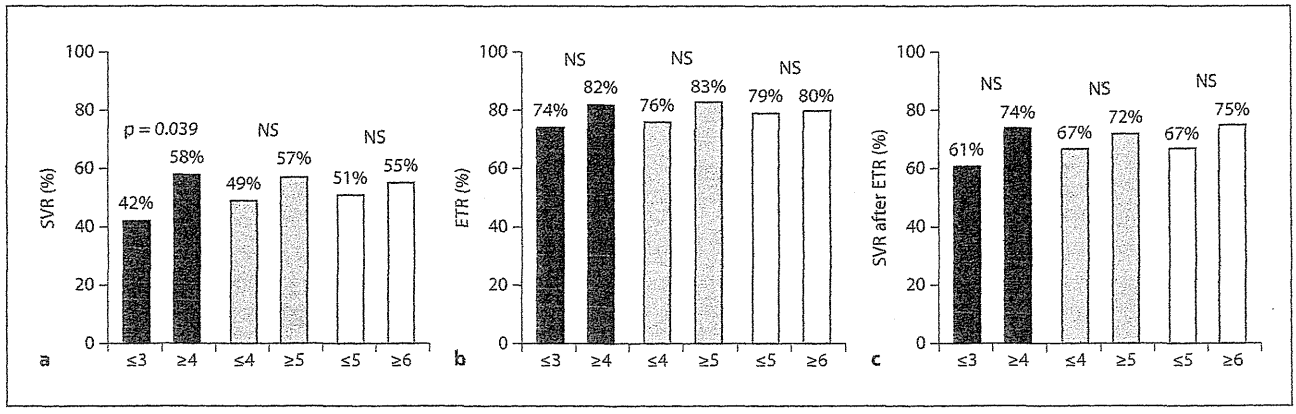

Fig. 5. Treatment response according to the number of aa substitutions in NS5A-IRRDR. **a** A significantly higher proportion of patients with ≥4 (58%) aa substitutions showed SVR compared to patients with ≤3 (42%) ($p = 0.039$), and it was useful as predictor of SVR to categorize into two groups of ≤4 and ≥5 aa substitutions by univariate analysis. **b**, **c** ETR and SVR after ETR rates were not significantly different according to the number of aa substitutions in IRRDR.

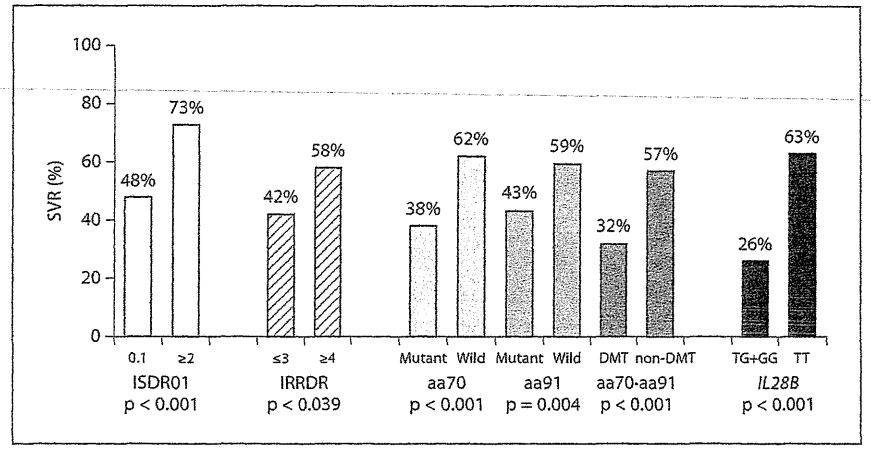

Fig. 6. SVR rate according to aa substitution in core/NS5A region and genetic variation near *IL28B* by univariate analysis.

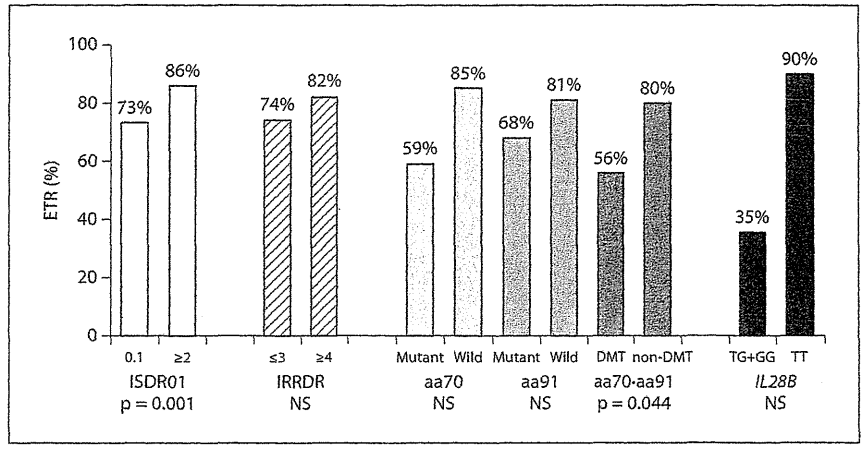

Fig. 7. ETR rate according to aa substitution in core/NS5A region and genetic variation near *IL28B* by univariate analysis.

Table 2. Factors associated with SVR to 48-week IFN + ribavirin combination therapy in patients infected with HCV-1b, identified by multivariate analysis

Factor	Category	OR (95% CI)	p
rs8099917 genotype	1: TG+GG 2: TT	1 16.7 (4.54–61.3)	<0.001
Gender	1: Female 2: Male	1 10.5 (3.47–32.3)	<0.001
ISDR of NS5A	1: WT 2: Non-WT	1 5.68 (1.22–26.3)	0.027

Only variables that achieved statistical significance ($p < 0.05$) on multivariate logistic regression are shown.

Table 3. Factors associated with ETR response to 48-week IFN + ribavirin combination therapy in patients infected with HCV-1b, identified by multivariate analysis

Factor	Category	OR (95% CI)	p
rs8099917 genotype	1: TG+GG 2: TT	1 18.2 (6.29–52.6)	<0.001
Level of viremia log IU/ml	1: ≥6.0 2: <6.0	1 9.20 (2.59–32.6)	0.001
Core aa 70	1: Gln70 (His70) 2: Arg70	1 4.68 (1.65–13.3)	0.004
Serum albumin g/dl	1: <3.9 2: ≥3.9	1 3.08 (1.11–8.47)	0.030

Only variables that achieved statistical significance ($p < 0.05$) on multivariate logistic regression are shown.

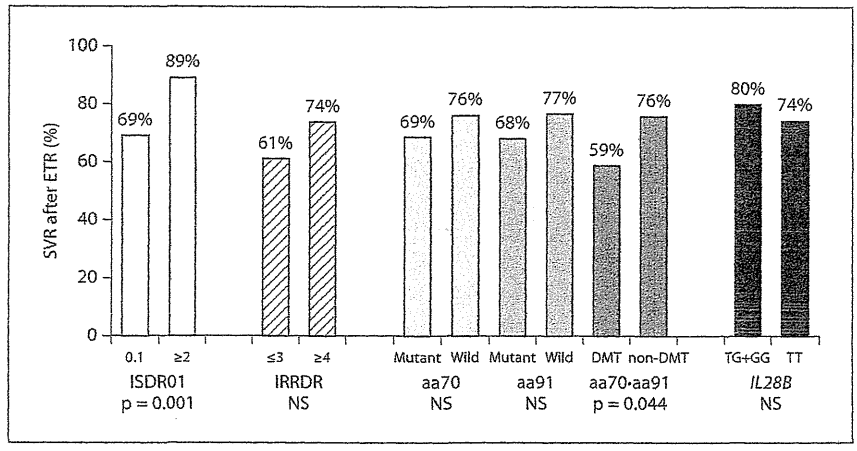

Fig. 8. SVR after ETR rate according to aa substitution in core/NS5A region and genetic variation near *IL28B* by univariate analysis.

substitution in the core/NS5A region and genetic variation near *IL28B* by univariate analysis.

Multivariate analysis that included the above variables identified 4 parameters that independently influenced ETR: genetic variation in rs8099917 (genotype TT; $p < 0.001$), level of viremia (<6.0 log IU/ml; $p = 0.001$), substitution of aa 70 (Arg70; $p = 0.004$), and albumin (≥3.9 g/dl; $p = 0.030$) (table 3).

Predictors of SVR after ETR as Determined by Uni- and Multivariate Analyses

Univariate analysis identified 11 parameters that influenced SVR after ETR: gender (male sex; $p < 0.001$), age (<55 years; $p < 0.001$), ribavirin dose (≥11.0 mg/kg; $p = 0.025$), leukocyte count (≥4,500/mm^3; $p = 0.033$), hemoglobin (≥14.0 g/dl; $p = 0.025$), platelet count (≥15.0 × 10^4/mm^3; $p = 0.001$), level of viremia (<6.0 log IU/ml; $p = 0.020$), total cholesterol (<170 mg/dl; $p = 0.017$), α-fetoprotein (<10 μg/l; $p = 0.004$), substitution of aa 70 and 91 (Arg70 and/or Leu91; $p = 0.044$), and the number of aa substitutions in ISDR (non-WT; $p = 0.001$). Figure 8 shows the SVR after ETR rate according to aa substitution in the core/NS5A region and genetic variation near *IL28B* by univariate analysis.

Multivariate analysis that included the above variables identified 6 parameters that independently influenced the SVR after ETR: gender (male sex; $p < 0.001$), ribavirin dose (≥11.0 mg/kg; $p = 0.002$), the number of aa substitutions in ISDR (non-WT; $p = 0.012$), substitution of aa 70 and 91 (Arg70 and/or Leu91; $p = 0.023$), platelet count (≥15.0 × 10^4/mm^3; $p = 0.033$), and α-fetoprotein (<10 μg/l; $p = 0.042$) (table 4).

Comparison of Factors Associated with Treatment Efficacy Identified by Multivariate Analysis

Table 5 shows the variables that achieved statistical significance on multivariate logistic regression for each evaluation of treatment efficacy. Rs8099917 genotype was an important predictor of ETR and SVR. With regard to viral factors, core region was an important predictor of ETR, and SVR after ETR. ISDR was an important predictor of SVR, and SVR after ETR. Level of viremia was an important predictor of ETR. Thus, genetic variation near *IL28B* and viral factors (core region, ISDR, and level of viremia) were important predictors of treatment efficacy. Furthermore, gender, α-fetoprotein, albumin, and platelet count were also identified as other important predictors of treatment efficacy, in addition to genetic variation near *IL28B* and viral factors.

Discussion

Using multivariate analysis, the present study identified viral- (aa substitutions in core aa 70/91 and NS5A-ISDR/IRRDR) and host-related factors (genetic variation near *IL28B* gene) that influenced treatment efficacy to 48-week IFN/ribavirin combination therapy, which is in agreement with recent findings [22, 23]. Identification of these viral and host factors before the start of IFN/ribavirin combination therapy should help to select better therapeutic regimens, including triple therapy of telaprevir/PEG-IFN/ribavirin [24–26], for those patients who are less likely to achieve SVR.

According to the number of substitutions in ISDR, a previous report showed that levels of viremia were sig-

Table 4. Factors associated with SVR in patients who achieved ETR response to 48-week IFN + ribavirin combination therapy in patients infected with HCV-1b, identified by multivariate analysis

Factor	Category	OR (95% CI)	p
Gender	1: Female 2: Male	1 4.27 (2.15–8.55)	<0.001
Ribavirin dose, mg/kg	1: <11.0 2: ≥11.0	1 2.95 (1.48–5.86)	0.002
ISDR of NS5A	1: WT 2: Non-WT	1 4.00 (1.35–11.8)	0.012
Core aa 70 and 91	1: Gln70 (His70) and Met91 2: Arg70 and/or Leu91	1 2.96 (1.16–7.52)	0.023
Platelet count $\times 10^4/mm^3$	1: <15.0 2: ≥15.0	1 2.19 (1.07–4.50)	0.033
α-Fetoprotein μg/l	1: ≥10 2: <10	1 2.66 (1.04–6.80)	0.042

Only variables that achieved statistical significance ($p < 0.05$) on multivariate logistic regression are shown.

Table 5. Comparison of factors associated with efficacy of 48-week IFN + ribavirin combination therapy in patients infected with HCV-1b, identified by multivariate analysis

Factor	ETR response (at 48 weeks)	SVR after ETR response	SVR
IL28B	rs8099917 p < 0.001, 18.2 (6.29–52.6)[a]		rs8099917 p < 0.001, 16.7 (4.54–61.3)[a]
Virus	Core aa 70 p = 0.004, 4.68 (1.65–13.3)[a]	Core aa 70 and 91 p = 0.023, 2.96 (1.16–7.52)[a]	
	Level of viremia p = 0.001, 9.20 (2.59–32.6)[a]	ISDR p = 0.012, 4.00 (1.35–11.8)[a]	ISDR p = 0.027, 5.68 (1.22–26.3)[a]
Others	Albumin p = 0.030, 3.08 (1.11–8.47)[a]	α-Fetoprotein p = 0.042, 2.66 (1.04–6.80)[a]	
		Platelet count p = 0.033, 2.19 (1.07–4.50)[a]	
		Gender p < 0.001, 4.27 (2.15–8.55)[a]	Gender p < 0.001, 10.5 (3.47–32.3)[a]
		Ribavirin dose p = 0.002, 2.95 (1.48–5.86)[a]	

Only variables that achieved statistical significance ($p < 0.05$) on multivariate logistic regression are shown.
[a] OR (95% CI).

nificantly lower in patients with non-WT of ISDR than in those with WT [8]. The present study indicated that substitution of IRRDR and core aa 91, in addition to substitution of ISDR, also significantly influenced levels of viremia. Furthermore, there was a significant positive correlation between the number of aa substitutions in ISDR and those in IRRDR, and the number of aa substitutions in ISDR/IRRDR of patients with Leu91 was significantly higher than that of patients with Met91. To our knowledge, this is the first report of the relationship between viremia levels and aa substitutions in core region/ISDR/IRRDR. This result might be interpreted to mean

that core aa 91/ISDR/IRRDR might be associated with viremia levels involved in resistance to combination therapy. Further studies that examine the functional impact of aa substitutions to combination therapy should be conducted to confirm the above finding.

The present results showed that α-fetoprotein, albumin, platelet count, and gender were predictors of virological response to IFN/ribavirin combination therapy. Previous data indicated that absence of advanced liver fibrosis was a positive predictor of SVR to IFN monotherapy and IFN/ribavirin combination therapy [2, 3, 13, 27–29], and that advanced liver fibrosis was usually associated with higher levels of α-fetoprotein, and lower levels of albumin and platelet count [1, 3, 30–32]. Furthermore, gender is also a predictor of treatment response to IFN/ribavirin combination therapy [2, 3, 14]. In the present study based on a large number of patients, histopathological changes in the liver and gender were identified as independent predictors of virological response, in addition to genetic variation near *IL28B* and viral factors (core region, ISDR, and level of viremia).

In a previous study, multivariate analysis identified core region, gender, and stage of liver fibrosis as parameters that independently influenced the SVR of patients who achieved early virological response, but ISDR was not entered into uni- and multivariate analysis [3]. To our knowledge, the present study based on multivariate analysis is the first report to identify ISDR as pretreatment predictor of SVR after ETR to combination therapy. Interestingly, ISDR was not a predictor of ETR, but was a significant predictor of SVR to combination therapy. Thus, the underlying mechanisms of failure to develop SVR in those patients who achieve HCV-RNA negativity remain unclear. Further studies that examine the impact of aa substitutions of ISDR to combination therapy should be conducted to confirm the above finding.

One limitation of the present study was that aa substitutions in areas other than the core region and NS5A-ISDR/IRRDR of the HCV genome were not examined. Other limitations were differences in host factors including race [24, 33, 34] and differences in viral factors, such as the distribution of HCV-1a or -1b, and geographic diversities of HCV-1b [35]. Further large-scale prospective studies are necessary to investigate whether the present results relate to the efficacy of 48-week IFN/ribavirin combination therapy, and further understanding of the complex interaction between virus- and host- related factors should facilitate the development of more effective therapeutic regimens.

Acknowledgement

This study was supported in part by a Grant-in-Aid from the Ministry of Health, Labor and Welfare, Japan.

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Complicated Relationships of Amino Acid Substitution in Hepatitis C Virus Core Region and *IL28B* Genotype Influencing Hepatocarcinogenesis

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The impact of amino acid (aa) 70 substitution in the core region on hepatocarcinogenesis and survival for liver-related death in patients of hepatitis C virus (HCV) genotype 1b (HCV-1b), who had not received antiviral therapy, is unknown. The relationships among aa 70 substitution, *IL28B* genotype, and hepatocarcinogenesis are also not clear. A total of 1,181 consecutive HCV-infected patients, who had not received antiviral therapy, were included in a follow-up study to determine predictive factors of hepatocarcinogenesis and survival for liver-related death. The cumulative hepatocarcinogenesis rates in HCV-1b of Gln70(His70) (glutamine (histidine) at aa 70) were significantly higher than those in HCV-1b of Arg70 (arginine at aa 70) and HCV-2a/2b. The cumulative survival rates for liver-related death in HCV-1b of Gln70(His70) were significantly lower than those in HCV-1b of Arg70 and HCV-2a/2b. Multivariate analysis identified gender (male), age (≥60 years), albumin (<3.9 g/dL), platelet count (<15.0 × 10^4/mm^3), aspartate aminotransferase (≥67 IU/L), and HCV subgroup (HCV-1b of Gln70(His70)) as determinants of both hepatocarcinogenesis and survival rates for liver-related death. In HCV-1b patients, the cumulative change rates from Arg70 to Gln70(His70) by direct sequencing were significantly higher than those from Gln70(His70) to Arg70. In patients of Arg70 at the initial visit, the cumulative change rates from Arg70 to Gln70(His70) in *IL28B* rs8099917 non-TT genotype were significantly higher than those in the TT genotype. *Conclusion:* Substitution of aa 70 in the core region of HCV-1b is an important predictor of hepatocarcinogenesis and survival for liver-related death in HCV patients who had not received antiviral therapy. The *IL28B* genotype might partly affect changes over time of dominant amino acid in core aa 70 of HCV-1b. (HEPATOLOGY 2012;56:2134-2141)

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) and high viral loads account for more than 70% of HCV infections, making it difficult to treat patients with chronic hepatitis C.[3]

Despite numerous lines of epidemiologic evidence connecting HCV infection and the development of HCC, it remains controversial whether HCV itself plays a direct role or an indirect role in the pathogenesis of HCC.[4] It has become evident that HCV core region has oncogenic potential through the use of transgenic mice, but the clinical impact of the core region on hepatocarcinogenesis is still unclear.[5] Previous reports indicated that amino acid (aa) substitutions at position 70 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

Abbreviations: aa, amino acid; ALT, alanine aminotransferase; AST, aspartate aminotransferase; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; PEG/IFN, pegylated interferon.

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Received May 1, 2012; Revised May 30, 2012; accepted June 17, 2012.

Supported in part by Grants-in-Aid for scientific research and development from the Ministry of Health, Labor and Welfare and Ministry of Education Culture Sports Science and Technology, Government of Japan.

of telaprevir/PEG-IFN/ribavirin,[6-9] and also affects hepatocarcinogenesis.[10-13] These reports support the findings of oncogenic potential by core region from the clinical aspect. However, its impact on hepatocarcinogenesis and survival for liver-related death in patients of HCV-1b who had not received antiviral therapy is still unknown.

The *IL28B* genotype is a poor predictor of virological response to PEG-IFN/ribavirin combination therapy and triple therapy of telaprevir/PEG-IFN/ribavirin[9,14-17] and is reported to be associated with HCC, although its impact on HCC is controversial.[18-21] Furthermore, treatment-resistant substitution of core aa 70 (glutamine/histidine at aa 70 (Gln70/His70)), which might affect hepatocarcinogenesis, was significantly more frequent in patients with treatment-resistant genotype (non-TT) than -sensitive genotype (TT) at *IL28B* rs8099917.[21-23] Thus, the significant linkage between substitution of aa 70 and *IL28B* genotype had been shown, but it is not clarified whether the existence of a complex interaction between the virus and host might affect hepatocarcinogenesis.

The present study included 1,181 consecutive HCV-infected patients who had not received antiviral therapy. The aims of the study were: (1) To evaluate the impact of aa substitutions in the core region of HCV-1b on hepatocarcinogenesis and survival for liver-related death; and (2) To investigate the association of *IL28B* genotype and time-dependent aa changes in the core region of HCV-1b.

Patients and Methods

Patients. Among 2,799 consecutive HCV-infected patients in whom antiviral therapy (IFN monotherapy or IFN plus ribavirin combination therapy) was not induced between December 1962 and November 2010 at Toranomon Hospital, 1,181 were selected in the present study based on the following criteria. (1) Positive for anti-HCV (third-generation enzyme immunoassay, Chiron, Emerville, CA) and positive for HCV RNA (nested polymerase chain reaction [PCR]), at the initial visit. (2) Patients without HCC at the initial visit. (3) Patients infected with single genotype of HCV-1b, 2a, or 2b. (4) In HCV-1b, patients analyzed aa substitutions of the core region by direct sequencing, one or more times from the initial visit. (5) Patients negative for hepatitis B surface antigen (radioimmunoassay, Dainabot, Tokyo, Japan). (6) Patients free of coinfection with human immunodeficiency virus. (7) Patients free of other types of chronic liver disease, including hemochromatosis, Wilson's disease, primary biliary cirrhosis, alcoholic liver disease, autoimmune liver disease, inherited liver disease including alpha-1 antitrypsin deficiency, and hepatic venous outflow block. (8) Patients who consented to the study.

Table 1. Profiles and laboratory data at the initial visit of 1,181 patients infected with HCV, who had not received antiviral therapy

Demographic data	
Number of patients	1,181
Sex (male/female)	608/573
Age (years)*	60 (20-93)
History of blood transfusion	526 (49.2%)
Family history of liver disease	201 (20.3%)
Lifetime cumulative alcohol intake (>500 kg)	110 (10.8%)
Laboratory data*	
Total bilirubin (mg/dl)	0.7(0.1-20.0)
Aspartate aminotransferase (IU/l)	71 (13-1,052)
Alanine aminotransferase (IU/l)	88 (4-1,210)
Albumin (g/dl)	4.1 (1.0-5.5)
Hemoglobin (g/dl)	14.0 (7.8-18.0)
Platelet count ($\times 10^4/mm^3$)	15.3 (2.6-52.9)
HCV genotype (Ib / 2a or 2b)	750/431
Levels of viremia (high viral load)	757 (74.4%)
Amino acid substitutions in the HCV genotype Ib	
Core aa 70 (arginine / glutamine (histidine))	431/319
Core aa 91 (leucine / methionine)	482/268

Data are number and percentages of patients, except those denoted by *, which represent the median (range) values.

Table 1 summarizes the profiles and laboratory data at the initial visit of 1,181 patients infected with HCV who had not received antiviral therapy. They did not receive antiviral therapy based on concerns about adverse effects, lack of time for treatment, physician recommendation based on the appearance of depression and cardiopulmonary disease, lower levels of aspartate aminotransferase (AST) / alanine aminotransferase (ALT), or elderly patients. They included 608 males and 573 females, aged 20 to 93 years (median, 60 years). The median follow-up time from the initial visit until death or until the last visit was 9.0 years (range, 0.0-37.7

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DOI 10.1002/hep.25949

Potential conflict of interest: Norio Akuta has received speakers' bureau from MSD K.K., and holds a right to get some loyalty from SRL. Inc.. Hiromitsu Kumada has received speakers' bureau from MSD K.K., Mitsubishi Tanabe Pharma, Dainippon Sumitomo Pharma, Bristol-Myers Squibb, and holds a right to get some loyalty from SRL. Inc.. Fumitaka Suzuki has received speakers' bureau from Bristol-Myers Squibb. The other authors have nothing to disclose.

years). The study protocol was approved by the Human Ethics Review Committee of the institution.

Laboratory Investigations. Blood samples were frozen at −80°C within 4 hours of collection and were not thawed until used for testing. Anti-HCV, HCV RNA, HCV genotype, and aa substitutions of the HCV-1b core region were assayed using stored frozen sera. HCV genotype was determined by PCR using a mixed primer set derived from nucleotide sequences of the NS5 region.[24] HCV RNA quantitative analysis was measured by branched DNA assay v. 2.0 (Chiron), AMPLICOR GT HCV Monitor v. 2.0 using the 10-fold dilution method (Roche Molecular Systems, Pleasanton, CA), or COBAS TaqMan HCV test (Roche Diagnostics, Tokyo, Japan). High viral load of viremia levels was defined as branched DNA assay ≥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 <1.0 Meq/mL, AMPLICOR GT HCV Monitor <100 × 10^3 IU/mL, or COBAS TaqMan HCV test <5.0 log IU/mL.

Detection of Amino Acid Substitutions in Core Regions of HCV-1b. In the present study, aa substitutions of the core region of HCV-1b were analyzed by direct sequencing. HCV RNA was extracted from serum samples and reverse transcribed with 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 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, 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).

With the use of HCV-J (accession no. D90208) as a reference,[25] the dominant sequence of 1-191 aa in the core protein of HCV-1b was determined by direct sequencing and then compared with the consensus sequence constructed on 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).[6] Especially, patients were classified into three HCV subgroups according to HCV genotype in combination with aa substitutions in HCV-1b core region (HCV-1b of Arg70, HCV-1b of Gln70(His70), and HCV-2a/2b).

Determination of IL28B Genotype. IL28B rs8099917 was genotyped by the Invader assay, TaqMan assay, or direct sequencing, as described.[26,27]

Follow-Up and Diagnosis of HCC. Follow-up of patients was made on a monthly to trimonthly basis after the initial visit. Imaging diagnosis was made one or more times per year with ultrasonography, computed tomography, or magnetic resonance imaging. During this time, liver-related death, which included HCC, cholangiocellular carcinoma, liver failure, or esophageal variceal bleeding, was also evaluated.

Statistical Analysis. The cumulative rates of hepatocarcinogenesis, survival for liver-related death, and amino acid changes in the core region were calculated using the Kaplan-Meier technique; differences between the curves were tested using the log-rank test. Statistical analyses of hepatocarcinogenesis, survival, and amino acid changes, according to groups, were calculated using the period from the initial visit. Stepwise Cox regression analysis was used to determine independent predictive factors that were associated with hepatocarcinogenesis and survival for liver-related death. The hazard ratio (HR) and 95% confidence interval (95% CI) was also calculated. Potential predictive factors associated with hepatocarcinogenesis and survival for liver-related death included the variables: sex, age, history of blood transfusion, family history of liver disease, lifetime cumulative alcohol intake, total bilirubin, AST, ALT, albumin, hemoglobin, platelet count, levels of viremia, and HCV subgroup according to HCV genotype in combination with aa substitution in core region. Variables that achieved statistical significance ($P < 0.05$) on univariate analysis were tested by multivariate Cox proportional hazard model to identify significant independent factors. Statistical comparisons were performed using the SPSS software (Chicago, IL). $P < 0.05$ by the two-tailed test were considered significant.