

# Association of Amino Acid Substitution Pattern in Core Protein of Hepatitis C Virus Genotype 1b High Viral Load and Non-Virological Response to Interferon-Ribavirin Combination Therapy

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## Key Words

Hepatitis C virus · Genotype 1b · Albumin · Core region · Interferon sensitivity-determining region · Interferon · Ribavirin · Non-virological responder · Viral kinetics

## Abstract

**Objective:** Patients with high titer ( $\geq 100$  kIU/ml) of hepatitis C virus (HCV) genotype 1b do not achieve highly sustained virological response rates to combination therapy with interferon plus ribavirin. Non-virological responders (NVRs, namely ultimate resistant cases) who do not achieve HCV-RNA negativity during treatment are also encountered. We investigated the pretreatment virological features of NVRs. **Methods:** We evaluated 50 consecutive Japanese adults with high titer of HCV genotype 1b who received combination therapy for 48 weeks. We investigated the pretreatment substitution patterns in amino acids 1–191 of the core region and amino acids 2209–2248 of NS5A, and early viral kinetics. **Results:** Overall, a non-virological response was noted in 12 (24%) patients. Multivariate analysis identified serum albumin  $<3.9$  g/dl, substitutions of amino acid 70 in the core region, and substitutions of amino

acid 91 as independent and significant factors associated with a non-virological response. Especially, substitutions of arginine (R) by glutamine (Q) at amino acid 70, and/or leucine (L) by methionine (M) at amino acid 91 were significantly more common in NVRs. The falls in HCV-RNA levels during treatment in patients with specific substitutions in the core region were significantly less than in those without such substitutions. **Conclusions:** Our results suggest that serum albumin and amino acid substitution patterns in the core region in patients with high titers of HCV genotype 1b may have an effect on combination therapy in NVRs. Further large-scale studies are required to examine the role of amino acid substitutions specific to a non-virological response to combination therapy.

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## Introduction

Hepatitis C virus (HCV) usually causes chronic infection which can result in liver cirrhosis and hepatocellular carcinoma (HCC) [1–4]. The aims of interferon (IFN) therapy for chronic hepatitis C include a reduction in the risk of development of HCC and liver-related death by

viral clearance, and then by normalization of alanine aminotransferase (ALT) even if viral clearance cannot be achieved [5].

The most effective initial therapy for viral clearance is the combination of IFN and ribavirin (RBV) administered for 48 weeks [6, 7]. In Japan, about 70% of patients with chronic hepatitis C are infected with HCV genotype 1b, and a sustained virological response (SVR) to IFN monotherapy for 24 weeks is as low as 10–20% in patients with genotype 1b infection [8–11]. Moreover, patients with a high titer of genotype 1b ( $\geq 100$  kIU/ml) do not achieve high SVR rates ( $<50\%$ ), even when the most effective combination treatment (IFN plus RBV) is administered for 48 weeks [6, 7]. Furthermore, in genotype 1b, we also often encounter non-virological responders (NVRs) who do not achieve HCV-RNA negativity as determined by polymerase chain reaction (PCR) during treatment, compared with only 1.0% (non-virological response rate) of patients infected with genotype 2a and treated with IFN monotherapy [12]. The underlying mechanism(s) of the different virological responses to treatment in patients with 1b strain infection is still unclear. Hence, in the present study, we investigated the pretreatment virological features of NVRs.

The present study included 50 consecutive Japanese adults with chronic hepatitis C of genotype 1b and a high viral load who received combination therapy for 48 weeks. The aims of the study were: (1) to investigate the rate of non-virological responses in this group; (2) to analyze the predictive factors associated with a non-virological response, including pretreatment virological features, and (3) to examine the pretreatment virological features associated with early viral kinetics. Previous studies have shown that the HCV core region might be associated with resistance to IFN therapy involving the Jak-STAT signaling cascade [13–16], and have also shown that the number of substitutions in amino acids 2209–2248 (IFN-sensitivity determining region, ISDR, of NS5A in HCV genotype 1b) [17, 18] might be associated with the efficacy of IFN therapy and viral load. Therefore, we analyzed the amino acid substitutions of the core region and NS5A in patients with genotype 1b and high viral load to identify the virus-related factors apart from the genotype and viral load.

## Materials and Methods

### Study Population

Fifty-seven HCV-infected adult Japanese patients were consecutively recruited into the study protocol of combination therapy with IFN (peginterferon (PEG)-IFN $\alpha$ -2b or IFN $\alpha$ -2b) plus RBV for

48 weeks between 2001 and 2004 at Toranomon Hospital, Tokyo, Japan. Among these, 50 patients were selected in the present study based on the following criteria. (1) They were negative for hepatitis B surface antigen (radioimmunoassay, Dainabot, Tokyo, Japan), positive for anti-HCV (third-generation enzyme immunoassay, Chiron Corp, Emerville, Calif., USA), and positive for HCV-RNA qualitative analysis with PCR (Amplicor, Roche Diagnostic Systems, Pleasanton, Calif., USA). (2) They were naive to RBV therapy. (3) They were infected with HCV genotype 1b alone. (4) Each had a high viral load ( $\geq 100$  kIU/ml) by quantitative analysis of HCV-RNA with PCR (Amplicor HCV-RNA kit, version 2.0, Roche Diagnostics) within the preceding 2 months of enrolment. (5) Each had chronic hepatitis, without cirrhosis or HCC, as confirmed by biopsy examination within the preceding 12 months of enrolment. (6) They had abnormal serum ALT levels (the upper limit of normal for ALT, 45 IU/l) within the preceding 2 months of enrolment. (7) In each patient, the hemoglobin (Hb) concentration was  $\geq 12.0$  g/dl, platelet count  $\geq 100 \times 10^3/\text{mm}^3$ , and neutrophil count  $\geq 1.5 \times 10^3/\text{mm}^3$  within the preceding 2 months of enrolment. (8) Their body weight was  $>40$  kg. (9) All were free of co-infection with human immunodeficiency virus. (10) None had been treated with antiviral or immunosuppressive agents within the preceding 3 months of enrolment. (11) None was an alcoholic; lifetime cumulative alcohol intake was  $<500$  kg (mild to moderate alcohol intake). (12) None had diabetes, other forms of hepatitis, such as hemochromatosis, Wilson disease, primary biliary cirrhosis, alcoholic liver disease, and autoimmune liver disease. (13) None of the females was pregnant or lactating. (14) All accepted treatment for 24 weeks or more as outlined in the study protocol, as well as repeated evaluation of HCV-RNA levels during treatment (at least once every month). (15) Each signed a consent form of the study protocol that had been approved by the Human Ethics Review Committee of Toranomon Hospital.

With regard to the treatment protocol, 34 (68.0%) patients received the PEG-IFN $\alpha$ -2b treatment protocol at dose of 1.5  $\mu\text{g}/\text{kg}$  subcutaneously each week plus oral RBV at 600–800 mg/day for 48 weeks. The remaining 16 (32.0%) patients received 6 million units of IFN $\alpha$ -2b intramuscularly each day for 48 weeks (6 times per week for initial 2 weeks, followed by 3 times per week for 46 weeks), and oral RBV at a dose of 600–800 mg/day for 48 weeks. The RBV dose was adjusted according to body weight (600 mg for weight  $\leq 60$  kg, and 800 mg for weight  $>60$  kg).

Table 1 summarizes the profiles and data of the 50 patients at the commencement of combination therapy with IFN plus RBV. They included 31 men and 19 women, aged 20–65 (median 53) years. The median total duration of treatment was 48 (range 28–48) weeks. In 14 of the 50 (28.0%) patients, the dose of RBV was reduced during treatment due to a fall in Hb concentration.

Patients who remained positive for HCV-RNA based on quantitative and/or qualitative analyses with PCR during and at the end of combination therapy were defined as NVRs (namely ultimate resistant cases), while the other patients who could achieve negative HCV-RNA by qualitative analysis with PCR during and/or at the end of treatment were defined as virological responders (VRs).

### Laboratory Tests

Blood samples were obtained at least once every month before, during, and after treatment, and were analyzed for ALT and HCV-RNA levels. The serum samples were frozen at  $-80^\circ\text{C}$  within 4 h of collection and were thawed at the time of measurement. HCV

genotype was determined by PCR using a mixed primer set derived from nucleotide sequences of NS5 region [19]. HCV-RNA levels were measured quantitatively by PCR (Amplicor HCV-RNA kit, version 2.0, Roche Diagnostics) at least once every month before, during, and after therapy. The lower limit of the assay was 0.5 kIU/ml. Samples collected during and after therapy that showed undetectable levels of HCV-RNA (<0.5 kIU/ml) were checked also by qualitative PCR (Amplicor, Roche Diagnostic Systems), which has a higher sensitivity than quantitative analysis, and the results are expressed as positive or negative. The lower limit of the assay was 100 copies/ml.

#### Histopathological Examination of Liver Biopsies

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), fixed in 10% formalin, and stained with hematoxylin and eosin, Masson's trichrome, silver impregnation, and periodic acid-Schiff after diastase digestion. All specimens for examinations contained 6 or more portal areas. Histopathological diagnosis was confirmed by an experienced liver pathologist (H.K.) who was blinded to the clinical data. Chronic hepatitis was diagnosed based on histological assessment according to the scoring system of Desmet et al. [20]. Hepatocyte steatosis was graded as either none (absent), mild (<1/3 of hepatocytes involved), moderate (>1/3 but <2/3 of hepatocytes involved), or severe (>2/3 of hepatocytes involved) [21].

#### Nucleotide Sequencing of the Core and NS5A Gene

We determined the sequences of amino acids 1–191 in the core and amino acids 2209–2248 (ISDR) in the NS5A by the direct sequencing method using pretreatment sera of 50 patients. These sequences were compared with the consensus sequence of genotype 1b, which was determined by comparing the sequences obtained in this study and prototype sequence (HCV J) [22]. 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 PCR using the following primers. (a) Nucleotide sequences of the core region: the first-round PCR was performed with CC11 (sense, 5'-GCC ATA GTG GTC TGC GGA AC-3') and e14 (antisense, 5'-GGA GCA GTC CTT CGT GAC ATG-3') primers, and the second-round PCR with CC9 (sense, 5'-GCT AGC CGA GTA GTG TT-3') and e14 (antisense) primers. (b) Nucleotide sequences of ISDR in NS5A: the first-round PCR was performed with ISDR1 (sense, 5'-ATG CCC ATG CCA GGT TCC AG-3') and ISDR2 (antisense, 5'-AGC TCC GCC AAG GCA GAA GA-3') primers, and the second-round PCR with ISDR3 (sense, 5'-ACC GGA TGT GGC AGT GCT CA-3') and ISDR4 (antisense, 5'-GTA ATC CGG GCG TGC CCA TA-3') primers (hemi-nested PCR and nested PCR). All samples were initially denatured at 95°C for 15 min. The 35 cycles of amplification were set as follows: denaturation for 1 min at 94°C, annealing of primers for 2 min at 55°C, and extension for 3 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. 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 termina-

**Table 1.** Patient profile and laboratory data at commencement of combination therapy with interferon plus ribavirin for 48 weeks in 50 patients infected with HCV genotype 1b

Demographic data	
Number	50
Sex, M/F	31/19
Age, years <sup>a</sup>	53 (20–65)
History of blood transfusion	14 (28.0%)
Family history of liver disease	16 (32.0%)
Body mass index, kg/m <sup>2a</sup>	23.2 (18.7–32.0)
Laboratory data <sup>a</sup>	
Serum alanine aminotransferase, IU/l	97 (35–276)
Serum albumin, g/dl	3.8 (3.1–4.2)
Hemoglobin, g/dl	14.4 (12.0–17.4)
Platelet count, × 10 <sup>4</sup> /mm <sup>3</sup>	17.4 (10.1–30.9)
ICG R15, % <sup>b</sup>	13 (7–41)
Serum iron, µg/dl	140 (52–308)
Serum ferritin, µg/l	150 (<10–644)
Creatinine clearance, ml/min	101 (46–142)
Viremia level, KIU/ml	710 (49–2,800)
Number of amino acid substitutions in ISDR (0/1–3/≥ 4)	27/20/3
Histological findings	
Stage (F1/F2/F3) <sup>c</sup>	31/15/4
Hepatocyte steatosis (none/mild/moderate/severe)	3/40/7/0
Treatment	
PEG-IFNα-2b/IFNα-2b	34/16
Ribavirin dose, mg/kg <sup>a</sup>	11.3 (9.7–14.2)

ALT levels were abnormal (the upper limit of normal for ALT; 45 IU/l) and viremia levels were high titer (≥ 100 kIU/ml), when all patients were recruited in this study. Normal reference ranges: 3.9–5.2 g/dl for albumin.

<sup>a</sup> Expressed as median (range).

<sup>b</sup> ICG R15: indocyanine green retention rate at 15 min.

<sup>c</sup> Stage of chronic hepatitis by Desmet et al. [20].

tion sequencing was performed with the Big Dye Deoxy Terminator Cycle Sequencing kit (Perkin-Elmer, Tokyo).

To avoid false-positive results, the procedures recommended by Kwok and Higuchi [23] to prevent contamination were strictly applied to these PCR assays. No false-positive results were observed in this study.

#### Viral Kinetic Study

Viral kinetic study was evaluated at three time points (4, 8 and 12 weeks during treatment). Falls in HCV-RNA levels from baseline were expressed using log<sub>10</sub> of viral loads at each time point, in comparison with the pretreatment viral load. For data analysis, we used the log<sub>10</sub> of the cutoff value (500 IU/ml) for HCV-RNA values below the limit of detection.

### Statistical Analysis

Non-parametric tests were used to analyze the decline in HCV-RNA levels and amino acid substitutions in HCV core and NS5A between the each groups, including the Mann-Whitney U test,  $\chi^2$  test and Fisher's exact probability test. Univariate and multivariate logistic regression analyses were used to determine the factors that significantly contributed to a non-virological response. We also calculated the odds ratios and 95% confidence intervals (95% CI). All p values of <0.05 by the two-tailed test were considered significant. Variables that achieved statistical significance ( $p < 0.05$ ) or marginal significance ( $p < 0.10$ ) on univariate analysis were entered into multiple logistic regression analysis to identify significant independent factors. Potential predictive factors associated with NVR included the following variables: sex, age, history of blood transfusion, familial history of liver disease, body mass index, ALT, albumin, Hb, platelet count, indocyanine green retention rate at 15 min (ICG R15), serum iron, serum ferritin, creatinine clearance, viremia level, pathological staging, hepatocyte steatosis, type of IFN, RBV dose according to body weight, treatment term, dose reduction, and pretreatment amino acid substitution in the core and ISDR of NS5A. Statistical analyses were performed using the SPSS software (SPSS Inc., Chicago, Ill., USA).

## Results

### Virological Response Rates by Combination Therapy

The virological response could be evaluated in all 50 patients. In this study, 38 of 50 (76.0%) patients achieved a virological response while the remaining 12 (24.0%) patients were considered NVRs.

### Predictive Factors Associated with a Non-Virological Response in Multivariate Analysis

We then analyzed the data of the whole population sample to determine those factors that could predict a non-virological response. Univariate analysis identified 5 parameters that tended to or significantly influenced the non-virological response. These included serum albumin ( $p = 0.008$ ), presence of amino acid substitution in HCV core in the pretreatment sample (substitution of amino acid 70,  $p = 0.003$ , and amino acid 91,  $p = 0.044$ ), RBV dose according to body weight ( $p = 0.044$ ), and serum ferritin ( $p = 0.095$ ).

Multivariate analysis identified three parameters that independently influenced the non-virological response, including serum albumin ( $p = 0.004$ ), substitutions of amino acids 70 ( $p = 0.013$ ) and 91 ( $p = 0.016$ ; table 2).

### Treatment Efficacy according to Substitution Patterns in Amino Acids of HCV Core

Figure 1 shows the sequences of amino acids 61–110 of the HCV core in 50 patients at the commencement of combination therapy. Substitutions at amino acid 70 of

**Table 2.** Factors associated with non-virological response to combination therapy with interferon plus ribavirin for 48 weeks in 50 patients infected with HCV genotype 1b, identified by multivariate analysis

Factor	Category	Odds ratio (95% confidence interval)	p
Albumin, g/dl	1: <3.9	1	0.004
	2: $\geq 3.9$	0.009 (0.000–0.227)	
Substitution of aa 70	1: Absent	1	0.013
	2: Present	22.2 (1.905–258.3)	
Substitution of aa 91	1: Absent	1	0.016
	2: Present	19.5 (1.737–219.3)	

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

the HCV core were significantly more frequent in NVRs ( $n = 8$ , 66.7%) than VRs ( $n = 7$ , 18.4%;  $p = 0.003$ ). Similarly, substitutions at amino acid 91 were significantly more frequent in NVRs ( $n = 9$ , 75.0%) than VRs ( $n = 14$ , 36.8%;  $p = 0.044$ ). Furthermore, dual substitutions at amino acids 70 and 91 were significantly more frequent in NVRs ( $n = 5$ , 41.7%) than VRs ( $n = 5$ , 13.2%;  $p = 0.046$ ). Thus, substitutions at amino acid(s) 70 and/or 91 were found in all 12 (100%) NVRs while only 16 (42.1%) of the VRs had such substitutions ( $p < 0.001$ ). There were no significant differences in other substitution sites and treatment efficacy between NVR and VR groups (table 3).

At amino acid 70, the substitution in which arginine (R) was replaced by glutamine (Q) was significantly more frequent in NVRs ( $n = 7$ , 58.3%) than VRs ( $n = 5$ , 13.2%;  $p = 0.004$ ). At amino acid 91, the substitution in which leucine (L) was replaced by methionine (M) was significantly more frequent in NVRs ( $n = 9$ , 75.0%) than VRs ( $n = 14$ , 36.8%;  $p = 0.044$ ). At amino acid 110, the substitution in which threonine (T) was replaced by asparagine (N) was significantly more frequent in NVRs ( $n = 3$ , 25.0%) than VRs ( $n = 2$ , 5.3%;  $p = 0.082$ ). Substitutions Q–M instead of R–L at amino acids 70 and 91 were significantly more frequent in NVRs ( $n = 5$ , 41.7%) than VRs ( $n = 3$ , 7.8%;  $p = 0.014$ ). Thus, 11 (91.7%) NVRs and 16 (42.1%) VRs ( $p = 0.003$ ) had a substitution of Q at amino acid 70 and/or M at amino acid 91. There were no significant differences in other substitution patterns and treatment efficacy between NVRs and VRs (table 3).

	70	80	90	100	110	Efficacy
Consensus	RRQPIPKARR	PEGRTWAQPG	YPWPLYGNEG	LGWAGWLLSP	RGSRPSWGPT	
HCJ	-----	-----	-----	M-----	-----	
Case 1	----- <b>Q</b> -----	-----	-- <b>L</b> -----	<b>M</b> -----	-----	NVR
2	-----	----- <b>D</b> -----	-----	<b>M</b> -----	-----	NVR
3	-----	-----	-----	<b>M</b> -----	----- <b>N</b> -----	NVR
4	-----	-----	-----	<b>M</b> -----	-----	NVR
5	-----	-----	-----	<b>M</b> -----	-----	NVR
6	----- <b>Q</b> -----	----- <b>A</b> -----	-----	<b>M</b> -----	----- <b>N</b> -----	NVR
7	----- <b>Q</b> -----	----- <b>A</b> -----	-----	-----	----- <b>S</b> -----	NVR
8	----- <b>Q</b> -----	----- <b>A</b> -----	-----	-----	-----	NVR
9	----- <b>Q</b> -----	----- <b>P</b> -----	-----	<b>M</b> -----	-----	NVR
10	----- <b>Q</b> -----	----- <b>A</b> -----	-----	<b>M</b> -----	----- <b>N</b> -----	NVR
11	----- <b>Q</b> -----	----- <b>A</b> -----	-----	<b>M</b> -----	-----	NVR
12	----- <b>H</b> -----	----- <b>A</b> -----	-----	-----	-----	NVR
13	-----	-----	-----	<b>M</b> -----	-----	VR
14	-----	----- <b>A</b> -----	-----	-----	----- <b>N</b> -----	VR
15	-----	-----	-----	<b>M</b> -----	-----	VR
16	----- <b>H</b> -----	----- <b>D</b> -----	-----	<b>M</b> -----	-----	VR
17	-----	----- <b>S</b> -----	-----	-----	H-----	VR
18	-----	----- <b>A</b> -----	-----	-----	-----	VR
19	-----	-----	-----	-----	-----	VR
20	-----	-----	-----	-----	H-----	VR
21	-----	----- <b>A</b> -----	-----	----- <b>T</b> -----	-----	VR
22	-----	----- <b>A</b> -----	-----	-----	----- <b>S</b> -----	VR
23	-----	-----	-----	-----	-----	VR
24	-----	-----	-----	-----	-----	VR
25	-----	-----	-----	-----	-----	VR
26	-----	----- <b>A</b> -----	-----	-----	-----	VR
27	-----	----- <b>A</b> -----	-----	-----	-----	VR
28	-----	----- <b>V</b> -----	-----	<b>M</b> -----	----- <b>N</b> -----	VR
29	----- <b>Q</b> -----	----- <b>A</b> -----	-----	<b>M</b> -----	-----	VR
30	----- <b>Q</b> -----	-----	-----	-----	-----	VR
31	----- <b>Q</b> -----	----- <b>A</b> -----	-----	<b>M</b> -----	-----	VR
32	----- <b>H</b> -----	-----	-----	<b>M</b> -----	-----	VR
33	-----	-----	-----	<b>M</b> -----	-----	VR
34	-----	-----	-----	<b>M</b> -----	----- <b>N</b> -----	VR
35	-----	----- <b>A</b> -----	-----	-----	-----	VR
36	-----	----- <b>A</b> -----	-----	-----	-----	VR
37	-----	-----	-----	-----	-----	VR
38	-----	----- <b>P</b> -----	-----	-----	-----	VR
39	-----	-----	-----	<b>M</b> -----	----- <b>N</b> -----	VR
40	----- <b>Q</b> -----	----- <b>A</b> -----	-----	-----	----- <b>N</b> -----	VR
41	-----	-----	-----	<b>M</b> -----	H----- <b>N</b> -----	VR
42	-----	-----	-----	-----	----- <b>N</b> ----- <b>S</b> -----	VR
43	-----	-----	-----	<b>M</b> -----	-----	VR
44	----- <b>Q</b> -----	-----	-----	<b>M</b> -----	-----	VR
45	-----	-----	-----	-----	-----	VR
46	-----	----- <b>A</b> -----	-----	-----	----- <b>N</b> -----	VR
47	-----	-----	-----	-----	-----	VR
48	-----	----- <b>A</b> -----	-----	-----	-----	VR
49	-----	-----	-----	<b>M</b> -----	----- <b>A</b> -----	VR
50	-----	----- <b>A</b> -----	-----	-----	-----	VR

**Fig. 1.** Sequences of amino acids 61–110 in the core region at the commencement of combination therapy in 50 patients infected with high HCV viral load genotype 1b. Dashes indicate amino acids identical to the consensus sequence of genotype 1b, and substituted amino acids are shown by standard single-letter codes. The amino acid patterns at positions that are probably associated with sensitivity to therapy are shown in boldface characters. NVR = Non-virological responder; VR = virological responder.

*Viral Kinetics according to Substitution Patterns in Amino Acids of HCV Core*

Table 4 shows HCV-RNA levels at 4, 8, and 12 weeks relative to baseline as a function of pretreatment amino acid substitutions in the core region. The fall in HCV-RNA level at each time point was significantly lower in

patients with specific substitution patterns (Q at amino acid 70, M at amino acid 91, N at amino acid 110, Q–M at amino acid 70 and 91, Q at amino acid 70 and/or M at amino acid 91) than in those without them.

**Table 3.** Amino acid substitutions in the core region in non-virological responders (NVR) and virological responders (VR) to combination therapy of interferon plus ribavirin for 48 weeks in 50 patients infected with HCV genotype 1b

	NVR (n = 12)	VR (n = 38)	p*
Presence of substitution site			
aa 70	8 (66.7%)	7 (18.4%)	0.003
aa 91	9 (75.0%)	14 (36.8%)	0.044
aa 70 and 91	5 (41.7%)	5 (13.2%)	0.046
aa 70 and/or 91	12 (100%)	16 (42.1%)	<0.001
Presence of substitution pattern			
Q at aa 70	7 (58.3%)	5 (13.2%)	0.004
M at aa 91	9 (75.0%)	14 (36.8%)	0.044
N at aa 110	3 (25.0%)	2 (5.3%)	0.082
Q-M at aa 70 and 91	5 (41.7%)	3 (7.8%)	0.014
Q at aa 70 and/or M at aa 91	11 (91.7%)	16 (42.1%)	0.003

Q = Glutamine; M = methionine; N = asparagine; aa = amino acid.  
\* NVR vs. VR (Fisher's exact probability test).

**Table 4.** Decline levels of HCV-RNA from baseline at 4, 8 and 12 weeks according to the amino acid substitutions in the core region during combination therapy of interferon plus ribavirin for 48 weeks in 50 patients infected with HCV genotype 1b

Presence of substitution pattern	Decline levels of HCV-RNA from baseline, log <sub>10</sub> IU/ml <sup>1</sup>		
	4 weeks	8 weeks	12 weeks
Q at aa 70			
Absent <sup>2</sup>	2.49 (-0.024 to 3.41)	3.02 (0.25 to 3.41)	2.98 (0.30 to 3.45)
Present	0.58 (0.11 to 3.13) ] <sup>a</sup>	1.18 (-0.095 to 3.16) ] <sup>b</sup>	1.99 (0.34 to 3.19) ] <sup>c</sup>
M at aa 91			
Absent	2.49 (0.12 to 3.41)	3.14 (1.69 to 3.41)	3.13 (0.49 to 3.45)
Present	0.85 (-0.024 to 3.16) ] <sup>d</sup>	1.56 (-0.095 to 3.41) ] <sup>e</sup>	2.40 (0.30 to 3.41) ] <sup>f</sup>
N at aa 110			
Absent	2.36 (0.10 to 3.41)	3.02 (-0.095 to 3.41)	2.96 (0.48 to 3.45)
Present	0.28 (-0.024 to 0.86) ] <sup>g</sup>	0.32 (0.25 to 1.43) ] <sup>h</sup>	0.70 (0.30 to 2.46) ] <sup>i</sup>
Q-M at aa 70 and 91			
Absent	2.49 (-0.024 to 3.41)	3.04 (0.25 to 3.41)	2.98 (0.30 to 3.45)
Present	0.58 (0.11 to 2.34) ] <sup>j</sup>	0.50 (-0.095 to 2.34) ] <sup>k</sup>	1.99 (0.34 to 3.19) ] <sup>l</sup>
Q at aa 70 and/or M at aa 91			
Absent	2.49 (0.88 to 3.41)	3.11 (1.69 to 3.41)	3.18 (2.40 to 3.45)
Present	0.85 (-0.024 to 3.16) ] <sup>m</sup>	2.01 (-0.095 to 3.41) ] <sup>n</sup>	2.40 (0.30 to 3.41) ] <sup>o</sup>

Q = Glutamine; M = methionine; N = asparagine; aa = amino acid.

<sup>1</sup> Decline levels of HCV-RNA from baseline are shown in log<sub>10</sub> of viral loads at each time point in comparison to pretreatment viral loads. For HCV-RNA quantitative values below the limit of detection, we used the log<sub>10</sub> of the cutoff value (500 IU/ml) for data analysis. Data are expressed as median (range).

<sup>2</sup> Absent vs. Present of substitution pattern (Mann-Whitney U test): <sup>a</sup> p = 0.025; <sup>b</sup> p = 0.019; <sup>c</sup> p = 0.011; <sup>d</sup> p = 0.049; <sup>e</sup> p = 0.001; <sup>f</sup> p = 0.007; <sup>g</sup> p = 0.010; <sup>h</sup> p = 0.002; <sup>i</sup> p = 0.004; <sup>j</sup> p = 0.018; <sup>k</sup> p = 0.001; <sup>l</sup> p = 0.019; <sup>m</sup> p = 0.028; <sup>n</sup> p = 0.006; <sup>o</sup> p = 0.001.

## Discussion

The main finding of the present study was that resistance to PEG-IFN $\alpha$ -2b+RBV or IFN $\alpha$ -2b+RBV combination therapy in patients with chronic hepatitis C genotype 1b and high viral load was partly influenced by serum albumin, substitutions of amino acids 70 and 91.

We previously showed that serum albumin was a negative predictor of SVR to IFN monotherapy in HCV patients, based on multivariate analysis [12]. Serum proteins including albumin are synthesized by hepatocytes, and falls in their concentrations usually reflect decreased hepatic synthesis although changes in plasma volume could also contribute to such falls. Advanced liver fibrosis is usually associated with decreased hepatic synthesis and low levels of serum albumin [24]. On the other hand, the absence of advanced liver fibrosis is a predictor of SVR to IFN monotherapy and combination therapy of IFN/RBV [11, 25–27]. This report on VRs showed that a milder form of liver fibrosis was not a positive predictor of response to combination therapy, compared with high levels of serum albumin [24]. These discrepant findings may be due to one or more factors. The first reason is probably related to the method used for evaluation; the degree of liver fibrosis roughly reflects liver function but can only be assessed using a three-stage (F1, F2, F3) system, in contrast to the serum albumin level. Thus, serum albumin might reflect liver function more sensitively than the degree of liver fibrosis. Furthermore, this finding showed that the ability of the liver to synthesize serum proteins including albumin might contribute to the observed response to treatment more than the degree of liver fibrosis. The second reason is probably related to the design of our study based on comparison between a virological and a non-virological response, rather than a SVR and a non-SVR. Our study based on multivariate analysis is the first to identify serum albumin as a predictor of a non-virological response in patients on 48-week IFN/RBV combination therapy.

IFN- $\alpha$  and IFN- $\beta$  bind to the type-I IFN receptor, and one major pathway in type-I IFN signaling involves the Jak-STAT signaling cascade [13, 28–37]. Previous studies reported that the HCV core region might be associated with resistance to the antiviral actions of IFN therapy. Blindenbacher et al. [14] showed that STAT signaling was strongly inhibited in liver cells of HCV core transgenic mice. Bode et al. [15] showed that HCV core protein induced the expression of the suppressor of cytokine signaling-3 and inhibited activation, tyrosine phosphorylation, and nuclear translocation of STAT1, which

might impair the antiviral actions of IFNs in HepG2 cells. Furthermore, Mélen et al. [16] indicated that IFN-induced nuclear accumulation of STAT1 was almost completely blocked and STAT2 was partially blocked in cell lines expressing high levels of HCV core protein. Our study identified amino acid substitutions in HCV core as a predictor of a non-virological response to 48-week IFN/RBV combination therapy based on multivariate analysis. This result suggests that substitutions of amino acids in the HCV core region might be associated with resistance to the antiviral actions of IFN therapy involving the Jak-STAT signaling cascade.

Since combination therapy could induce hemolytic anemia and possibly other major side effects [6], it is important to identify resistant patients, especially NVRs among non-sustained virological responders, early during therapy with the intent of revising the treatment regimen. In fact, we were able to revise or terminate treatment before completion of the full course of combination therapy for 48 weeks and spare patients from receiving unnecessary treatment based on consideration of risks/benefits. Our study indicated that falls in HCV-RNA levels from baseline were significantly lower in patients with specific pretreatment amino acid substitution patterns in the HCV core. Thus, our study identified pretreatment virological features associated with early viral kinetics during combination therapy with IFN/RBV. Further studies are required to explore the relationship between virological features and differences in viral kinetics.

In conclusion, our results suggest that albumin levels and amino acid substitution patterns in the core region in patients with a high titer of HCV genotype 1b might determine a non-virological response to combination therapy. One limitation of this study was that we did not examine other viral factors, such as amino acid substitutions in areas other than the core region and ISDR of HCV genome, as well as other host factors such as IFN-inducible protein kinase, MxA and 2',5'-OAS protein [28–31, 37–42], although they should be investigated together with other factors in future studies. Moreover, further large-scale prospective studies are necessary to investigate whether our results also explain resistance to IFN-RBV combination therapy.

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