

**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 <sup>4</sup> /mm <sup>3</sup> )	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
γ-GTP (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).

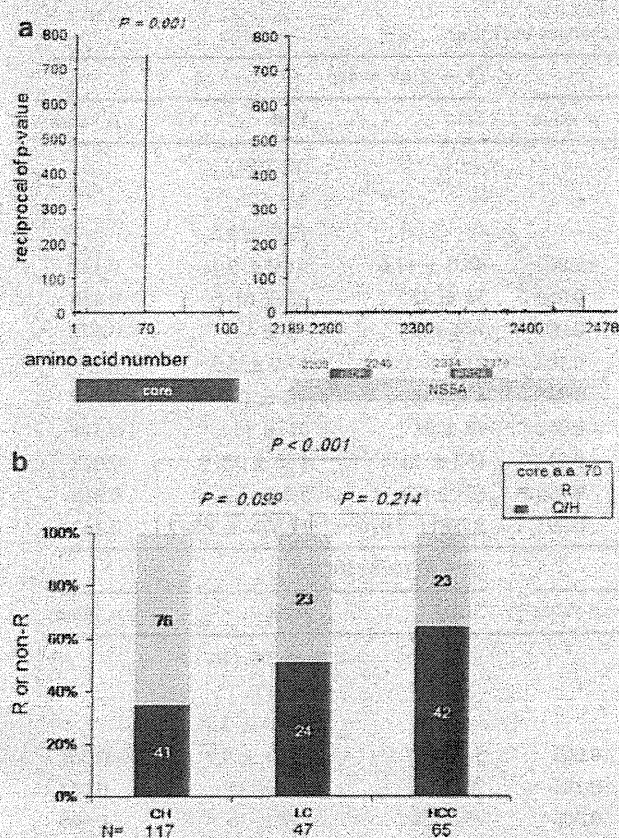
#### Core and NS5A sequences in patients with and without HCC

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 <sup>4</sup> /mm <sup>3</sup> )	11.3 ± 5.8	15.3 ± 6.2	<0.001
Albumin (g/dL)	3.6 ± 0.5	4.4 ± 2.9	0.025
γ-GTP (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, γ-GTP, 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

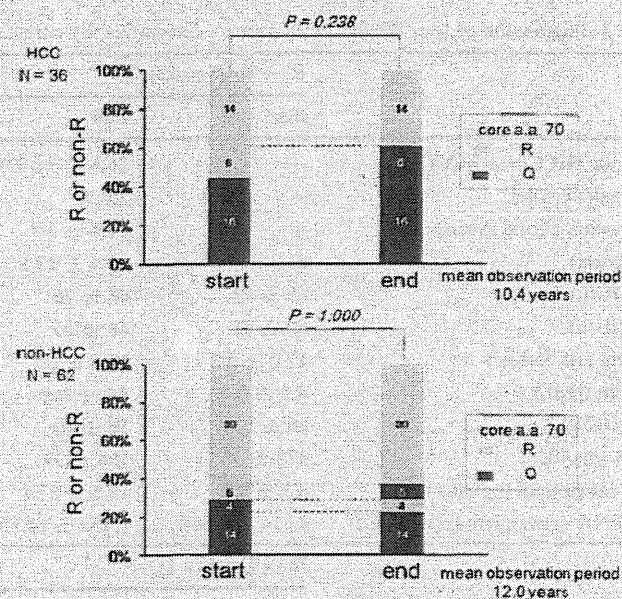


**Fig. 2** Core and NS5A sequences in additional patients were studied. **a** Using sera from 230 additional patients at the time of diagnosis, amino acid usage was compared between HCC and non-HCC patients for the part of core and the NS5A region, and this difference is shown as the bar height expressed 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



**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)

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.

**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 <sup>-4</sup> /mm <sup>3</sup> )	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
γ-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 <sup>-3</sup> /mm <sup>3</sup> )	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
γ-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.

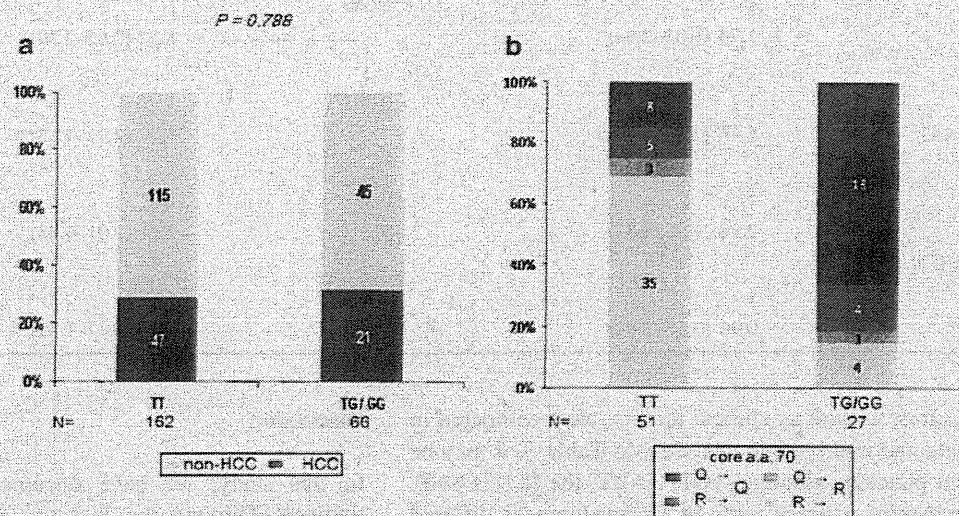
The IL28B SNP and its association with core a.a. 70 and disease progression

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, γGTP 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 <sup>3</sup> /mm <sup>3</sup> )	13.8 ± 5.8	14.3 ± 6.6	0.632
Albumin (g/dL)	4.2 ± 3.0	4.0 ± 0.5	0.528
γ-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/L)	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
IRRD	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 ( <i>N</i> = 228)		Multivariate analysis ( <i>N</i> = 228)	
	Odds ratio (95% CI)	<i>p</i> value	Odds ratio (95% CI)	<i>p</i> 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 <sup>4</sup> /mm <sup>3</sup> )				
>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 ( <i>N</i> = 228)		Multivariate analysis ( <i>N</i> = 228)	
	Odds ratio (95% CI)	<i>p</i> value	Odds ratio (95% CI)	<i>p</i> 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 <sup>4</sup> /mm <sup>3</sup> )				
>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)	
γ-GTP (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.

**Acknowledgements** This study was supported in part by a Grant-in-Aid scientific research fund of the Ministry of Education, Science, Sports and Culture number 20390206 and in part by a Grant-in-Aid

from the Ministry of Health, Labour, and Welfare of Japan (H19-kanen-002).

## References

1. Kiyosawa K, Sodeyama T, Tanaka E, et al. Interrelationship of blood transfusion, non-A, non-B hepatitis and hepatocellular carcinoma: analysis by detection of antibody to hepatitis C virus. *Hepatology* 1990;12:671–675
2. Enomoto N, Sakuma I, Asahina Y, et al. Mutations in the non-structural protein 5A gene and response to interferon in patients with chronic hepatitis C virus 1b infection. *N Engl J Med* 1996;334:77–81
3. Akuta N, Suzuki F, Sezaki H, et al. 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. *Intervirology* 2005;48:372–380
4. Hung CH, Chen CH, Lee CM, et al. Association of amino acid variations in the NS5A and E2-PcPHD region of hepatitis C virus 1b with hepatocellular carcinoma. *J Viral Hepat* 2008;15:58–65
5. El-Shamy A, Nagano-Fujii M, Sasase N, Imoto S, Kim SR, Hotta H. Sequence variation in hepatitis C virus nonstructural protein 5A predicts clinical outcome of pegylated interferon/ribavirin combination therapy. *Hepatology* 2008;48:38–47
6. Moriya K, Nakagawa K, Santa T, et al. Oxidative stress in the absence of inflammation in a mouse model for hepatitis C virus-associated hepatocarcinogenesis. *Cancer Res* 2001;61:4365–4370
7. Okuda M, Li K, Beard MR, et al. Mitochondrial injury, oxidative stress, and antioxidant gene expression are induced by hepatitis C virus core protein. *Gastroenterology* 2002;122:366–375
8. Nishina S, Hino K, Korenaga M, et al. Hepatitis C virus-induced reactive oxygen species raise hepatic iron level in mice by reducing hepcidin transcription. *Gastroenterology* 2008;134(1):226–238
9. Bartosch B, Thimme R, Blum HE, Zoulim F. Hepatitis C virus-induced hepatocarcinogenesis. *J Hepatol* 2009;51:810–820
10. Tsai WL, Chung RT. Viral hepatocarcinogenesis. *Oncogene* 2010;29:2309–2324
11. Moriya K, Fujie H, Shintani Y, et al. The core protein of hepatitis C virus induces hepatocellular carcinoma in transgenic mice. *Nat Med* 1998;4:1065–1067
12. Akuta N, Suzuki F, Kawamura Y, et al. Amino acid substitutions in the hepatitis C virus core region are the important predictor of hepatocarcinogenesis. *Hepatology* 2007;46:1357–1364
13. Franco S, Gimenez-Barcons M, Puig-Basagoiti F, et al. Characterization and evolution of NS5A quasispecies of hepatitis C virus genotype 1b in patients with different stages of liver disease. *J Med Virol* 2003;71:195–204
14. Gimenez-Barcons M, Franco S, Suarez Y, et al. High amino acid variability within the NS5A of hepatitis C virus (HCV) is associated with hepatocellular carcinoma in patients with HCV-1b-related cirrhosis. *Hepatology* 2001;34:158–167
15. Nagayama K, Kurosaki M, Enomoto N, Miyasaka Y, Marumo F, Sato C. Characteristics of hepatitis C viral genome associated with disease progression. *Hepatology* 2000;31:745–750
16. Fukuhara T, Taketomi A, Motomura T, et al. Variants in IL28B in liver recipients and donors correlate with response to peginterferon and ribavirin therapy for recurrent hepatitis C. *Gastroenterology* 2010;139:1577–1585
17. Tanaka Y, Nishida N, Sugiyama M, et al. Genome-wide association of IL28B with response to pegylated interferon-alpha and ribavirin therapy for chronic hepatitis C. *Nat Genet* 2009;41:1105–1109

18. Suppiah V, Moldovan M, Ahlenstiel G, et al. IL28B is associated with response to chronic hepatitis C interferon-alpha and ribavirin therapy. *Nat Genet* 2009;41:1100–1104
19. Ge D, Fellay J, Thompson AJ, Simon JS, et al. Genetic variation in IL28B predicts hepatitis C treatment-induced viral clearance. *Nature* 2009;461:399–401
20. Thomas DL, Thio CL, Martin MP, et al. Genetic variation in IL28B and spontaneous clearance of hepatitis C virus. *Nature* 2009;461:798–801
21. Ogura S, Akuta N, Hirakawa M, et al. Virological and biochemical features in elderly HCV patients with hepatocellular carcinoma: amino acid substitutions in HCV core region as predictor of mortality after first treatment. *Intervirology* 2009;52:179–188
22. Nakamoto S, Imazeki F, Fukui K, et al. Association between mutations in the core region of hepatitis C virus genotype 1 and hepatocellular carcinoma development. *J Hepatol* 2010;52:72–78
23. Kobayashi M, Akuta N, Suzuki F, et al. Influence of amino-acid polymorphism in the core protein on progression of liver disease in patients infected with hepatitis C virus genotype 1b. *J Med Virol* 2010;82:41–48
24. Hu Z, Muroyama R, Kowatari N, Chang J, Omata M, Kato N. Characteristic mutations in hepatitis C virus core gene related to the occurrence of hepatocellular carcinoma. *Cancer Sci* 2009;100:2465–2468
25. Akuta N, Suzuki F, Kawamura Y, et al. Substitution of amino acid 70 in the hepatitis C virus core region of genotype 1b is an important predictor of elevated alpha-fetoprotein in patients without hepatocellular carcinoma. *J Med Virol* 2008;80:1354–1362



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

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Received: 21 April 2011 / Accepted: 22 July 2011  
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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

**Electronic supplementary material** The online version of this article (doi:10.1007/s12072-011-9306-7) contains supplementary material, which is available to authorized users.


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Published online: 18 August 2011

 Springer

[1]. Among these NS3/4A PIs, telaprevir, boceprevir, SCH446211, danoprevir (ITMN-191), naldaprevir (SCH900518), 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 NSSB 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<sup>®</sup>, Schering-Plough, Tokyo, Japan) plus RBV (REBETOL<sup>®</sup>, 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-IFN $\alpha$ -2b, 1.5  $\mu$ g/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<sup>™</sup> 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