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ORIGINAL ARTICLE

# Analysis of viral amino acids sequences and the IL28B SNP influencing the development of hepatocellular carcinoma in chronic hepatitis C

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**Abstract**

*Background and aims* The association between hepatitis C virus (HCV) sequences with interleukin 28B (IL28B) single-nucleotide polymorphism (SNP) in the development of hepatocellular carcinoma (HCC) has not been well clarified.

*Methods* Complete HCV open-reading frame sequences were determined in 20 patients developing HCC and 23 non-HCC patients with HCV-1b infection in two distant time points. An additional 230 patients were studied cross-sectionally for core and NS5A sequences with HCC development. Among them, 98 patients with available samples were investigated for changes in viral core sequences over time. Finally, IL28B SNPs and HCC development were investigated in 228 patients.

*Results* During observation period (HCC for 10.8 years, and non-HCC for 11.1 years), changes in core a.a. 70 and three amino acid positions in NS5A were characteristics of the patients developing HCC. In 230 patients, Q (glutamine) or H (histidine) to R (arginine) ratio at core a.a. 70 was significantly higher in the HCC group (HCC group 43:22 vs. non-HCC group 66:99, $p = 0.001$). A change in core R70Q was observed over time in 11 patients associated with a decrease in platelets ($p = 0.005$) and albumin ($p = 0.005$), while a Q70R change was observed in 4 patients without associated changes in platelets (nonsignificant) and albumin (nonsignificant). IL28B SNP showed significant correlation with the core a.a. 70 residue. There was no evident link between IL28B SNPs and the occurrence of HCC.

*Conclusions* Hepatitis C virus core a.a. 70 residue is associated with liver disease progression and is independent factor for HCC development in genotype-1b infection. IL28B SNPs are related to core a.a. 70 residue, but not to HCC. The functional relevance of core a.a. 70 residue in hepatitis C pathogenesis should be further investigated.

**Keywords** HCV · HCC · Core · IL28B

## Introduction

Hepatitis C virus (HCV) infection is a major risk factor for hepatocellular carcinoma (HCC). Chronic HCV infection can result in liver cirrhosis (LC) and HCC over the course of 20–30 years [1]. However, the rate of progression is variable; some patients remain for a long time with persistently normal ALT values, while others progress rapidly to LC and HCC.

Viral factors, host factors, and their interplay appear to play an important role in determining the progression of chronic hepatitis C to LC and HCC. In terms of viral factors, most previous clinical studies have focused on searching for HCV regions correlated with the response to interferon (IFN)-based therapy. In those analyses, correlation between amino acid substitutions and treatment response have been reported for the IFN sensitivity

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M. Miura and S. Maekawa have contributed equally to this study.

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determining region in nonstructural (NS)5A [2], a.a. 70 and 91 in core [3], the PKR/eIF-2alpha phosphorylation homology domain (PePHD) in envelope (E)2 [4], and the IFN–ribavirin resistance determining region (IRRDR) in NS5A [5].

Regarding the viral factors related to disease progression, among the various HCV proteins, the core protein has been thought widely to contribute because it has been shown experimentally to affect multiple cellular functions, in addition to the evidence from clinical studies [6–10]. Core protein modifies cellular apoptosis, oncogenic signaling, reactive oxygen species formation, lipid metabolism, transcriptional activation, transformation, and immune reactivity. Core protein has oncogenic potential in transgenic mice [11]. In contrast, fewer clinical studies to date have systematically investigated the correlation between the variability of HCV regions and disease progression. However, some of those limited clinical studies reported a correlation between amino acid substitutions in core or NS5A with disease progression [12–15]. Despite those reports, few studies to date support the correlation. Moreover, it is unclear whether those viral sequences change during disease progression or how the disease activity is modified by those viral sequences in the long course of chronic hepatitis.

On the other hand, regarding host factors, recent reports disclosed a significant correlation between polymorphisms in the IL28B gene and responses to pegylated-IFN plus ribavirin therapy for HCV patients [16–19]. This single-nucleotide polymorphism (SNP) also showed significant correlation with natural HCV clearance [20]. However, it remains unknown whether the IL28B SNP is related to disease progression or the development of HCC.

In this study, we first undertook the analysis to identify the viral regions related to disease progression and HCC development through the analysis of complete HCV open-reading frame (ORF) sequences. Because some regions in HCV core and NS5A showed characteristic changes over time in patients developing HCC during the observation period, we proceeded further to analyze the contribution of those regions to disease progression, in association with time and with the IL28B SNP.

## Patients and methods

### Patients

This study is based on the analysis of two groups of patients, 43 in Group 1 and 230 patients in Group 2.

In the first part, we tried to characterize and extract viral sequences specific to disease progression through the analysis of complete HCV ORFs (Fig. 1a). In particular, we focused our investigation on the changes in viral sequences over time in association with disease progression by comparing HCV sequences of two sufficiently distant time points. With this aim, we determined to investigate patients with a history of IFN therapy, because those patients often were followed long-term with preservation of old and recent sera. However, we excluded sustained

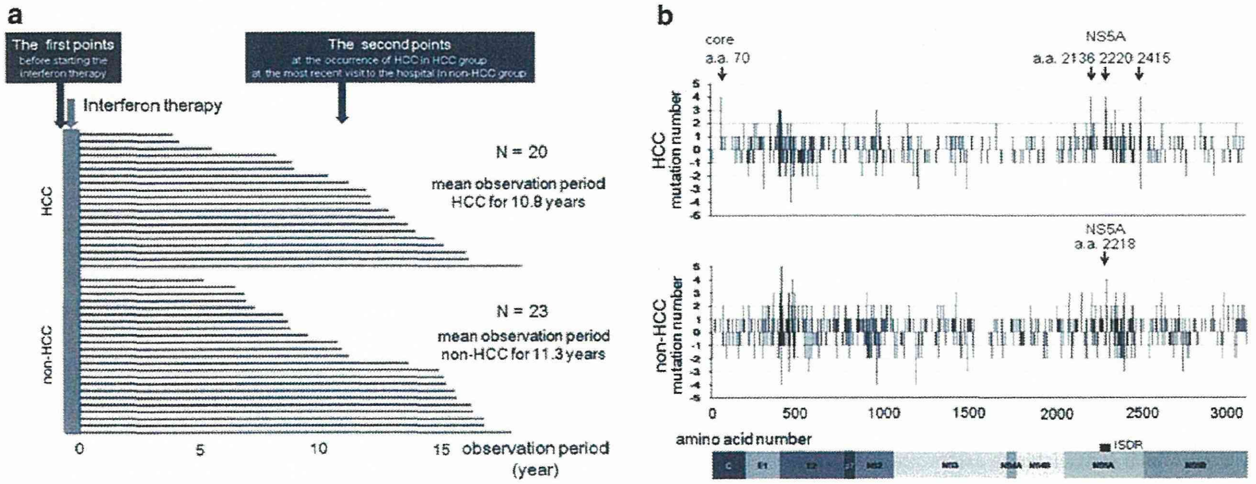

**Fig. 1** **a** A total of 43 patients were analyzed for complete HCV ORF sequences. They were all non-responders to the previous IFN therapy. Twenty patients developed HCC during the observation period, while the 23 patients did not. HCV ORF sequences were determined for the paired samples and the predicted amino acid changes were compared in each patient. **b** Specific HCV amino acid changes associated with disease progression was evaluated by the analysis of the full-length viral ORF during the observation period for each patient according to the following rules: *1* +1 point for consensus to non-consensus, *2* −1 point for non-consensus to consensus, *3* 0 point for non-consensus to non-consensus. These points were added together and are shown for HCC and non-HCC patients. Patient with later HCC development (*upper panel*). Non-HCC patients (*lower panel*)

virologic response (SVR) patients because we thought that the viral clearance leads to improvement of the liver disease and, therefore, viral regions influencing the IFN response would be extracted as affecting the course of disease. Between March 1992 and April 2004, 273 consecutive patients with HCV-1b infection were given IFN monotherapy at Yamanashi University Hospital, and 133 were followed long-term. A total of 65 patients showed SVR, while 68 showed non-SVR. Among these 68 non-SVRs, 43 patients were included in the study because laboratory data and sera were available from the two distant time points (Group 1). Twenty patients developed HCC during the observation period, while the remaining 23 did not. Regarding the sera, the first time point for both groups was before starting IFN therapy, while the second points were at the occurrence of HCC for the HCC group and at the most recent visit to the hospital for the non-HCC group (Fig. 1a).

An additional 230 HCV-1b patients were recruited (Group 2) as the second study group. They were mainly outpatients at Yamanashi University Hospital and were selected randomly from those with stored sera at the time of disease diagnosis. Sixty-five had HCC, while the remaining 165 did not. They all were positive for HCV RNA at the time of study, although 74 patients had a history of IFN therapy. Parts of the core and NS5A sequences were determined at HCC onset in 65 HCC patients and at the most recent visit to the hospital in 165 non-HCC patients. Because historical sera around 10 years before were also available for 55 of these 230 patients, HCV sequence analysis was also performed for core and NS5A at those previous time points in those patients.

From these two study groups, 228 patients (68 HCCs and 160 non-HCCs) with available genomic DNAs were examined to determine the IL28B SNP.

All the patients studied all fulfilled following criteria: (1) Negative for hepatitis B surface antigen. (2) No other forms of hepatitis, such as primary biliary cirrhosis, autoimmune liver disease, or alcoholic liver disease. (3) Free of coinfection with human immunodeficiency virus. (4) A signed consent was obtained for the study protocol that had been approved by Human Ethics Review Committee of Yamanashi University Hospital.

### Complete and partial HCV ORF sequence determination by direct sequencing from sera

HCV RNA extraction, complementary DNA synthesis and amplification by two-step nested PCR from serum samples were done using the specific primers for full HCV ORF or partial viral regions as described previously [15]. PCR amplicons were sequenced directly by Big Dye Terminator Version 3.1 (ABI, Tokyo, Japan) with universal M13 forward and reverse primers using an ABI prism 3130 sequencer (ABI). Generated sequence files were assembled using Vector NTI software (Invitrogen, Tokyo, Japan) and base-calling errors were corrected following inspection of the chromatogram.

### IL28B SNP analysis

Human genomic DNA was extracted from peripheral blood using a blood DNA extraction kit (QIAGEN, Tokyo, Japan) according to the manufacturer's protocol. The allele typing of each DNA sample was performed by real-time PCR with a model 7500 (ABI) using FAM-labeled SNP primer for the locus rs8099917 (ABI).

### Statistical analysis

Statistical differences in the parameters, including all available patients' demographic, biochemic, hematologic, and virologic data, were determined between different groups of patients by Student's *t* test for numerical variables and Fisher's exact probability test for categorical variables. Odds ratios and their 95% confidence intervals were used to quantify the level of association. All *p* values of <0.05 by the two-tailed test were considered significant throughout. Multiple logistic regression analyses were used to identify the independent variables influencing core a.a. 70 residue and HCC development. Because most variables used for the analyses were generally considered to correlate with the disease progression, we entered all the variables into the multiple logistic regression analysis even if some of them did not reach significant differences in individual univariate analysis.

## Results

### Comparing complete HCV amino acid sequences between patients with and without HCC

The clinical characteristics of the 43 patients (Group 1) analyzed for HCV ORF changes over time are shown in Table 1. At the start of observation, clinical characteristics did not differ significantly between the HCC group and the non-HCC group. The mean observation period was comparable between the two groups and was 10.8 years for the HCC group and 11.3 years for non-HCC group ($p = 0.745$). On the other hand, platelets ($p < 0.001$), albumin ($p < 0.001$), and AFP ($p = 0.001$) became significantly lower or higher in the HCC group at the end of observation (Table 1).

We proceeded to investigate viral amino acid changes during the course of disease in each patient to determine

**Table 1** Patient characteristics in Group 1

| | At the start of observation | | | At the end of observation | | |
|---|---|---|---|---|---|---|
| | HCC ($N = 20$) | Non-HCC ($N = 23$) | $p$ value | HCC ($N = 20$) | Non-HCC ($N = 23$) | $p$ value |
| Observation period (years) | | | | 10.8 ± 3.6 | 11.3 ± 3.8 | 0.745 |
| Sex (male/female) | 11/9 | 12/11 | 0.999 | 11/9 | 12/11 | 0.999 |
| Age (years) | 51.5 ± 8.0 | 50.0 ± 9.9 | 0.604 | 61.7 ± 10.0* | 61.0 ± 10.9* | 0.818 |
| Stage of fibrosis (F1/2/3/4) | 1/7/6/6 | 5/11/4/3 | 0.190 | N/A | N/A | – |
| AST (IU/L) | 102 ± 114 | 74 ± 40 | 0.695 | 71 ± 36* | 51 ± 30 | 0.048 |
| ALT (IU/L) | 124 ± 86 | 104 ± 71 | 0.411 | 69 ± 47* | 52 ± 31* | 0.159 |
| Platelets ($10^{-4}$/mm$^3$) | 16.2 ± 4.8 | 18.3 ± 6.2 | 0.217 | 9.7 ± 3.9* | 15.3 ± 5.1 | <0.001 |
| Albumin (g/dL) | 4.1 ± 0.4 | 4.1 ± 0.2 | 0.639 | 3.6 ± 0.4 | 4.1 ± 0.5 | <0.001 |
| γGTP (IU/L) | 90 ± 60 | 71 ± 46 | 0.275 | 69 ± 59 | 45 ± 38* | 0.114 |
| T.Chol (mg/dL) | 169 ± 28 | 156 ± 22 | 0.110 | 146 ± 21 | 164 ± 5,108 | 0.086 |
| Alpha-fetoprotein (ng/mL) | 10.5 ± 6.8 | 9.3 ± 10.8 | 0.695 | 42.4 ± 41.1 | 4.7 ± 2.7 | 0.001 |
| HCV RNA concentration (kIU/mL) | 706 ± 696 | 614 ± 1,181 | 0.760 | 3,325 ± 415* | 4,508 ± 5,108* | 0.426 |

* Factors with significant changes over time (<0.05)

whether specific amino acid changes related to disease progression could be identified. First, the consensus amino acid was determined at each amino acid position in the HCV ORF after determination of all sequences in these 43 patients. Amino acid changes were determined according to the following rules to highlight directional changes according to disease progression: When an amino acid changed from the consensus to the non-consensus during the observation period, we scored +1 point. Conversely, a change from the non-consensus to the consensus scored −1 point. We scored 0 point for a change from one non-consensus amino acid to another. As shown in Fig. 1b, directional amino acid changes were observed throughout the HCV genome to some degree both in patients with and without HCC development during the clinical course of almost 10 years, and frequent substitutions in E2 hypervariable region were common in both groups. On the other hand, in patients with HCC development, as many as four directional changes were observed at core a.a. 70 and at three amino acid positions of NS5A (Fig. 1b, upper panel). In contrast, in patients without HCC, the significant change ($n = 4$) was observed at a.a. 2,218 of NS5A when E2 hypervariable region was excluded (Fig. 1b, lower panel).

### Core and NS5A sequences in patients with and without HCC

Because the first analysis suggested that the patients with later HCC development might accumulate specific mutations in core and NS5A at the time of HCC occurrence, additional sequences were analyzed from 230 HCV-1b patients to confirm the result. The clinical backgrounds of the additional 230 patients are shown in Table 2 (Group 2).

**Table 2** Patient characteristics in Group 2

| | HCC ($N = 65$) | Non-HCC ($N = 165$) | $p$ value |
|---|---|---|---|
| Observation period (years) | | | |
| Sex (male/female) | 42/23 | 76/89 | 0.018 |
| Age (years) | 68.2 ± 9.2 | 62.4 ± 11.7 | <0.001 |
| AST (IU/L) | 66 ± 35 | 41 ± 21 | <0.001 |
| ALT (IU/L) | 67 ± 47 | 44 ± 47 | <0.001 |
| Platelets ($10^{-4}$/mm$^3$) | 11.3 ± 5.8 | 15.3 ± 6.2 | <0.001 |
| Albumin (g/dL) | 3.6 ± 0.5 | 4.4 ± 2.9 | 0.025 |
| γGTP (IU/L) | 59 ± 53 | 38 ± 40 | 0.001 |
| T.Chol (mg/dL) | 153 ± 30 | 165 ± 31 | 0.004 |
| Alpha-fetoprotein (ng/mL) | 302 ± 1,670 | 10 ± 25 | 0.025 |
| HCV RNA concentration (kIU/mL) | 5,400 ± 13,574 | 7,990 ± 8,512 | 0.104 |

All patients were positive for HCV RNA. Between the HCC (65 patients) and non-HCC (165 patients) groups, HCC patients were older ($p < 0.001$) and more frequently tended to be males ($p = 0.018$). Moreover, AST, ALT, γGTP, and AFP were significantly higher, and platelets, albumin, and cholesterol were significantly lower in the HCC group. Different predicted amino acids in the core and NS5A regions, between the two groups, are demonstrated in Fig. 2a. The ratio of the core a.a. 70Q (glutamine) or H (histidine) to R (arginine) was significantly higher with the existence of HCC as demonstrated in Fig. 2a (left panel). On the other hand, evident correlations were not confirmed between mutations in NS5A and disease progression (Fig. 2a, right panel). The ratio of Q or H

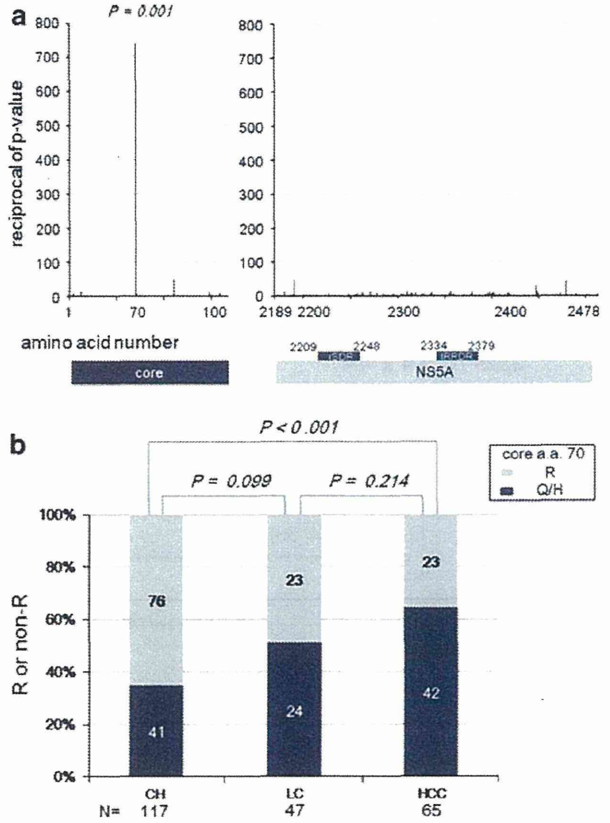

**Fig. 2** Core and NS5A sequences in additional patients were studied. **a** Using sera from 230 additional patients at the time of diagnosis, amino acid usage was compared between HCC and non-HCC patients for the part of core and the NS5A region, and this difference is shown as the *bar height* expressed as reciprocal *p* values. **b** In 230 patients, the association between polymorphisms of core a.a. 70 and the state of liver disease (chronic hepatitis, LC, or HCC) is shown

to R progressively increased in patients in the three major groups of disease activity: chronic hepatitis, cirrhosis, and HCC (Fig. 2b). The association between the disease progression and core a.a. 70 polymorphism also was observed irrespective of IFN-based therapy (data not shown).

## Changes in core a.a. 70 over time in patients with and without HCC

We then examined changes in core a.a. 70 over time in association with disease progression (Fig. 3). For this analysis, 55 patients from Group 2, for whom sera from two distant time points were available, were added to the 43 patients in Group 1 and a total of 98 patients were enrolled. When they were classified into two groups according to later HCC onset, the mean observation period was comparable between the groups, 10.4 years for the HCC group and 12 years for the non-HCC group. The occurrence of core a.a. 70Q was 61% (22/36) at the time of HCC onset in the HCC group (Fig. 3, upper panel), but 31% (19/62) for the non-HCC group (Fig. 3, lower panel). In contrast, it was 44% (16/36) at the start of observation in the HCC group (Fig. 3, upper panel) and 29% (18/62) for the non-HCC group (Fig. 3, lower panel). Regarding the core a.a. 70 changes over time, R was dominant throughout the observation period in the non-HCC group (71% at the start and 69% at the end), while the dominant amino acid changed from R (56%) to Q (61%) in the HCC group, so that the core a.a. 70 residues of 17% of the HCC patients changed from R to Q during the course of HCC development. In other words, 6 of 22 patients with 70Q at the onset of HCC had 70R originally (6/22, 27%), while 0 of 14 (0%) with 70R at the most recent observation time had 70Q at the beginning. There were no patients with core a.a. 70H throughout the observation period in this population. The result demonstrates that the relationship between 70Q and HCC development is significant at the time of HCC development. At the start of observation, there was also a tendency that the patients with 70Q compared with 70R develop HCC. However, this difference did not reach statistical significance as shown in Supplementary Fig. 1.

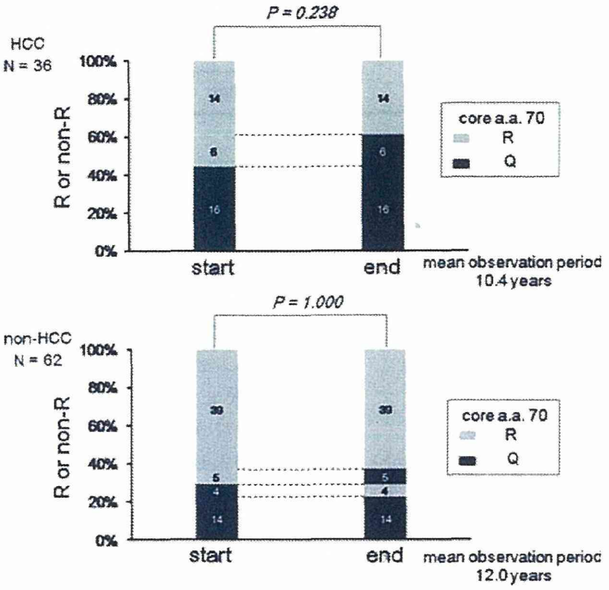

**Fig. 3** Changes in core a.a. 70 over time were studied. In 98 patients available for the analysis of 2, sufficiently distant time points, amino acid changes of core a.a. 70 were investigated over time in each patient group. Patients with later HCC development (*upper panel*). Non-HCC patients (*lower panel*)

## Core a.a. 70 changes over time and their association with disease progression

These 98 patients were classified into four groups according to the pattern of core a.a. 70 change (Table 3) and their

**Table 3** Progression of liver disease in 98 patients categorized by core a.a. 70 changes over time

| | R → R ($N = 53$) | | | Q → R ($N = 4$) | | |
|---|---|---|---|---|---|---|
| | Start | End | $p$ value | Start | End | $p$ value |
| HCC rate (HCC/non-HCC) | – | 26.4% (14/39) | – | – | 0% (0/4) | – |
| Sex (male/female) | – | 25/28 | – | – | 4/0 | – |
| Observation period (years) | – | 11.1 ± 3.4 | – | – | 12.9 ± 3.5 | – |
| Age (years) | 51.3 ± 11.7 | 62.4 ± 12.1 | <0.001 | 48.0 ± 11.6 | 61.0 ± 9.1 | 0.128 |
| AST (IU/L) | 68 ± 73 | 48 ± 26 | 0.066 | 56 ± 32 | 83 ± 61 | 0.456 |
| ALT (IU/L) | 80 ± 71 | 48 ± 35 | 0.003 | 114 ± 71 | 96 ± 42 | 0.678 |
| Platelets ($10^{-4}/\text{mm}^3$) | 17.0 ± 5.8 | 15.0 ± 6.7 | 0.104 | 21.3 ± 3.9 | 17.2 ± 5.2 | 0.251 |
| Albumin (g/dL) | 4.1 ± 0.4 | 3.9 ± 0.6 | 0.225 | 4.4 ± 0.4 | 4.3 ± 0.4 | 0.647 |
| $\gamma$GTP (IU/L) | 56 ± 51 | 38 ± 40 | 0.052 | 95 ± 51 | 61 ± 46 | 0.371 |
| T.Chol (mg/dL) | 172 ± 36 | 158 ± 33 | 0.032 | 152 ± 14 | 175 ± 32 | 0.222 |
| Alpha-fetoprotein (ng/mL) | 8.3 ± 9.5 | 12.5 ± 22.1 | 0.202 | 6.0 ± 6.0 | 5.2 ± 2.2 | 0.816 |
| HCV RNA concentration (kIU/mL) | 4,634 ± 8,509 | 7,070 ± 14,159 | 0.291 | 5,798 ± 7,970 | 13,676 ± 1,881 | 0.162 |
| | R → Q ($N = 11$) | | | Q → Q ($N = 30$) | | |
| | Start | End | $p$ value | Start | End | $p$ value |
| HCC rate (HCC/non-HCC) | | 54.5% (6/5) | | | 53.3% (16/14) | |
| Sex (male/female) | | 6/5 | | | 13/17 | |
| Observation period (years) | | 13.7 ± 1.65 | | | 10.8 ± 3.5 | |
| Age (years) | 56.4 ± 7.5 | 69.3 ± 9.3 | 0.002 | 54.6 ± 8.5 | 64.9 ± 9.9 | <0.001 |
| AST (IU/L) | 62 ± 47 | 46 ± 12 | 0.285 | 79 ± 51 | 60 ± 31 | 0.087 |
| ALT (IU/L) | 100 ± 69 | 37 ± 15 | 0.008 | 95 ± 58 | 59 ± 36 | 0.006 |
| Platelets ($10^{-4}/\text{mm}^3$) | 17.7 ± 3.9 | 11.8 ± 4.8 | 0.005 | 16.3 ± 6.5 | 11.9 ± 5.6 | 0.007 |
| Albumin (g/dL) | 4.2 ± 0.2 | 3.8 ± 0.4 | 0.005 | 4.1 ± 0.3 | 3.8 ± 0.5 | 0.009 |
| $\gamma$GTP (IU/L) | 73 ± 53 | 33 ± 16 | 0.025 | 101 ± 55 | 71 ± 65 | 0.065 |
| T.Chol (mg/dL) | 157 ± 21 | 144 ± 27 | 0.245 | 163 ± 28 | 150 ± 32 | 0.100 |
| Alpha-fetoprotein (ng/mL) | 7.1 ± 4.3 | 97.8 ± 63.6 | 0.267 | 20.8 ± 50.0 | 35.1 ± 54.7 | 0.295 |
| HCV RNA concentration (kIU/mL) | 2,415 ± 3,163 | 2,349 ± 1,851 | 0.957 | 2,869 ± 3,984 | 3,229 ± 4,026 | 0.731 |

clinical characteristics were investigated. Significant decreases of platelets ($p = 0.007$) and albumin ($p = 0.009$) were observed in the Q unchanged group during the observation period, but not in the R unchanged group ($p = 0.104$ and 0.225, respectively). Because platelets and albumin are markers of liver disease progression, it was considered that the Q unchanged group progressed rapidly with frequent HCC occurrence (53%, 16/30) while the R unchanged group showed stable disease with less frequent HCC occurrence (26%, 14/53). In contrast, the R to Q group showed progressive disease ($p = 0.005$ and 0.005, respectively) similar to the Q unchanged group, while the Q to R group showed stable disease similar to the R unchanged group ($p = 0.251$ and 0.647, respectively), demonstrating that amino acid changes of core a.a. 70 were significantly associated with disease progression.

### The IL28B SNP and its association with core a.a. 70 and disease progression

Next, the association between the state of liver disease and IL28B SNP was analyzed for a total of 228 patients through the analysis of the rs8099917 locus. Among them, 162 patients (71%) had the major homozygous TT alleles, while 66 patients (29%) had the minor homozygous or heterozygous alleles (GG/TG). Although some patients had a history of IFN therapy, all patients were positive for HCV RNA at the time of study. The clinical characteristics related to disease progression were compared, as shown in Table 4. Each group consisted of patients with similar distributions of age and sex. Though most clinical factors showed no evident differences in these groups, $\gamma$GTP was high ($p = 0.020$) and HCV RNA concentration was apt to be low ($p = 0.085$) in TG/GG group. Moreover, the ratio

**Table 4** Patient characteristics classified by IL28B SNPs at the time of diagnosis

| | TT ($N = 162$) | TG/GG ($N = 66$) | $p$ value |
|---|---|---|---|
| Sex (male/female) | 81/81 | 32/34 | 0.951 |
| Age (years) | 63.3 ± 10.7 | 63.8 ± 11.9 | 0.735 |
| Platelets ($10^{-4}$/mm$^3$) | 13.8 ± 5.8 | 14.3 ± 6.6 | 0.632 |
| Albumin (g/dL) | 4.2 ± 3.0 | 4.0 ± 0.5 | 0.528 |
| $\gamma$GTP (IU/L) | 41 ± 39 | 55 ± 49 | 0.020 |
| T.Chol (mg/dl) | 162 ± 31 | 157 ± 31 | 0.237 |
| HCV RNA concentration (kIU/ml) | 7,576 ± 10,292 | 5,069 ± 6,701 | 0.085 |
| Alpha-fetoprotein (ng/ml) | 39.0 ± 152.2 | 27.3 ± 48.3 | 0.555 |
| AST (IU/L) | 49.6 ± 29.6 | 51.9 ± 30.5 | 0.607 |
| ALT (IU/IL) | 54.5 ± 53.9 | 51.6 ± 37.7 | 0.689 |
| Core a.a. 70R/(Q/H) | 106/56 | 17/49 | <0.001 |
| Core a.a. 91L/(M/C) | 108/54 | 38/28 | 0.253 |
| ISDR | 1.2 ± 2.0 | 0.9 ± 1.5 | 0.164 |
| IRRDR | 5.1 ± 2.4 | 4.7 ± 2.2 | 0.207 |
| HCC −/+ | 115/47 | 45/21 | 0.788 |
| IFN −/+ | 95/67 | 34/32 | 0.402 |

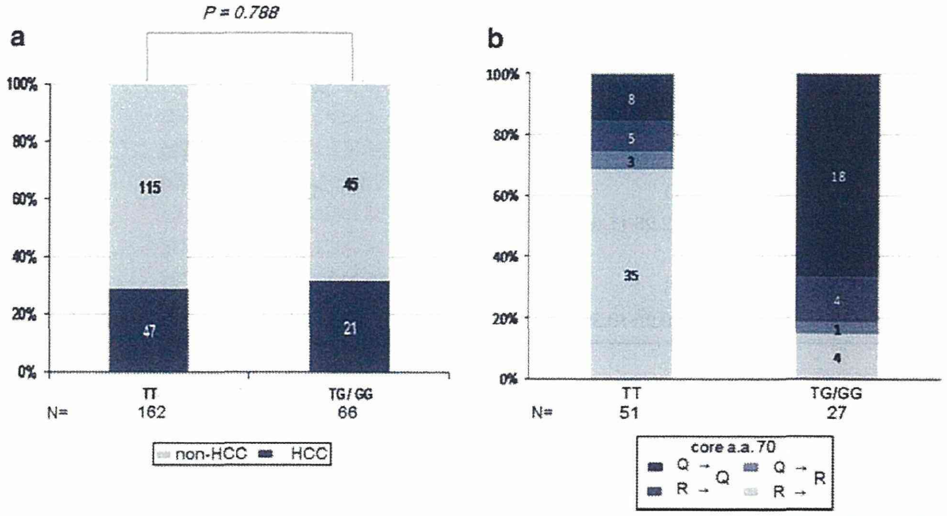

**Fig. 4** **a** Association between the state of liver disease and IL28B SNP. **b** Time-dependent core a.a. 70 changes and its relation to IL28B SNP was investigated in 78 patients

of R/(Q/H) at core a.a. 70 was significantly higher in those with the TT alleles than in those with TG/GG ($p < 0.001$). In association of IL28B SNP with HCC development, there was no evident relationship as demonstrated in Fig. 4.

### IL28B SNP and time-dependent core a.a. 70 changes

In Fig. 4b, it is demonstrated that the direction of time-dependent core a.a. 70 change was influenced by IL28B SNPs. In IL28B TG/GG patients, 4 (50%) out of 8 patients with the initial core a.a. 70R changed into 70Q, while only 1 (5%) out of 19 patients with the initial core a.a. 70Q changed into 70R, demonstrating that core a.a. 70 tended to change into Q over time in IL28B TG/GG patients ($p = 0.034$). On the other hand, there was no evident changing direction in IL28B TT patients; 5 (13%) out of 40 patients with the initial core a.a. 70R changed into 70Q, while 3 (27%) out of 11 patients with the initial core a.a. 70Q changed into 70R ($p = 0.45$).

### Multivariate analysis for independent factors influencing core a.a. 70

To investigate further the relationship between core a.a. 70, the IL28B SNP, and HCC development, we divided the patients according to the specification of core a.a. 70 and

**Table 5** Factors related to polymorphism of core a.a. 70

| Variables | Univariate analysis ($N = 228$) | | Multivariate analysis ($N = 228$) | |
|---|---|---|---|---|
| | Odds ratio (95% CI) | $p$ value | Odds ratio (95% CI) | $p$ value |
| Sex | | | | |
| Female | 1 | 0.415 | 1 | 0.812 |
| Male | 1.23 (0.74–2.01) | | 1.08 (0.58–1.99) | |
| Age (years) | | | | |
| <65 | 1 | 0.216 | 1 | 0.855 |
| ≥65 | 1.39 (0.82–2.35) | | 1.06 (0.57–1.96) | |
| Platelets ($10^{-4}$/mm$^3$) | | | | |
| >12 | 1 | 0.004 | 1 | 0.844 |
| ≤12 | 1.76 (1.03–2.99) | | 1.07 (0.53–2.16) | |
| Albumin (g/dL) | | | | |
| >4 | 1 | 0.002 | 1 | 0.300 |
| ≤4 | 2.28 (1.33–3.91) | | 1.46 (0.71–3.00) | |
| γGTP (IU/L) | | | | |
| <41 | 1 | 0.003 | 1 | 0.299 |
| ≥41 | 2.32 (1.32–4.09) | | 1.42 (0.73–2.79) | |
| ALT (IU/L) | | | | |
| <41 | 1 | 0.040 | 1 | 0.573 |
| ≥41 | 1.74 (1.03–2.94) | | 1.22 (0.62–2.39) | |
| IL28B | | | | |
| TT | 1 | <0.001 | 1 | <0.001 |
| TG or GG | 5.46 (2.88–10.30) | | 5.74 (2.91–11.31) | |
| HCC | | | | |
| − | 1 | <0.001 | 1 | 0.046 |
| + | 2.98 (1.65–5.37) | | 2.21 (1.01–4.83) | |
| Previous IFN therapy | | | | |
| − | 1 | 0.874 | 1 | 0.644 |
| + | 0.96 (0.57–1.62) | | 0.87 (0.47–1.59) | |

those factors, as well as clinical factors, were compared in univariate and multivariate analyses. In Table 5, it may be seen that platelets, albumin, γGTP, ALT, the IL28B SNP, and number of patients with HCC development differed significantly between the two groups in univariate analysis. In contrast, successive multivariate analysis demonstrated that the number of patients with HCC development ($p = 0.046$) and the IL28B SNP ($p < 0.001$) were extracted as independent variables correlated with the core a.a. 70 residue (Table 5).

### Multivariate analysis for independent factors influencing HCC development

To disclose factors influencing HCC development, multivariate analysis was performed. As shown in Table 6, age, albumin, and core a.a. 70 residue were extracted as independent factors. On the other hand, IL28B SNP was not extracted as one of those factors.

## Discussion

In this study, we have documented several important findings. Through the investigation of HCV sequences, including complete HCV ORFs analysis, we have shown that the core a.a. 70 residue and its changes over time are associated with the disease progression as well as HCC development in genotype-1b HCV infection. Specifically, core a.a. 70Q/H was associated with HCC development and disease progression; core a.a. 70 often changed with time and R70Q substitutions were associated with progressive disease, while Q70R substitutions were associated with the stable disease. Moreover, we have shown that the IL28B SNP and core a.a. 70 showed significant linkage. In contrast, we have also shown that HCC development and disease progression were not apparently correlated with the IL28B SNP.

Recently, core amino acids have been reported in several studies to be associated with HCC [12, 21–25]. In

**Table 6** Factors related to influencing HCC development

| Variables | Univariate analysis ($N = 228$) | | Multivariate analysis ($N = 228$) | |
|---|---|---|---|---|
| | Odds ratio (95% CI) | $p$ value | Odds ratio (95% CI) | $p$ value |
| Sex | | | | |
| Female | 1 | 0.161 | 1 | 0.190 |
| Male | 1.50 (0.85–2.67) | | 1.69 (0.77–3.71) | |
| Age (years) | | | | |
| <65 | 1 | 0.006 | 1 | 0.004 |
| ≥65 | 2.30 (1.28–4.16) | | 3.26 (1.46–7.25) | |
| Platelets ($10^{-4}$/mm$^3$) | | | | |
| >12 | 1 | <0.001 | 1 | 0.021 |
| ≤12 | 5.82 (3.11–10.88) | | 2.59 (1.16–5.82) | |
| Albumin (g/dL) | | | | |
| >4 | 1 | <0.001 | 1 | <0.001 |
| ≤4 | 13.75 (6.69–28.24) | | 7.73 (3.53–16.94) | |
| γGTP (IU/L) | | | | |
| <41 | 1 | <0.001 | 1 | 0.122 |
| ≥41 | 3.09 (1.70–5.62) | | 1.87 (0.85–4.13) | |
| ALT (IU/L) | | | | |
| <41 | 1 | <0.001 | 1 | 0.109 |
| ≥41 | 3.88 (2.06–7.31) | | 1.98 (0.86–4.56) | |
| IL28B | | | | |
| TT | 1 | 0.626 | 1 | 0.290 |
| TG or GG | 1.17 (0.13–2.17) | | 0.63 (0.27–1.49) | |
| Core a.a. 70 | | | | |
| R | 1 | <0.001 | 1 | 0.029 |
| Q/H | 2.91 (1.61–5.26) | | 2.44 (1.09–5.44) | |
| Previous IFN therapy | | | | |
| – | 1 | 0.949 | 1 | 0.331 |
| + | 0.98 (0.55–1.74) | | 1.46 (0.68–3.16) | |

these studies, patients with core a.a. 70Q/H frequently developed HCC with exacerbation of liver damage. In this analysis, we confirmed the previous findings. However, because this association might be a reflection of the core-dependent IFN sensitivity differences often reported in recent studies [12, 22, 25], we restricted the analysis to patients, who were unable to clear HCV RNA previously through IFN-based therapy. Moreover, we also confirmed the relationship of the core sequences and disease development among the populations without a previous history of IFN therapy (data not shown). These findings strongly confirmed the role of core a.a. 70 in disease progression, independent of any IFN response.

It is a focus of interest how the core sequence evolves with time or with the course of disease. If the core sequences were fixed throughout the course of disease, HCV with core 70Q might be an "oncogenic" virus, while HCV with core 70R might be "non-oncogenic", and the initial viral sequence might forecast future liver disease. In this study, we have demonstrated that core sequences changed in 15% (15/98) of patients during the observation period of around 10 years. Among these changes, R70Q ($N = 11$) was more common than Q70R ($N = 4$). Interestingly, changes in this region were significantly associated with disease activity or HCC development, although patients with R70Q substitutions were significantly more likely to have exacerbation of the disease and Q70R substitutions were associated with the stable disease. These results demonstrate that the core a.a. 70 residue is not fixed, but often changes with time during the course of disease in close association with disease progression and HCC development. Although the molecular mechanism of their interaction needs further exploration, this result highlights the important clinical and basic implications for the association between host and virus.

The importance of the IL28B SNP has been demonstrated recently in HCV infection in terms of a correlation with treatment outcome of pegylated-IFN plus ribavirin therapy [16–19]. The contribution of the IL28B SNP to the outcome of therapy was confirmed in successive studies, although the mechanism remains under investigation. On this basis, we sought to investigate the impact of the IL28B SNP on disease progression and HCC development, separate from the IFN-based treatment response. As shown in Table 4, we compared the clinical features between the two groups (IL28B GG/TG vs. IL28B TT). Importantly, this comparison disclosed a significant correlation between the core a.a. 70 polymorphisms and the IL28B SNP ($p < 0.001$) and confirmed the existence of a complex interaction between the host and the virus in chronic HCV infection. According to the result, patients with IL28B TG/GG were more likely to be infected with HCV with core a.a. 70Q/H than with core a.a. 70R and vice versa. Although the molecular mechanisms of their relationship remain unknown, it could be speculated that the IL28B SNP has an influence on the viral core sequences, because the host IL28B SNP remains fixed and cannot be influenced by the viral core sequence.

On the other hand, we observed no evident association between the IL28B SNP and HCC development. This was rather unexpected because it is considered that the IL28B SNP has a significant influence on the core a.a. 70 residue. Therefore, to clarify the correlation among core a.a. 70, IL28B, and HCC development, we undertook multivariate analysis to extract the independent variables affecting the core 70 residue. As demonstrated in Table 5, the IL28B SNP and the development of HCC were extracted as variables independently correlated with the core a.a. 70 residue. The result indicates that the core a.a. 70 residue was not only influenced by the IL28B SNP, but also by factors strongly related to HCC development, independent of the IL28B SNP. When considering the result, it is not strange if there is no direct relationship between IL28B SNP and HCC development. In contrast, multivariate analysis undertaken for disclosing factors influencing HCC development revealed that core a.a. 70 residue was a variable independently associated with HCC development other than age, albumin, or platelets even though the IL28B SNP was not extracted (Table 6). However, further comprehensive studies are warranted to disclose the molecular mechanisms for the complicated relationships among core a.a. 70, IL28B, and HCC development.

In conclusion, we have shown that core a.a. 70 was closely associated with disease progression and, often, changes of that residue were accompanied by temporal changes in liver damage, in close relationship with the IL28B SNP.

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ORIGINAL ARTICLE

# Characterization of naturally occurring protease inhibitor-resistance mutations in genotype 1b hepatitis C virus patients

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**Abstract**

*Background and aims* Protease inhibitor (PI)-resistant hepatitis C virus (HCV) variants may be present in substantial numbers in PI-untreated patients according to recent reports. However, influence of these viruses in the clinical course of chronic hepatitis C has not been well characterized.

*Methods* The dominant HCV nonstructural 3 (NS3) amino acid sequences were determined in 261 HCV genotype 1b-infected Japanese patients before pegylated interferon plus ribavirin (PEG-IFN/RBV) therapy, and investigated the patients' clinical characteristics as well as treatment responses including sustained virological response (SVR) rate. HCV-NS3 sequences were also determined in 39 non-SVR patients after completion of the therapy.

*Results* Four single mutations (T54S, Q80K, I153V, and D168E) known to confer PI resistance were found in 35 of 261 patients (13.4%), and double mutations (I153V plus T54S/D168E) were found in 6 patients (2.3%). Responses to PEG-IFN/RBV therapy did not differ between patients with and without PI-resistance mutations (mutation group, SVR 48%; wild-type group, SVR 40%; $P = 0.38$). On the other hand, two mutations appeared in two non-SVR patients after PEG-IFN/RBV therapy (I153V and E168D, 5.1%).

*Conclusions* PI-resistance-associated NS3 mutations exist in a substantial proportion of untreated HCV-1b-infected patients. The impact of these mutations in the treatment of PIs is unclear, but clinicians should pay attention to avoid further development of PI resistance.

**Keywords** HCV · Protease inhibitor · Naturally occurring viral resistance mutations

## Introduction

Hepatitis C virus (HCV) infects more than 170 million persons worldwide and thus represents a global health problem. At least 130 million infected individuals are chronic carriers of HCV and are at significant risk of developing liver cirrhosis and hepatocellular carcinoma [1]. The current standard treatment with pegylated interferon plus ribavirin (PEG-IFN/RBV) is complicated by frequent adverse reactions, and a sustained virologic response (SVR) can be achieved only in 50% of patients infected with the most prevalent genotype 1 [2]. In Japan, since 70% of patients are infected with intractable genotype 1b HCV, more effective treatments are urgently required.

A promising approach is the development of specifically targeted antiviral therapies for hepatitis C (STAT-C). HCV-specific protease inhibitors (PIs) target an essential step in HCV replication by blocking the nonstructural 3/4A (NS3/4A) protease-dependent cleavage of the HCV polyprotein

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[1]. Among these NS3/4A PIs, telaprevir, boceprevir, SCH446211, danoprevir (ITMN-191), nalaprevir (SCH900 518), and TMC435 are now under clinical trials [1, 3–7]. In PROVE1 and PROVE2 studies [3, 4] undertaken in North America and Europe, the SVR rate was favorable (67 and 69%, respectively) in a triple therapy regimen including telaprevir. In addition, some studies have suggested that shortening of treatment duration may be possible for patients who achieve a rapid virologic response (RVR) [8, 9].

However the sole use of STAT-C drugs, such as PIs, promotes production and selection of drug-resistant variants in patients experiencing viral rebound during treatment [3, 10, 11] as well as in HCV replicon experiments [11, 12]. Therefore, these drugs should be used in combination with the PEG-IFN/RBV to prevent the appearance of drug-resistant variants. However, Kuntzen et al. [13] demonstrated the presence of these drug-resistant variants in high frequencies (8.6–16.2%) by population-based sequencing in patients not treated with the drugs [1, 13]. Gaudieri et al. [14] have suggested that regions of NS3 protease and NS5B polymerase are likely to be under HLA immune pressure and therapeutic selection, and that drug-resistant variants may occur naturally to escape the immune system. These observations seem quite astonishing and troubling, since a substantial number of patients may not respond to the new therapies such as STAT-C drugs.

In the present study, to assess the prevalence of NS3 mutations conferring PI resistance in HCV genotype 1b-infected Japanese patients who had not been previously treated with PIs, as well as to assess the influence of those mutations in response to PEG-IFN/RBV therapy, the dominant HCV-NS3 sequences were determined in 261 HCV-1b patients before starting the PEG-IFN/RBV therapy.

## Methods

### Patients

Serum samples were acquired from 261 HCV genotype 1b-infected adult Japanese patients before combination therapy with PEG-IFN (PEGINTRON®, Schering-Plough, Tokyo, Japan) plus RBV (REBETOL®, Schering-Plough) between 2004 and 2008 at the University of Yamanashi, Musashino Red Cross Hospital and Kanazawa University. The therapy was administered according to the standard PEG-IFN/RBV treatment protocol established for Japanese patients by a hepatitis study group of the Ministry of Health, Labor, and Welfare, Japan. Specifically, the patients were subcutaneously administered PEG-IFNα-2b, 1.5 μg/kg body weight, once weekly and RBV 600–800 mg daily per os for 48 weeks. These patients were not infected with human immunodeficiency virus (HIV). The study was approved by the ethics committees of all participating universities and the hospital, and the protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in a priori approval by the Institutional Review Board at Massachusetts General Hospital. Written informed consent was obtained from each study participant.

### Amplification and sequencing of full-length HCV genomes

Viral loads were determined using the Amplicor HCV RNA kit, version 2.0 (Roche Diagnostics, Tokyo, Japan) or the Cobas TaqMan test (Roche Diagnostics). HCV RNA was extracted from pretreatment serum samples by the AGPC method using Isogen (Wako, Osaka, Japan) according to the manufacturer's protocol. Complementary DNA was synthesised using Superscript II (Invitrogen, Tokyo, Japan) and random primers (Invitrogen), and then amplified by two-step nested PCR using the primers listed in Supplementary Table 1. All samples were initially denatured at 95°C for 7 min, followed by 40 cycles of amplification with denaturation at 95°C for 15 s, annealing at 55°C for 15 s, and extension at 72°C for 45 s using the BD Advantage™ 2 PCR Enzyme system (BD Biosciences Clontech, CA, USA). PCR amplicons were directly sequenced using BigDye Terminator version 3.1 (ABI, Tokyo, Japan) and universal M13 forward/reverse primers using an ABI prism 3130 sequencer (ABI).

### Sequence alignment and analysis

Sequences were determined in both directions, particularly for the ambiguous stretches, were assembled using the Vector NTI software (Invitrogen), and base-calling errors were corrected following the inspection of chromatograms. If mixed bases were detected as two different chromatogram peaks at the same residue, only the dominant base was called after evaluation of all overlapping fragments. A consensus sequence was generated from the alignment on the basis of the most common amino acid at each site.

### Determination of PI resistance mutations

Multiple viral NS3 mutations were observed in amino acid positions reported to confer PI resistance among 261 patients: V36, Q41, F43, T54, V55, Q80, R109, I153, R155, A156, D168, V170, and M175. NS3 amino acid mutations with proven PI resistance in previously published studies (Table 1) were designated as resistance proven mutations (e.g., V36M/A). Mutations in the PI-resistance site not known to confer drug resistance were designated resistance unproven mutations (e.g., V36I). Patients were allocated to two groups according to the presence of PI-resistance mutations (including resistance unproven mutations), and clinical characteristics including HCV RNA levels and responses to PEG-IFN/RBV therapy were compared. To assess the influence of PEG-IFN/RBV therapy on NS3 mutational status, posttreatment HCV-NS3 sequences in 39 of 58 non-SVR patients were also examined.

### Statistical analysis

Statistical differences in the data, including all available patients' demographic, biochemic, hematologic, and virologic data such as sequence variation factors, were determined among the various groups by Student's *t* test or Mann–Whitney *U* test for numerical variables and Fisher's exact probability test for categorical variables.

## Results

### Prevalence of dominant PI-resistance-associated nonstructural 3 mutations in untreated patients

Figure 1 shows the frequency of substitutions in 261 patients for each of 181 NS3 protease amino acid residues compared to the consensus sequence. A total of 41 resistance proven mutations were detected in 35 (13.4%) patients: T54S (14 patients, 5.4%), Q80K (1 patient, 0.4%), I153V (22 patients, 8.4%), D168E (4 patients, 1.5%), T54S plus I153V double mutation (4 patients, 1.5%), and I153V plus D168E double mutation (2 patients, 0.8%). The mutation number increased to 54 in 47 (18.0%) patients when resistance unproven mutations were included: V36I (2 patients, 0.8%), I153L (11 patients, 4.2%), and I153V plus V36I double mutation (2 patients, 1.5%). Double mutations were found in 7 patients (2.7%) (Table 1). Q80L was observed in 47 (18%) patients but these were excluded from consideration because a previous study demonstrated that this mutation does not confer resistance [15]. All mutations observed in this study would confer low- to moderate-level PI resistance according to previous studies [6, 15–19]. No mutations conferring high-level resistance such as R155 or A156 [11, 17, 19–22] were observed.

### Clinical characteristics of patients with PI-resistance mutations

Table 2 presents the characteristics of patients classified according to the presence of PI-resistance mutations

**Table 1** Prevalence of PI-resistance-associated NS3 mutations

| Drug-resistance mutations described in the literature | | | | Detected resistance mutations |
|---|---|---|---|---|
| NS3 residue | Resistance mutations | Drugs | References | Genotype 1b (*N* = 261), (%) |
| V36 | A, M, L, G, C | Telaprevir, Boceprevir | [1, 3, 4, 10, 11, 19, 31, 37] | I × 2 (0.8) |
| Q41 | R | ITMN-191, Boceprevir | [19] | |
| F43 | S, C | ITMN-191, Boceprevir, Telaprevir, TMC435 | [15, 19] | |
| T54 | A, S | Telaprevir, Boceprevir, SCH900518 | [1, 3, 10, 11, 19, 20, 31, 38] | **S × 14 (5.4)** |
| V55 | A | Boceprevir | [1] | |
| Q80 | R, K | TMC435 | [6, 15] | **K × 1 (0.4)** |
| R109 | K | SCH446211 | [17] | |
| I153 | V | SCH446211 | [17] | **V × 22 (8.4)**, L ×11 (4.2) |
| R155 | K, T, I, M, G, L, S, Q | Telaprevir, Boceprevir, ITMN-191, BILN2061, TMC435 | [1, 3, 4, 6, 10, 11, 15, 19, 20] | |
| A156 | S, T, V, I, G | Telaprevir, Boceprevir, ITMN-191, BILN2061, SCH446211, TMC435, SCH900518 | [1, 3, 4, 10, 11, 15, 17, 19, 20, 38] | |
| D168 | A, V, E, N, T, H | BILN2061, ITMN-191, TMC435 | [6, 15, 20] | **E × 4 (1.5)** |
| V170 | A | Telaprevir, Boceprevir | [1, 19, 20] | |
| M175 | L | Boceprevir | [39] | |
| Total number (%) of patients with resistance proven mutations | | | | 35 (13.4) |
| Total number (%) of patients with resistance proven and unproven mutations | | | | 47 (18.0) |

Amino acid mutations conferring PI resistance in the literatures and those observed in PI-treatment-naive patients in this study are indicated. Bold indicates resistance proven mutations, and the others indicate resistance unproven mutations

Double mutations found were as follows: V36I and I153V × 1, T54S and I153V × 4, I153V and D168E × 2 (including resistance unproven mutations). Age, sex ratio, body mass index, alanine aminotransferase (ALT) levels, serum albumin, platelet count, and fibrosis stage did not differ between the NS3 mutation and wild-type groups. No significant difference was observed between the two groups in the parameters of PEG-IFN/RBV treatment response, HCV sequence variations in interferon sensitivity determining region (ISDR), Core 70, interferon plus ribavirin resistance-determining region (IRRDR), or interleukin 28B (IL28B) single nucleotide polymorphism (SNP) (rs8099917; T/G and G/G vs. T/T) [23–30]. These clinical variables were also compared between the mutation group defined as resistance proven mutations and the wild-type group, but no notable differences were observed.

### Unimpaired in vivo fitness of viral strains with resistance mutations

Because most PI-resistance mutations described till date have been associated with reduced replicative capacity of varying degrees [1, 10, 11, 13, 17, 20–22, 31, 32], we examined viral replication levels in patients with drug-resistance mutations (Fig. 2). The estimated *P* value indicated no significant difference between the mutation (median 1,500 KIU/ml) and wild-type (median 1,800 KIU/ml) groups ($P = 0.69$). The results indicate that drug-resistant HCVs were not necessarily impaired in their ability to replicate in vivo. However, patients with double mutations ($N = 7$) tended to have low viral loads (median 1,200 KIU/ml) ($P = 0.09$).

### Resistance mutations and virologic response to PEG-IFN/RBV therapy

To determine the difference in virologic response to PEG-IFN/RBV therapy according to the PI mutation, frequency of HCV RNA levels below detection at 4 weeks (rapid viral response, RVR) and 12 weeks (complete early viral response, cEVR), and SVR rate (%) were investigated in each group. The frequency of HCV RNA levels below detection at 4 and 12 weeks was 14 and 50%, respectively, in the mutation group, and was 11 and 46%, respectively, in the wild-type group. The SVR rate was 48 and 40% in the mutation and wild-type groups, respectively ($P = 0.38$). No significant difference was observed between the two groups in any of the indexes investigated (Table 2). The time-dependent viral clearance rate during PEG-IFN/RBV therapy was estimated in 133 patients including 25 patients (19%) with PI-resistance mutations available for the analysis. Kaplan–Meier analysis demonstrated that HCV clearance did not differ between the two groups with and without resistance mutations (log-rank test, $P = 0.30$) (Fig. 3).

### Changes in nonstructural 3 amino acid sequence diversity during PEG-IFN/RBV therapy

Full-length NS3 protease sequences were determined in 39 non-SVR patients after PEG-IFN/RBV therapy. A single amino acid change at resistance-associated sites in two patients was observed. In one patient, isoleucine (Ile) at position 153 changed to valine (Val), and glutamic acid (Glu) changed to aspartic acid (Asp) at position 168 in the second (Fig. 4). At the nucleotide level, ATC (Ile) changed to GTC (Val) in I153V, and GAA (Glu) changed to GAC (Asp) in E168D. Both mutations were caused by one nucleotide exchange. No other changes were observed in the other 37 patients.

## Discussion

Here we report that in 18% (47/261) HCV genotype 1b-infected patients who had not been previously treated with NS3 PIs, the viral genome contained dominant amino acid mutations within the NS3 PI-resistance sites. Even after confining the data to established PI-resistance mutations, the mutation rate was still significant in 13.4% (35/261). No clinical differences were observed between patients

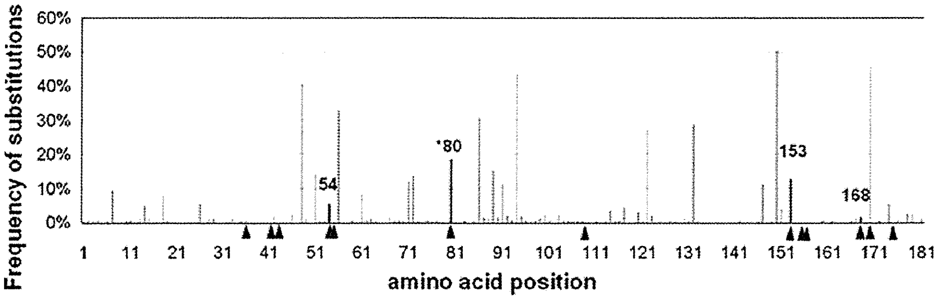

**Fig. 1** Frequency of polymorphic mutations for each of the 181 NS3 protease amino acid residues in 261 patients. *Arrowheads* indicate the sites reported to confer PI resistance. *Dark bars* denote the amino acid variations at the resistant sites in this study. **80*, we detected one resistant mutation (Q80K) and 47 (18%) non-resistant variations (Q80L) at the 80th residue

**Table 2** Characteristics of patients with or without HCV genomes harboring drug-resistance mutations

| Characteristics | Mutation type ($N = 47$) | Wild-type ($N = 214$) | $P$ value |
|---|---|---|---|
| Patients' characteristics | | | |
| Age, median (range) | 59 (46–72) | 57 (19–77) | 0.17 |
| Male, no. (%) | 26 (55) | 112 (52) | 0.70 |
| BMI, median (range) | 23.2 (15.5–31.9) | 22.8 (16.1–31.9) | 0.41 |
| ALT IU/ml | 81.3 ± 72.6[a] | 74.8 ± 51.9 | 0.93 |
| Serum albumin g/dl | 4.00 ± 0.37 | 4.01 ± 0.36 | 0.81 |
| Platelet count × $10^4$/μl | 15.8 ± 4.3 | 14.5 ± 4.8 | 0.18 |
| HCV RNA KIU/ml, median (range) | 1,500 (58–6,310) | 1800 (28–15,849) | 0.69 |
| Fibrosis, no. (%) | | | 0.97 |
| F0 | 0 (0) | 7 (3) | |
| F1 | 23 (50) | 89 (42) | |
| F2 | 9 (20) | 52 (24) | |
| F3 | 9 (20) | 40 (19) | |
| F4 | 5 (11) | 26 (12) | |
| IFN pre-treatment no. (%) | 15/40 (38)[b] | 66/172 (38) | 1.00 |
| IL28B (rs8099917) T/G or G/G no. (%) | 6/20 (30) | 19/67 (28) | 1.00 |
| Response to PEG-IFN/RBV therapy | | | |
| SVR total cases no. (%) | 22/46 (48) | 83/210 (40) | 0.38 |
| RVR in total cases no. (%) | 6/44 (14) | 22/195 (11) | 0.83 |
| cEVR in total cases no.(%) | 22/44 (50) | 92/200 (46) | 0.75 |
| SVR 48w treatment no. (%) | 16/29 (55) | 55/130 (42) | 0.29 |
| End of treatment response no. (%) | 26/41 (63) | 123/202 (61) | 0.91 |
| HCV genome sequence variation | | | |
| ISDR mutation $\leq$1 no. (%) | 32/46 (70) | 167/210 (80) | 0.21 |
| Core70 R no. (%) | 26/44 (59) | 136/210 (65) | 0.56 |
| IRRDR mutation >3 no. (%) | 25/38 (66) | 107/190 (56) | 0.34 |

[a] Mean ± SD

[b] Number/total number (%)

harboring viruses with and without these mutations. Moreover, no differences were observed in the responses of either group to PEG-IFN/RBV therapy.

Recent studies reported that significant number of patients who were never treated with PI possess viral sequences with PI-resistance-associated NS3 mutations. In these studies, the prevalence of PI-resistance mutations was determined to be 8.6–16.2% [13, 14], in HCV genotype 1- and 3-infected patients in European–American populations. These patients were often coinfected with HIV. Analysis of the public HCV databases (EuHCVdb and Los Alamos) also reported the presence of naturally occurring PI-resistance-associated NS3 mutations in worldwide isolates [33]. However, in vivo and in vitro studies demonstrated that most of the mutations observed conferred only low- to moderate-level PI resistance [7, 13, 14, 34, 35]. Regarding viral fitness, PI-resistant HCVs show lower fitness at varying degrees as revealed by in vitro studies [1, 10, 11, 17, 20–22, 31, 32], but HCV RNA levels in a clinical study did not differ significantly. The response to PEG-IFN/RBV therapy was almost comparable to that in HCV-infected patients without PI-resistance mutations either in HCV replicon experiments or in a clinical study of small number of treated patients [34].

The prevalence of 13.4% for PI-resistance-proven patients observed in the present study was almost comparable to the results of previous studies. Although HIV is known to increase HCV replication in coinfection with HCV [36], and HIV patients are often treated with the HIV-specific PIs, the HIV infection might not affect the natural occurrence of HCV-specific PI-resistance mutations since our studied patients were all proven to be free from coinfection with HIV infection. As shown in Table 1 and Fig. 1, I153 V (22/261, 8.4%), T54S (14/261, 5.4%), and D168E (4/261, 1.5%) were among the most prevalent PI-resistance-proven mutations in the present study. The most frequent mutation detected in our study I153V was reported to appear secondarily to the occurrence of R109K mutations in a HCV replicon system [17]. Although the role of this mutation is not understood, the I153V mutation on its own conferred SCH446211 resistance to the HCV replicon to a lesser degree [17]. Interestingly, I153V was often found in double mutations in our study, as shown in Fig. 2. This suggests analogy between in vitro and in vivo data. T54S and D168E, the other frequent mutations, have been also reported to occur as single dominant mutations in previous in vitro or in vivo studies in HCV genotype 1

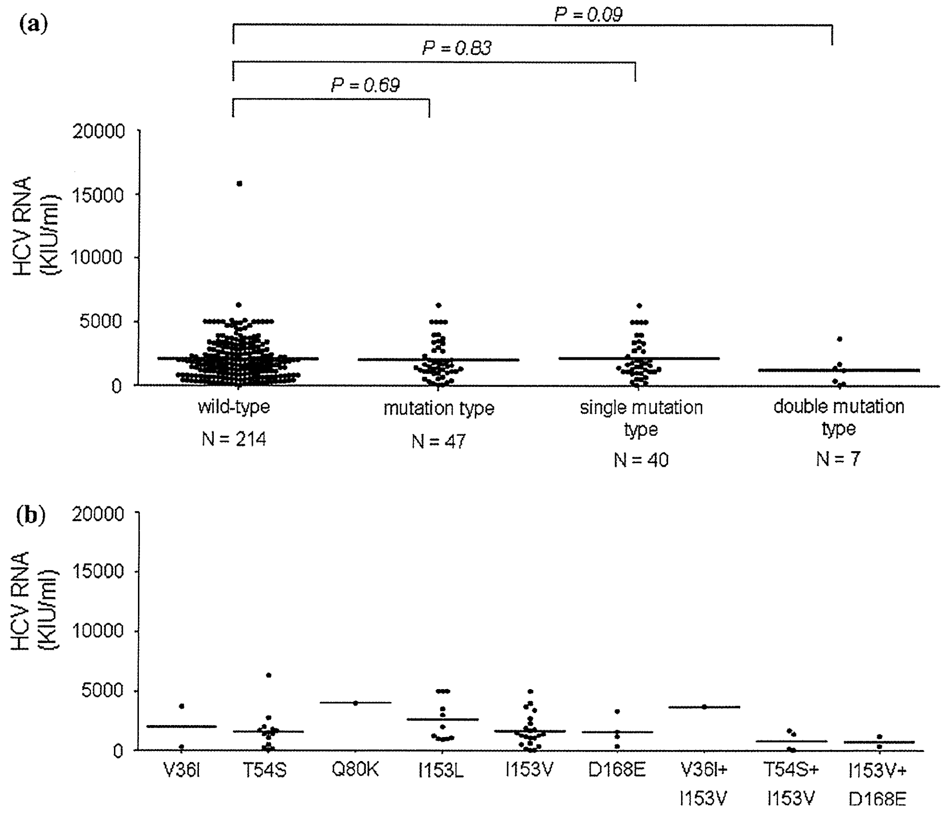

**Fig. 2** In vivo fitness of HCV with PI-resistance-associated NS3 mutations. HCV RNA levels were compared between patients with and without NS3 PI-resistance-associated mutations (**a**) and between patients with each resistance mutation (**b**). The estimated *P* value (Mann–Whitney *U* test) indicates no significant difference between the wild-type and other groups (wild-type vs. mutation type, wild-type vs. single mutation type, and wild-type vs. double mutation type). (Wild-type, $N = 214$; mutation type, $N = 47$; single mutation type, $N = 40$; double mutation type, $N = 7$; V36I, $N = 2$; T54S, $N = 14$; Q80K, $N = 1$; I153L, $N = 11$; I153V, $N = 22$; D168E, $N = 4$; E176A, $N = 1$; V36I + I153V, $N = 1$; T54S + I153V, $N = 4$, and I153V + D168E, $N = 2$)

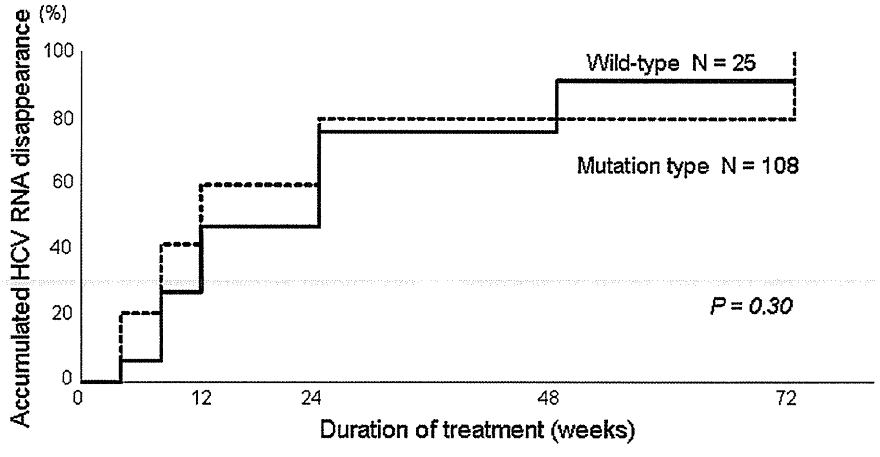

**Fig. 3** Comparison of virologic response to PEG-IFN/RBV therapy between HCV-infected patients with and without PI-resistance-associated NS3 mutations. Time-dependent HCV clearance rate analysis was based on serum HCV RNA positivity during PEG-IFN/RBV therapy for HCV isolates with resistance mutations or wild-type sequences. A total of 133 patients for whom the limit of viral genome detection could be determined were analyzed. Among this group, NS3 mutations were detected in 25 patients (19%). The estimated *P* value (log-rank test) shows no significant difference between the two groups ($P = 0.30$)

infections showing moderate degrees of resistance [16, 18, 19].

Most PI-resistance mutations described to date have been associated with varying degrees of reduced replicative capacity [10, 11, 17, 20–22, 31, 32]. In the present study, HCV RNA levels of those patients with low- to moderate-level resistance mutations were similar to those in patients in the wild-type groups, suggesting that in vitro viral fitness

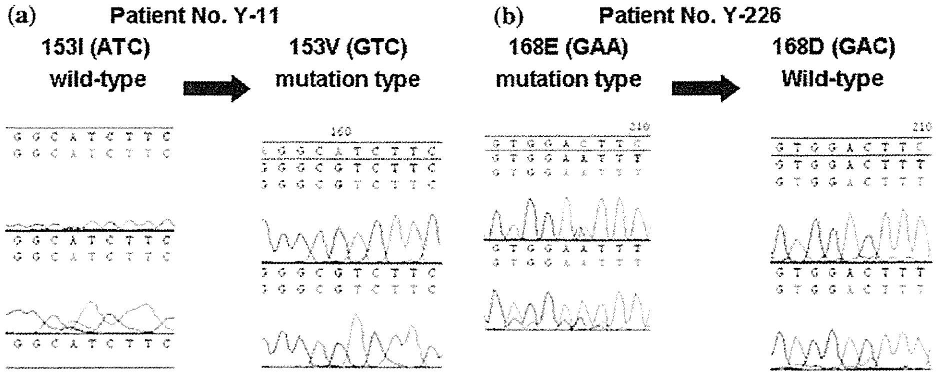

**Fig. 4** Appearance of PI-resistance-associated NS3 mutations during the PEG-IFN/RBV therapy. Chromatograms show part of the HCV NS3 sequence demonstrating PI-resistance mutations in two patients receiving therapy. **a** Site 153 isoleucine (Ile) (ATC) changed to valine (Val) (GTC), **b** Site 168 glutamic acid (Glu) (GAA) changed to aspartic acid (Asp) (GAC)

does not necessarily reflect in vivo viral fitness. This, however, does not rule out the possibility that some unknown compensatory viral mutations might have resulted in upregulation of reduced viral fitness. Interestingly, although the replicative capacity conferred by a single mutation seemed to be the same, the HCV RNA levels of double mutations were frequently low, suggesting that double mutations might weaken viral fitness.

In previous studies, clinical characteristics representing the state of liver disease other than HCV RNA levels were not studied in patients with PI-resistance mutations. In this study, we show that those clinical characteristics did not differ according to the presence of viral NS3 mutations. As shown in Table 2, age, sex ratio, fibrosis stage, ALT levels, serum albumin, platelet count, and past history of IFN pretreatment did not differ according to the presence of NS3 mutations. These results suggest that NS3 mutations occur independently of disease progression. Moreover, no evident differences were observed between viral and host factors known to affect IFN-based treatment responses. However, viral amino acid variations in the core and NS5A or the allelic frequency of IL28B SNPs, which were recently reported for the close relationship of responses to PEG-IFN/RBV therapy, did not differ between the two groups.

A significant outcome of the present study is the demonstration that PI-resistance mutations might not affect responses to PEG-IFN/RBV therapy. Previous in vitro studies demonstrated that HCV replicons harboring PI-resistance mutations were also sensitive to IFN treatment [31]. In addition, recent clinical studies also indicated that PI-resistance mutations were sensitive to the PEG-IFN/RBV [10, 34]. However, our analysis was more comprehensive because viral and host factors that contribute to treatment responses were simultaneously analyzed. A unique aspect of the present study is that we investigated the influence of the PEG-IFN/RBV treatment on the occurrence of new PI mutations by direct nucleotide sequencing, and were able to show that the PEG-IFN/RBV might not induce amino acid mutations.

Will the pre-existence of naturally occurring PI-resistance mutations have an influence on future treatment of HCV infections? Since new PIs are on the verge of clinical use, all clinicians should bear in mind the substantial numbers of HCV-infected patients with PI-resistance mutations. Although the degree of resistance is considered to be low or moderate in untreated patients, weak resistance might progress to more potent resistance with additional mutations, when PIs become widely used. Therefore, all clinicians need to be sufficiently prepared for the possibility of later onset of PI-resistance mutations that confer greater drug resistance and concomitant poorer responses to therapy. In SPRINT-1 study, the lead-in therapy was associated with a modestly lower rate of breakthrough than with no lead in [7]. Considering that PEG-IFN/RBV was equally effective for PI-resistant viruses, sufficient "lead-in" therapy before the administration of PIs could be an option in the forthcoming triple therapy modality.

In conclusion, we demonstrate here that PI-resistance-associated NS3 mutations exist in a substantial proportion of untreated HCV-1b-infected patients. Although the degree of resistance might not be strong, clinicians will need to consider this upon the introduction of triple therapy.

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