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Table 1. Baseline characteristics of all patients, and patients assigned to the model building or validation groups.

	All patients n = 496	Model group n = 331	Validation group n = 165
Gender: male	250 (50%)	170 (51%)	80 (48%)
Age (years)	57.1 ± 9.9	56.8 ± 9.7	57.5 ± 10.2
ALT (IU/L)	78.6 ± 60.8	78.1 ± 61.4	79.7 ± 59.6
GGT (IU/L)	59.3 ± 63.6	58.9 ± 62.0	60.2 ± 66.9
Platelets (10 ⁹ /L)	154 ± 53	153 ± 52	154 ± 56
Fibrosis: F3-4	121 (24%)	80 (24%)	41 (25%)
HCV-RNA: >600,000 IU/ml	409 (82%)	273 (82%)	136 (82%)
ISDR mutation: ≤1	220 (88%)	290 (88%)	145 (88%)
Core 70 (Arg/Gln or His)	293 (59%)/203 (41%)	197 (60%)/134 (40%)	96 (58%)/69 (42%)
Core 91 (Leu/Met)	299 (60%)/197 (40%)	200 (60%)/131 (40%)	99 (60%)/66 (40%)
<i>IL28B</i> : Minor allele	151 (30%)	101 (31%)	50 (30%)
SVR	194 (39%)	129 (39%)	65 (39%)
Relapse	152 (31%)	103 (31%)	49 (30%)
NVR	150 (30%)	99 (30%)	51 (31%)

ALT, alanine aminotransferase; GGT, gamma-glutamyltransferase; ISDR, interferon sensitivity determining region; Arg, arginine; Gln, glutamine; His, histidine; Leu, leucine; Met, methionine; Minor, heterozygote or homozygote of minor allele; SVR, sustained virological response; NVR, null virological response.

Japanese [6], European [7], and a multi-ethnic population [8,9]. The last three studies focused on the association of SNPs in the *IL28B* region with SVR [7–9] but we found a stronger association with NVR [6]. In addition to these host genetic factors, we have reported that mutations within a stretch of 40 amino acids in the NS5A region of HCV, designated as the IFN sensitivity determining region (ISDR), are closely associated with the virological response to IFN therapy: a lower number of mutations is associated with treatment failure [10–13]. Amino acid substitutions at positions 70 and 91 of the HCV core region (Core70, Core91) also have been reported to be associated with response to PEG-IFN/RBV therapy: glutamine (Gln) or histidine (His) at Core70 and methionine (Met) at Core91 are associated with treatment resistance [4,14]. The importance of substitutions in the HCV core and ISDR was confirmed recently by a Japanese multicenter study [15]. How these viral factors contribute to response to therapy is yet to be determined. For general application in clinical practice, host genetic factors and viral factors should be considered together.

Data mining analysis is a family of non-parametric regression methods for predictive modeling. Software is used to automatically explore the data to search for optimal split variables and to build a decision tree structure [16]. The major advantage of decision tree analysis over logistic regression analysis is that the results of the analysis are presented in the form of flow chart, which can be interpreted intuitively and readily made available for use in clinical practice [17]. The decision tree analysis has been utilized to define prognostic factors in various diseases [18–25]. We have reported recently its usefulness for the prediction of an early virological response (undetectable HCV-RNA within 12 weeks of therapy) to PEG-IFN/RBV therapy in chronic hepatitis C [26].

This study aimed to define the pre-treatment prediction of response to PEG-IFN/RBV therapy through the integrated analysis of host factors, such as the *IL28B* genetic polymorphism and various clinical covariates, as well as viral factors, such as mutations in the HCV core and ISDR and serum HCV-RNA load. In addition,

for the general application of these results in clinical practice, decision models for the pre-treatment prediction of response were determined by data mining analysis.

Materials and methods

Patients

This was a multicentre retrospective study supported by the Japanese Ministry of Health, Labor and Welfare. Data were collected from a total of 496 chronic hepatitis C patients who were treated with PEG-IFN alpha and RBV at five hospitals and universities throughout Japan. Of these, 98 patients also were included in the original GWAS analysis [6]. The inclusion criteria in this study were as follows (1) infection by genotype 1b, (2) lack of co-infection with hepatitis B virus or human immunodeficiency virus, (3) lack of other causes of liver disease, such as autoimmune hepatitis, and primary biliary cirrhosis, (4) completion of at least 24 weeks of therapy, (5) adherence of more than 80% to the planned dose of PEG-IFN and RBV for the NVR patients, (6) availability of DNA for the analysis of the genetic polymorphism of *IL28B*, and (7) availability of serum for the determination of mutations in the ISDR and substitutions of Core70 and Core91 of HCV. Patients received PEG-IFN alpha-2a (180 µg) or 2b (1.5 µg/kg) subcutaneously every week and were administered a weight adjusted dose of RBV (600 mg for <60 kg, 800 mg for 60–80 kg, and 1000 mg for >80 kg daily) which is the recommended dosage in Japan. Written informed consent was obtained from each patient and the study protocol conformed to the ethical guidelines of the Declaration of Helsinki and was approved by the institutional ethics review committee. The baseline characteristics are listed in Table 1. For the data mining analysis, 67% of the patients (331 patients) were assigned randomly to the model building group and 33% (165 patients) to the validation group. There were no significant differences in the clinical backgrounds between these two groups.

Laboratory and histological tests

Blood samples were obtained before therapy and were analyzed for hematologic tests and for blood chemistry and HCV-RNA. Sequences of ISDR and the core region of HCV were determined by direct sequencing after amplification by reverse-transcription and polymerase chain reaction as reported previously [4,11]. Genetic polymorphism in one tagging SNP located near the *IL28B* gene (rs8099917) was determined by the GWAS or DigiTag2 assay [27]. Homozygosity (GG) or heterozygosity (TG) of the minor sequence was defined as having the *IL28B* minor allele, whereas homozygosity for the major sequence (TT) was

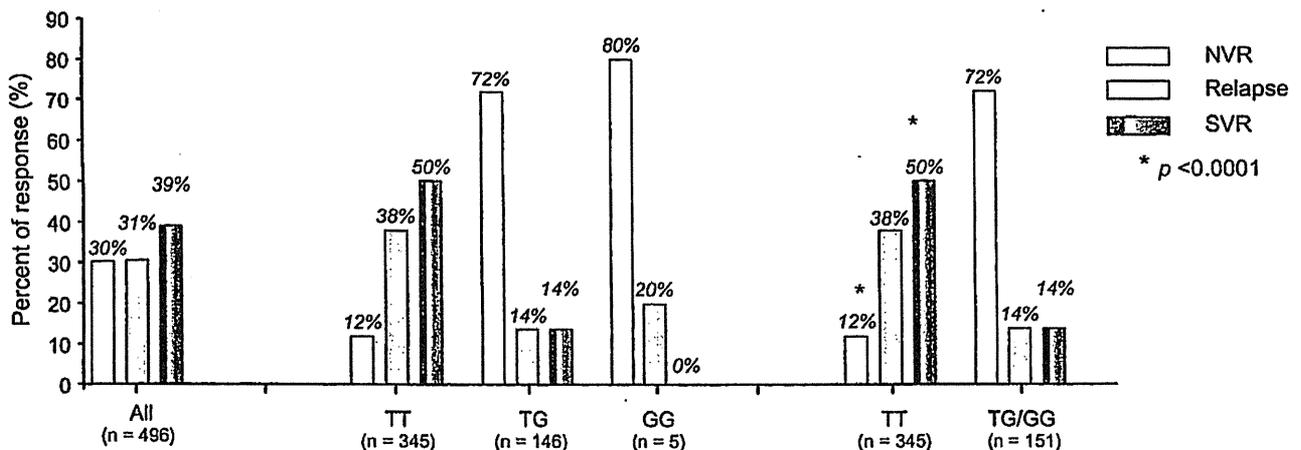


Fig. 1. Association between the IL28B genotype (rs8099917) and treatment response. The rates of response to treatment are shown for each rs8099917 genotype. The rate of null virological response (NVR), relapse, and sustained virological response (SVR) is shown. The p values are from Fisher's exact test. The rate of NVR was significantly higher ($p < 0.0001$) and the rate of SVR was significantly lower ($p < 0.0001$) in patients with the IL28B minor allele compared to those with the major allele.

defined as having the IL28B major allele. In this study, NVR was defined as a less than 2 log reduction of HCV-RNA at week 12 and detectable HCV-RNA by qualitative PCR with a lower detection limit of 50 IU/ml (Amplicor, Roche Diagnostic systems, CA) at week 24 during therapy. RVR (rapid virological response) and complete early virological response (cEVR) were defined as undetectable HCV-RNA at 4 weeks and 12 weeks during therapy and SVR was defined as undetectable HCV-RNA 24 weeks after the completion of therapy. Relapse was defined as reappearance of HCV-RNA after the completion of therapy. The stage of liver fibrosis was scored according to the METAVIR scoring system: F0 (no fibrosis), F1 (mild fibrosis: portal fibrosis without septa), F2 (moderate fibrosis: few septa), F3 (severe fibrosis: numerous septa without cirrhosis) and F4 (cirrhosis). Percentage of steatosis was quantified in 111 patients by determining the average proportion of hepatocytes affected by steatosis.

Statistical analysis

Associations between pre-treatment variables and treatment response were analyzed by univariate and multivariate logistic regression analysis. Associations between the IL28B polymorphism and sequences of HCV were analyzed by Fisher's exact test. SPSS software v.15.0 (SPSS Inc., Chicago, IL) was used for these analyses. For the data mining analysis, IBM-SPSS Modeler version 13.0 (IBM-SPSS Inc., Chicago, IL) software was utilized as reported previously [26]. The patients used for model building were divided into two groups at each step of the analysis based on split variables. Each value of each variable was considered as a potential split. The optimum variables and cut-off values were determined by a statistical search algorithm to generate the most significant division into two prognostic subgroups that were as homogeneous as possible for the probability of SVR. Thereafter, each subgroup was evaluated again and divided further into subgroups. This procedure was repeated until no additional significant variable was detected or the sample size was below 15. To avoid over-fitting, 10-fold cross validation was used in the tree building process. The reproducibility of the resulting model was tested with the data from the validation patients.

Results

Association between the IL28B (rs8099917) genotype and the PEG-IFN/RBV response

The rs8099917 allele frequency was 70% for TT ($n = 345$), 29% for TG ($n = 146$), and 1% for GG ($n = 5$). We defined the IL28B major allele as homozygous for the major sequence (TT) and the IL28B minor allele as homozygous (GG) or heterozygous (TG) for the minor sequence. The rate of NVR was significantly higher (72% vs. 12%, $p < 0.0001$) and the rate of SVR was significantly lower (14% vs. 50%, $p < 0.0001$) in patients with the IL28B minor allele compared to those with the major allele (Fig. 1).

Effect of the IL28B polymorphism, substitutions in the ISDR, Core70, and Core91 of HCV on time-dependent clearance of HCV

Patients were stratified according to their IL28B allele type, the number of mutations in the ISDR, the amino acid substitutions in Core70 and Core91, and the rate of undetectable HCV-RNA at 4, 8, 12, 24, and 48 weeks after the start of therapy were analyzed (Fig. 2A-D). The rate of undetectable HCV-RNA was significantly higher in patients with the IL28B major allele than the minor allele, in patients with two or more mutations in the ISDR compared to none or only one mutation, in patients with arginine (Arg) at Core70 rather than Gln/His, and in patients with leucine (Leu) at Core91 rather than Met. The difference was most significant when stratified by the IL28B allele type. The rate of RVR and cEVR was significantly more frequent in patients with the IL28B major allele compared with those with the IL28B minor allele: 9% vs. 3% for RVR ($p < 0.005$) and 57% vs. 11% for cEVR ($p < 0.0001$). These findings suggest that IL28B has the greatest impact on early virological response to therapy.

Association between substitutions in the ISDR and relapse after the completion of therapy

Patients were stratified according to the IL28B allele, number of mutations in the ISDR, and amino acid substitutions of Core70 and Core91, and the rate of relapse was analyzed (Fig. 3A and B). Among patients who achieved cEVR, the rate of relapse was significantly lower in patients with two or more mutations in the ISDR compared to those with only one or no mutations (15% vs. 31%, $p < 0.005$) (Fig. 3 B). On the other hand, the relapse rate was not different between the IL28B major and minor alleles within patients who achieved RVR (3% vs. 0%) or cEVR (28% vs. 29%) (Fig. 3A). Amino acid substitutions of Core70 and Core91 were not associated with the rate of relapse (data not shown).

Factors associated with response by multivariate logistic regression analysis

By univariate analysis, the minor allele of IL28B ($p < 0.0001$), one or no mutations in the ISDR ($p = 0.03$), high serum level of

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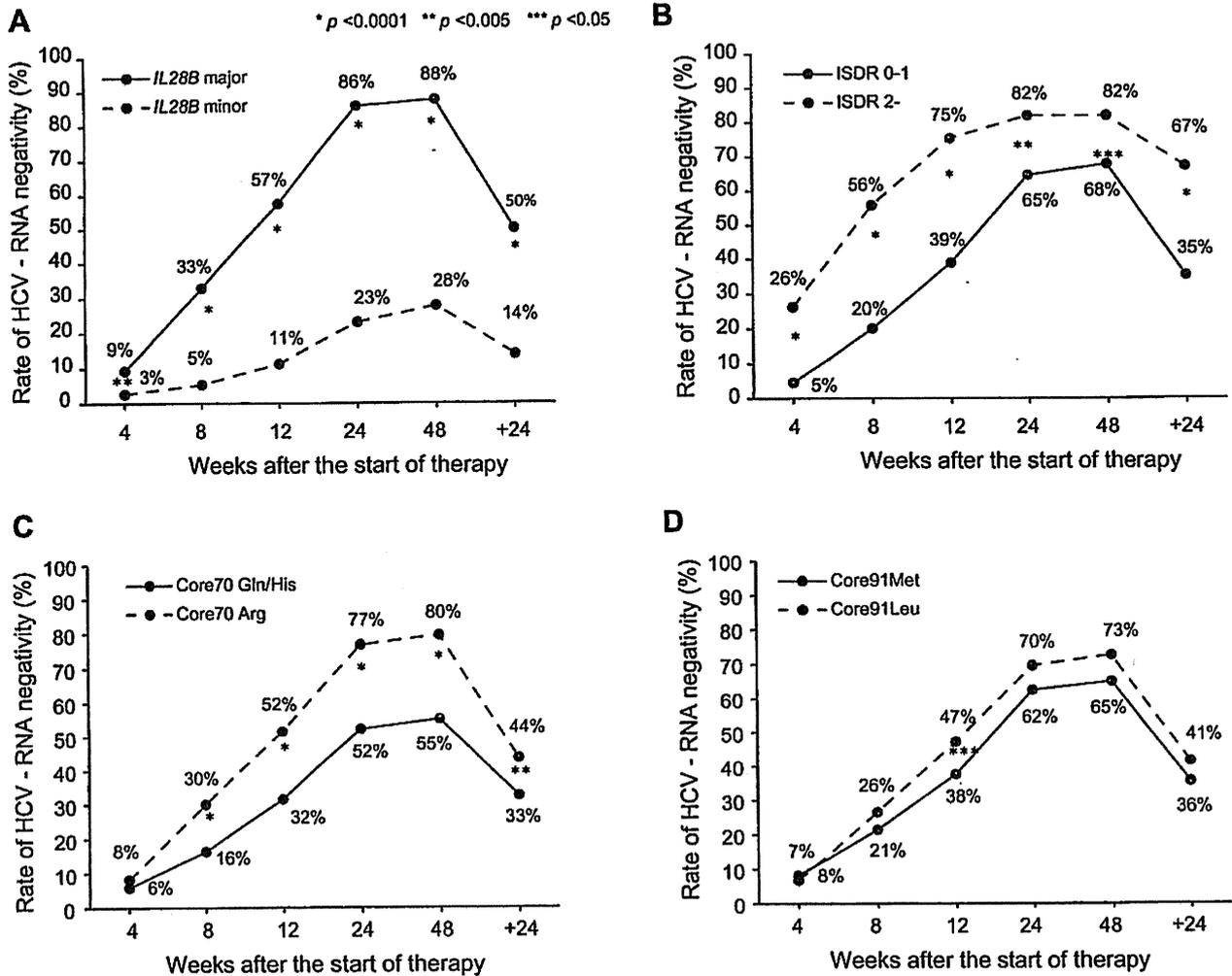


Fig. 2. Effect of *IL28B* mutations in the ISDR, Core70, and Core91 of HCV on time-dependent clearance of HCV. The rate of undetectable HCV-RNA was plotted for serial time points after the start of therapy (4, 8, 12, 24, and 48 weeks) and for 24 weeks after the completion of therapy. Patients were stratified according to (A) the *IL28B* allele (minor allele vs. major allele), (B) the number of mutations in the ISDR (0-1 mutation vs. 2 or more mutations), amino acid substitutions of (C) Core70 (Gln/His vs. Arg), and (D) Core91 (Met vs. Leu). The *p* values are from Fisher's exact test.

HCV-RNA ($p = 0.035$), Gln or His at Core70 ($p < 0.0001$), low platelet counts ($p = 0.009$), and advanced fibrosis ($p = 0.0002$) were associated with NVR. By multivariate analysis, the minor allele of *IL28B* (OR = 20.83, 95%CI = 11.63–37.04, $p < 0.0001$) was associated with NVR independent of other covariates (Table 2). Notably, mutations in the ISDR ($p = 0.707$) and at amino acid Core70 ($p = 0.207$) were not significant in multivariate analysis due to the positive correlation with the *IL28B* polymorphism ($p = 0.004$ for ISDR and $p < 0.0001$ for Core70, Fig. 4).

Genetic polymorphism of *IL28B* also was associated with SVR (OR = 7.41, 95% CI = 4.05–13.57, $p < 0.0001$) independent of other covariates, such as platelet counts, fibrosis, and serum levels of HCV-RNA. Mutation in the ISDR was an independent predictor of SVR (OR = 2.11, 95% CI = 1.06–4.18, $p = 0.033$) but the amino acid at Core70 was not (Table 3).

Factors associated with the *IL28B* polymorphism

Patients with the *IL28B* minor allele had significantly higher serum level of gamma-glutamyltransferase (GGT) and a higher

frequency of hepatic steatosis (Table 4). When the association between the *IL28B* polymorphism and HCV sequences was analyzed, Gln or His at Core70, that is linked to resistance to PEG-IFN and RBV therapy [4,14,15], was significantly more frequent in patients with the minor *IL28B* allele than in those with the major allele (67% vs. 30%, $p < 0.0001$) (Fig. 4). Other HCV sequences with an IFN resistant phenotype also were more prevalent in patients with the minor *IL28B* allele than those with the major allele: Met at Core91 (46% vs. 37%, $p = 0.047$) and one or no mutations in the ISDR (94% vs. 85%, $p = 0.004$) (Fig. 4).

Data mining analysis

Data mining analysis was performed to build a model for the prediction of SVR and the result is shown in Fig. 5. The analysis selected four predictive variables, resulting in six subgroups of patients. Genetic polymorphism of *IL28B* was selected as the best predictor of SVR. Patients with the minor *IL28B* allele had a lower probability of SVR and a higher probability of NVR than those with the major *IL28B* allele (SVR: 14% vs. 50%, NVR: 72% vs.

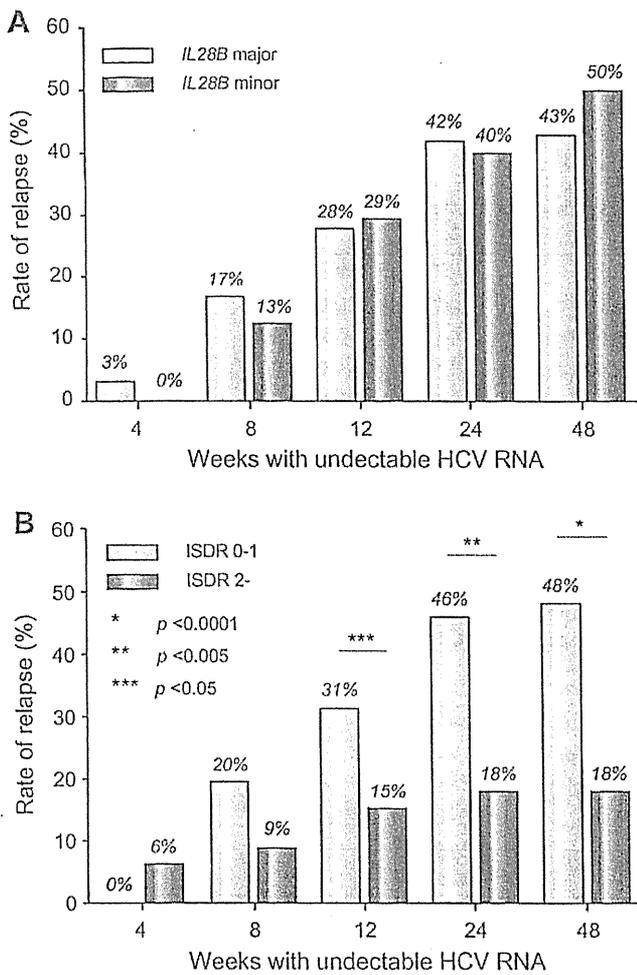


Fig. 3. Association between relapse and the *IL28B* allele or mutations in the ISDR. The rate of relapse was calculated for patients who had undetectable HCV-RNA at serial time points after the start of therapy (4, 8, 12, 24, and 48 weeks). Patients were stratified according to (A) the *IL28B* allele (minor allele vs. major allele) and (B) the number of mutations in the ISDR (0–1 mutation vs. 2 or more mutations). The *p* values are from Fisher's exact test.

12%). After stratification by the *IL28B* allele, patients with low platelet counts ($<140 \times 10^9/L$) had a lower probability of SVR and higher probability of NVR than those with high platelet counts ($\geq 140 \times 10^9/L$): for the minor *IL28B* allele, SVR was 7% vs. 19%, and NVR was 84% vs. 62%, and for the major *IL28B* allele, SVR was 32% vs. 66% and NVR was 16% vs. 8%. Among patients with the major *IL28B* allele and low platelet counts, those with two or more mutations in the ISDR had a higher probability of SVR and lower probability of relapse than those with one or no mutations in the ISDR (SVR: 75% vs. 27%, and relapse: 8% vs. 57%). Among patients with the major *IL28B* allele and high platelet counts, those with a low HCV-RNA titer ($<600,000$ IU/ml) had a higher probability of SVR and lower probability of NVR and relapse than those with a high HCV-RNA titer (SVR: 90% vs. 61%, NVR: 0% vs. 10%, and relapse: 10% vs. 29%). The sensitivity and specificity of the decision tree were 78% and 70%, respectively. The area under the receiver operating characteristic (ROC) curve of the model was 0.782 (data not shown). The pro-

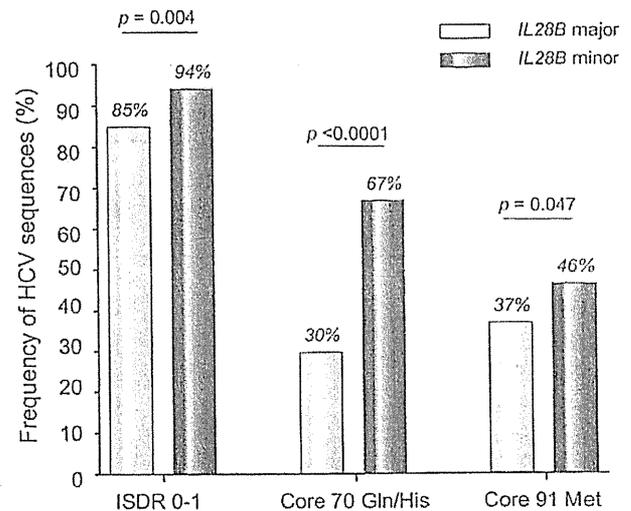


Fig. 4. Associations between the *IL28B* allele and HCV sequences. The prevalence of HCV sequences predicting a resistant phenotype to IFN was higher in patients with the minor *IL28B* allele than those with major allele. (A) 0 or 1 mutation in the ISDR of NS5A, (B) Gln or His at Core70, and (C) Met at Core91. *p* values are from Fisher's exact test.

portion of patients with advanced fibrosis (F3-4) was 39% (84/217) in patients with low platelet counts ($<140 \times 10^9/L$) compared to 13% (37/279) in those with high platelet counts ($\geq 140 \times 10^9/L$).

Validation of the data mining analysis

The results of the data mining analysis were validated with 165 patients who differed from those used for model building. Each patient was allocated to one of the six subgroups for the validation using the flow-chart form of the decision tree. The rate of SVR and NVR in each subgroup was calculated. The rates of SVR and NVR for each subgroup of patients were closely correlated between the model building and the validation patients ($r^2 = 0.99$ and 0.98) (Fig. 6).

Discussion

The rate of NVR after 48 weeks of PEG-IFN/RBV therapy among patients infected with HCV of genotype 1 is around 20–30%. Previously, there have been no reliable baseline predictors of NVR or SVR. Because more potent therapies, such as protease and polymerase inhibitor of HCV [28,29] and nitazoxanide [30], are in clinical trials and may become available in the near future, a pre-treatment prediction of the likelihood of response may be helpful for patients and physicians, to support clinical decisions about whether to begin the current standard of care or whether to wait for emerging therapies. This study revealed that the *IL28B* polymorphism was the overwhelming predictor of NVR and is independent of host factors and viral sequences reported previously. The *IL28B* encodes a protein also known as IFN-lambda 3, which is thought to suppress the replication of various viruses including HCV [31,32]. The results of the current study and the findings of the GWAS studies [6–9] may provide the rationale for developing diagnostic testing or an IFN-lambda based therapy for chronic hepatitis C in the future.

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Table 2. Factors associated with NVR analyzed by univariate and multivariate logistic regression analysis.

	Univariate			Multivariate		
	Odds ratio	95%CI	p value	Odds ratio	95%CI	p value
Gender: female	0.98	0.67-1.45	0.938	1.29	0.75-2.23	0.363
Age	1.01	0.97-1.01	0.223	0.99	0.97-1.02	0.679
ALT	1.00	1.00-1.00	0.867	1.00	0.99-1.00	0.580
GGT	1.004	1.00-1.01	0.029	1.00	1.00-1.00	0.715
Platelets	0.95	0.91-0.99	0.009	0.92	0.87-0.98	0.006
Fibrosis: F3-4	2.23	1.46-3.42	0.0002	1.97	1.09-3.57	0.025
HCV-RNA: $\geq 600,000$ IU/ml	1.83	1.05-3.19	0.035	2.49	1.17-5.29	0.018
ISDR mutation: ≤ 1	2.14	1.08-4.22	0.030	0.96	0.78-1.18	0.707
Core 70 (Gln/His)	3.23	2.16-4.78	<0.0001	1.41	0.83-2.42	0.207
Core 91 (Met)	1.39	0.95-2.06	0.093	1.21	0.72-2.04	0.462
IL28B: Minor allele	19.24	11.87-31.18	<0.0001	20.83	11.63-37.04	<0.0001

ALT, alanine aminotransferase; GGT, gamma-glutamyltransferase; ISDR, interferon sensitivity determining region; Gln, glutamine; His, histidine; Met, methionine; Minor allele, heterozygote or homozygote of minor allele.

Table 3. Factors associated with SVR analyzed by univariate and multivariate logistic regression analysis.

	Univariate			Multivariate		
	Odds ratio	95%CI	p value	Odds ratio	95%CI	p value
Gender: female	0.81	0.56-1.16	0.253	0.86	0.55-1.35	0.508
Age	0.97	0.95-0.99	0.0003	0.99	0.96-1.01	0.199
ALT	1.00	1.00-1.00	0.337	1.00	1.00-1.01	0.108
GGT	1.00	1.00-1.00	0.273	1.00	1.00-1.00	0.797
Platelets	1.12	1.01-1.16	<0.0001	1.13	1.08-1.19	<0.0001
Fibrosis: F0-2	2.64	1.65-4.22	<0.0001	1.87	1.07-3.28	0.029
HCV-RNA: <600,000 IU/ml	2.49	1.55-3.98	0.0001	2.75	1.55-4.90	0.001
ISDR mutation: $2\leq$	3.78	2.14-6.68	<0.0001	2.11	1.06-4.18	0.033
Core 70 (Arg)	1.61	1.11-2.28	0.012	0.84	0.52-1.35	0.470
Core 91 (Leu)	1.28	0.88-1.85	0.185	1.26	0.81-1.96	0.300
IL28B: Major allele	6.21	3.75-10.31	<0.0001	7.41	4.05-13.57	<0.0001

ALT, alanine aminotransferase; GGT, Gamma-glutamyltransferase; ISDR, interferon sensitivity determining region; Arg, arginine; Leu, leucine; Major allele, homozygote of major allele.

Among baseline factors, *IL28B* was the most significant predictor of NVR and SVR. Moreover, the *IL28B* allele type was also correlated with early virological response: the rate of RVR and cEVR was significantly high for the *IL28B* major allele compared to the *IL28B* minor allele: 9% vs. 3% for RVR and 57% vs. 11% for cEVR (Fig. 2). On the other hand, the relapse rate was not different between the *IL28B* genotypes within patients who achieved RVR or cEVR (Fig. 3). We believe that optimal therapy should be based on baseline features and a response-guided approach. Our findings suggest that the *IL28B* genotype is a useful baseline predictor of virological response which should be used for selecting the treatment regimen: whether to treat patients with PEG-IFN and RBV or to wait for more effective future therapy including direct acting antiviral drugs. On the other hand, baseline *IL28B* genotype might not be suitable for determining the treatment duration in patients who started PEG-IFN/RBV therapy

and whose virological response is determined because the *IL28B* genotype is not useful for the prediction of relapse. The duration of therapy should be personalized based on the virological response. Future studies need to explore whether the combination of baseline *IL28B* genotype and response-guided approach further improves the optimization of treatment duration.

The SVR rate in patients having the *IL28B* minor allele was 14% in the present study while it was 23% in Caucasians and 9% in African Americans in a study by McCarthy et al. [33]. On the other hand, the SVR rate in patients having the *IL28B* minor allele was 28% in genotypes 1/4 compared to 80% in genotypes 2/3 in a study by Rauch et al. [9]. These data imply that the impact of the *IL28B* polymorphism on response to therapy may be different in terms of race, geographical areas, or HCV genotypes, and that our data need to be validated in future studies including different populations and geographical areas before generalization.

Table 4. Factors associated with *IL28B* genotype.

	<i>IL28B</i> major allele n = 345	<i>IL28B</i> minor allele n = 151	p value
Gender: male	166 (48%)	84 (56%)	0.143
Age (years)	57 ± 10	57 ± 10	0.585
ALT (IU/L)	79 ± 60	78 ± 62	0.842
Platelets (10 ⁹ /L)	153 ± 54	155 ± 52	0.761
GGT (IU/L)	51 ± 45	78 ± 91	0.001
Fibrosis: F3-4	76 (22%)	45 (30%)	0.063
Steatosis:			
>10%	16/88 (18%)	13/23 (57%)	0.024
>30%	6/88 (7%)	6/23 (26%)	0.017
HCV-RNA: >600,000 IU/ml	284 (82%)	125 (83%)	1.000

ALT, alanine aminotransferase; GGT, gamma-glutamyltransferase.

Four GWAS studies have shown the association between a genetic polymorphism near the *IL28B* gene and response to PEG-IFN plus RBV therapy. The SNPs that showed significant association with response were rs12979860 [8] and rs8099917 [6,7,9]. There is a strong linkage-disequilibrium (LD) between these two SNPs as well as several other SNPs near the *IL28B* gene in Japanese patients [34] but the degree of LD was weaker in Caucasians and Hispanics [8]. Thus, the combination of SNPs is not useful for predicting response in Japanese patients but may improve the predictive value in patients other than Japanese who have weaker LD between SNPs.

Other significant predictors of response independent of *IL28B* genotype were platelet counts, stage of fibrosis, and HCV RVA load. A previous study reported that platelet count is a predictor of response to therapy [35], and the lower platelet count was related with advanced liver fibrosis in the present study. The association between response to therapy and advanced fibrosis independent of the *IL28B* polymorphism is consistent with a recent study by Rauch et al. [9].

There is agreement that the viral genotype is significantly associated with the treatment outcome. Moreover, viral factors such as substitutions in the ISDR of the NS5A region [10] or in the amino acid sequence of the HCV core [4] have been studied in relation to the response to IFN treatment. The amino acid Gln or His at Core70 and Met at Core91 are repeatedly reported to be associated with resistance to therapy [4,14,15] in Japanese patients but these data wait to be validated in different populations or other geographical areas. In this study, we confirmed that patients with two or more mutations in the ISDR had a higher rate of undetectable HCV-RNA at each time point during therapy. In addition, the rate of relapse among patients who achieved cEVR was significantly lower in patients with two or more mutations in ISDR compared to those with only one or no mutations (15% vs. 31%, *p* < 0.05). Thus, the ISDR sequence may be used to predict a relapse among patients who achieved virological response during therapy, while the *IL28B* polymorphism may be used to predict the virological response before therapy. A higher number of mutations in the ISDR are reported to have close association with SVR in Japanese [11–13,15,36] or Asian [37,38] populations but data from Western countries have been controversial [39–42]. A meta-analysis of 1230 patients including 525 patients from Europe has shown that there was a positive correlation

between the SVR and the number of mutations in the ISDR in Japanese as well as in European patients [43] but this correlation was more pronounced in Japanese patients. Thus, geographical factors may account for the different impact of ISDR on treatment response, which may be a potential limitation of our study.

To our surprise, these HCV sequences were associated with the *IL28B* genotype: HCV sequences with an IFN resistant phenotype were more prevalent in patients with the minor *IL28B* allele than those with the major allele. This was an unexpected finding, as we initially thought that host genetics and viral sequences were completely independent. A recent study reported that the *IL28B* polymorphism (rs12979860) was significantly associated with HCV genotype: the *IL28B* minor allele was more frequent in HCV genotype 1-infected patients compared to patients infected with HCV genotype 2 or 3 [33]. Again, patients with the *IL28B* minor allele (IFN resistant genotype) were infected with HCV sequences that are linked to an IFN resistant phenotype. The mechanism for this association is unclear, but may be related to an interaction between the *IL28B* genotype and HCV sequences in the development of chronic HCV infection as discussed by McCarthy et al., since the *IL28B* polymorphism was associated with the natural clearance of HCV [44]. Alternatively, the HCV sequence within the patient may be selected during the course of chronic infection [45,46]. These hypotheses should be explored through prospective studies of spontaneous HCV clearance or by testing the time-dependent changes in the HCV sequence during the course of chronic infection.

How these host and viral factors can be integrated to predict the response to therapy in future clinical practice is an important question. Because various host and viral factors interact in the same patient, predictive analysis should consider these factors in combination. Using the data mining analysis, we constructed a simple decision tree model for the pre-treatment prediction of SVR and NVR to PEG-IFN/RBV therapy. The classification of patients based on the genetic polymorphism of *IL28B*, mutation in the ISDR, serum levels of HCV-RNA, and platelet counts, identified subgroups of patients who have the lowest probabilities of NVR (0%) with the highest probabilities of SVR (90%) as well as those who have the highest probabilities of NVR (84%) with the lowest probability of SVR (7%). The reproducibility of the model was confirmed by the independent validation based on a second group of patients. Using this model, we can rapidly develop an

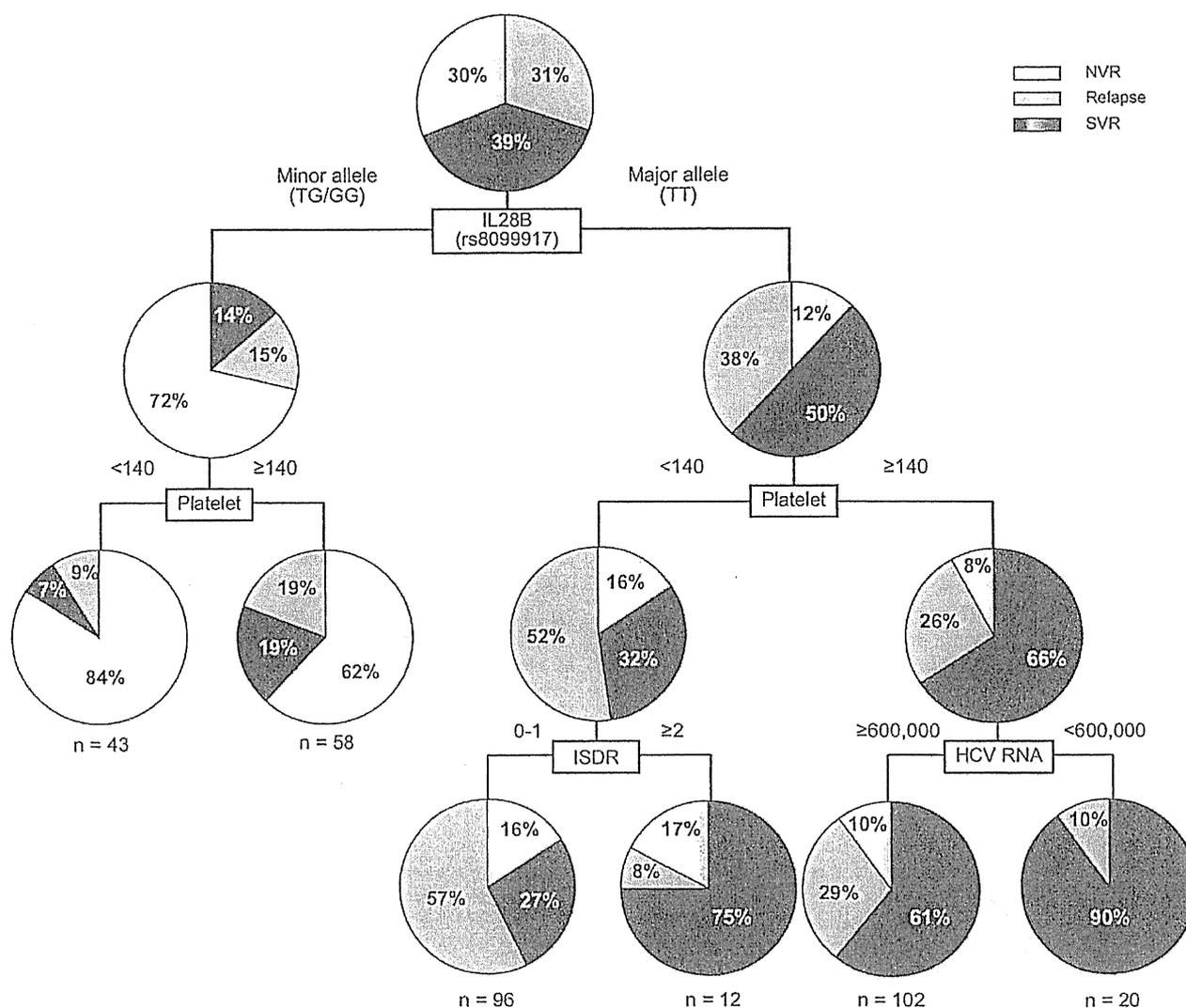


Fig. 5. Decision tree for the prediction of response to therapy. The boxes indicate the factors used for splitting. Pie charts indicate the rate of response for each group of patients after splitting. The rate of null virological response, relapse, and sustained virological response is shown.

estimate of the response before treatment, by simply allocating patients to subgroups by following the flow-chart form, which may facilitate clinical decision making. This is in contrast to the calculating formula, which was constructed by the traditional logistic regression model. This was not widely used in clinical practice as it is abstruse and inconvenient. These results support the evidence based approach of selecting the optimum treatment strategy for individual patients, such as treating patients with a low probability of NVR with current PEG-IFN/RBV combination therapy or advising those with a high probability of NVR to wait for more effective future therapies. Patients with a high probability of relapse may be treated for a longer duration to avoid a relapse. Decisions may be based on the possibility of a response against a potential risk of adverse events and the cost of the therapy, or disease progression while waiting for future therapy.

We have previously reported the predictive model of early virological response to PEG-IFN and RBV in chronic hepatitis C

[26]. The top factor selected as significant was the grade of steatosis, followed by serum level of LDL cholesterol, age, GGT, and blood sugar. The mechanism of association between these factors and treatment response was not clear at that time. To our interest, a recent study by Li et al. [47] has shown that high serum level of LDL cholesterol was linked to the *IL28B* major allele (CC in rs12979860). High serum level of LDL cholesterol was associated with SVR but it was no longer significant when analyzed together with the *IL28B* genotype in multivariate analysis. Thus, the association between treatment response and LDL cholesterol levels may reflect the underlining link of LDL cholesterol levels to *IL28B* genotype. Steatosis is reported to be correlated with low lipid levels [48] which suggest that *IL28B* genotypes may be also associated with steatosis. In fact, there were significant correlations between the *IL28B* genotype and the presence of steatosis in the present study (Table 4). In addition, the serum level of GGT, another predictive factor in our previous study, was signif-

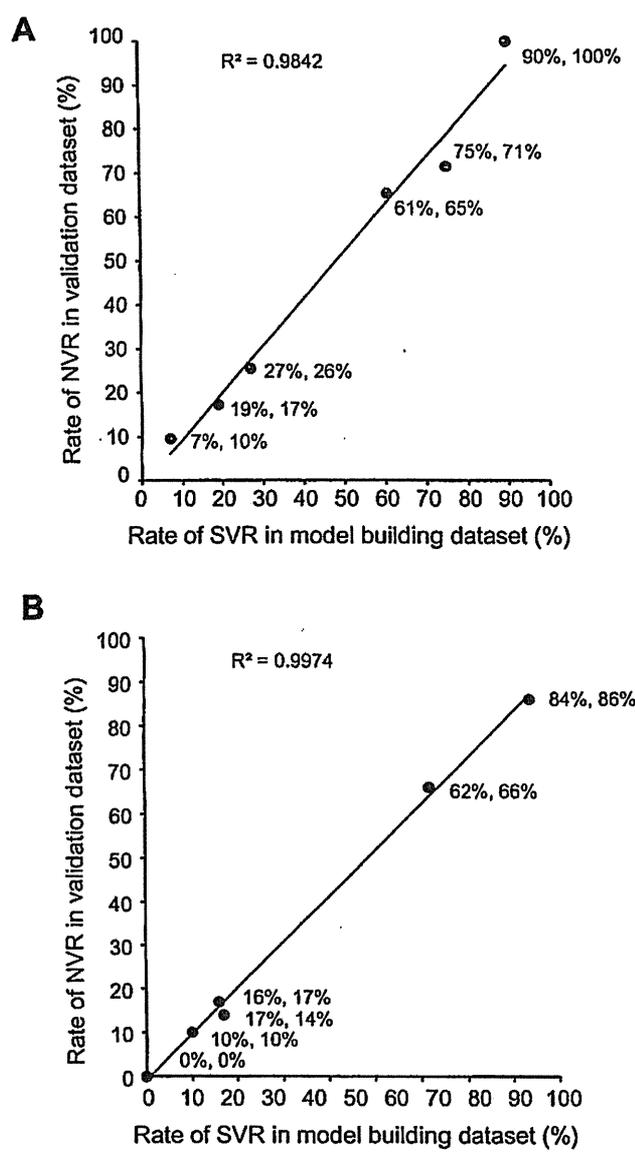


Fig. 6. Validation of the CART analysis. Each patient in the validation group was allocated to one of the six subgroups by following the flow-chart form of the decision tree. The rate of (A) sustained virological response (SVR) and (B) null virological response (NVR) in each subgroup was calculated and plotted. The X-axis represents the rate of SVR or NVR in the model building patients and the Y-axis represents those in the validation patients. The rate of SVR and NVR in each subgroup of patients is closely correlated between the model building and the validation patients (correlation coefficient: $r^2 = 0.98-0.99$).

icantly associated with *IL28B* genotype in the present study (Table 4). The serum level of GGT was significantly associated with NVR when examined independently but was no longer significant when analyzed together with the *IL28B* genotype. These observations indicate that some of the factors that we have previously identified may be associated with virological response to therapy through the underlining link to the *IL28B* genotype.

In conclusion, the present study highlighted the impact of the *IL28B* polymorphism and mutation in the ISDR on the pre-treatment prediction of response to PEG-IFN/RBV therapy. A decision model including these host and viral factors has the potential to

support selection of the optimum treatment strategy for individual patients, which may enable personalized treatment.

Conflict of interest

The authors who have taken part in this study declare that they do not have anything to disclose regarding funding or conflict of interest with respect to this manuscript.

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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), naldaprevir (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-IFN α -2b, 1.5 μ 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[™] 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				References	Detected resistance mutations Genotype 1b (<i>N</i> = 261), (%)
NS3 residue	Resistance mutations	Drugs			
V36	A, M, L, G, C	Telaprevir, Boceprevir	[1, 3, 4, 10, 11, 19, 31, 37]	1 × 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-naïve 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 (IRRD), 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

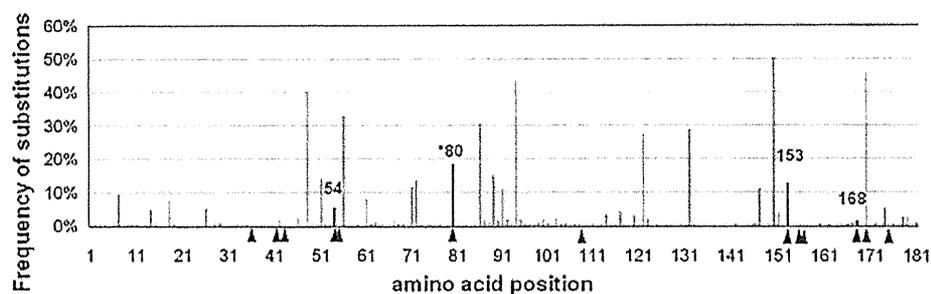


Fig. 1 Frequency of polymorphic mutations for each of the 181 NS3 protease amino acid residues in 261 patients. *Arrowheads* indicate the sites reported to confer PI resistance. *Dark bars* denote the amino acid

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 (<i>N</i> = 47)	Wild-type (<i>N</i> = 214)	<i>P</i> 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 ≤1 no. (%)	32/46 (70)	167/210 (80)	0.21
Core70 R no. (%)	26/44 (59)	136/210 (65)	0.56
IRRD R 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 coinfecting 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)

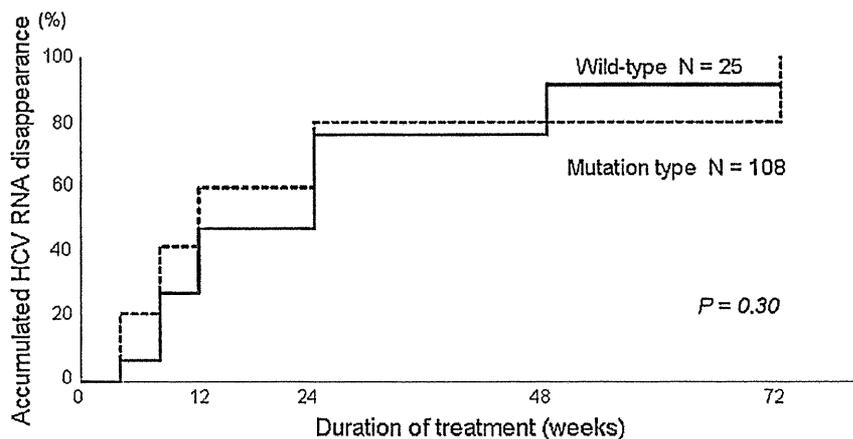
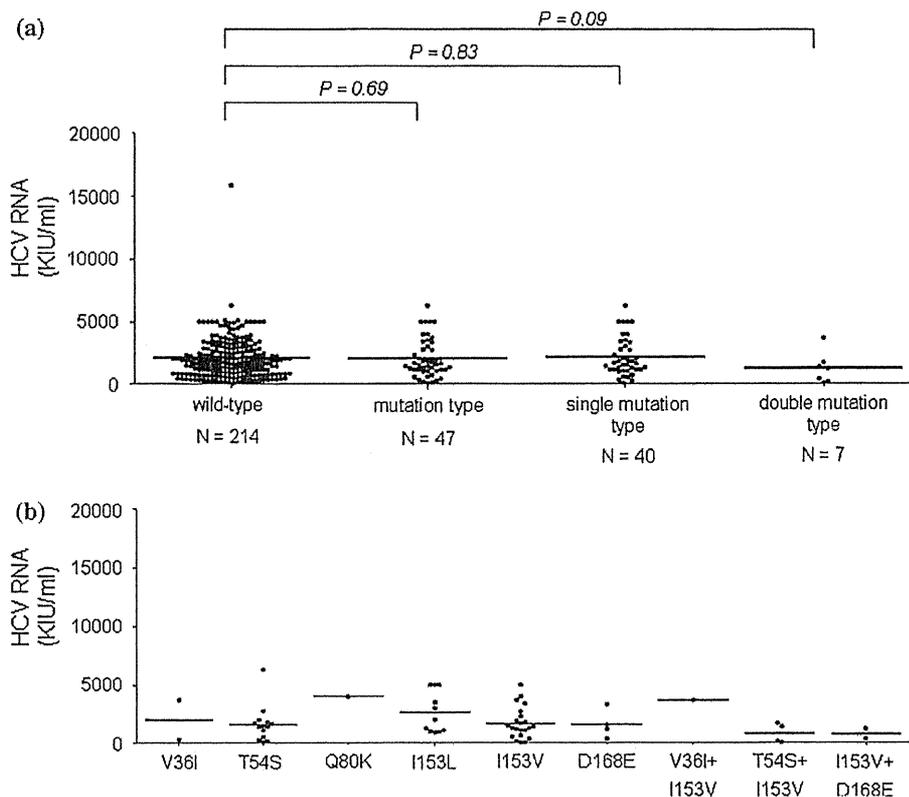


Fig. 3 Comparison of virologic response to PEG-IFN/RBV therapy between HCV-infected patients with and without PI-resistance-associated NS3 mutations. Time-dependent HCV clearance rate analysis was based on serum HCV RNA positivity during PEG-IFN/RBV therapy for HCV isolates with resistance mutations or wild-

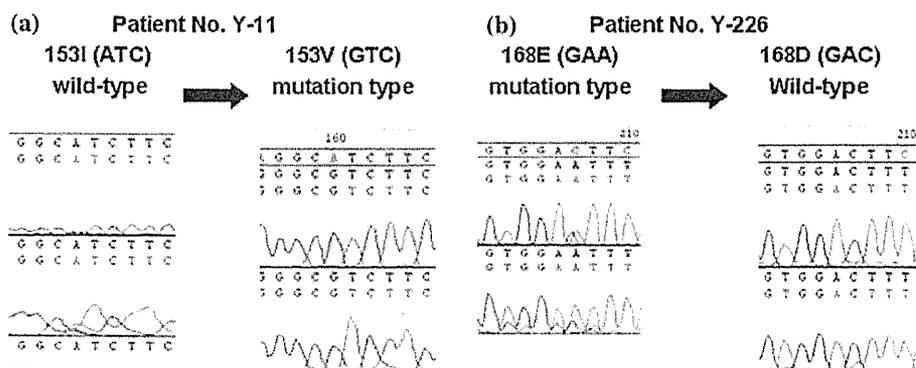
type sequences. A total of 133 patients for whom the limit of viral genome detection could be determined were analyzed. Among this group, NS3 mutations were detected in 25 patients (19%). The estimated *P* value (log-rank test) shows no significant difference between the two groups (*P* = 0.30)

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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Expression of Keratin 19 Is Related to High Recurrence of Hepatocellular Carcinoma after Radiofrequency Ablation

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Key Words

Hepatocellular carcinoma · Radiofrequency ablation · Recurrence · Keratin · Carcinogenesis · Needle biopsy · Hepatic progenitor cell

Abstract

Objective: Keratin (K) 19 positivity has been reported to be a useful predictive marker for recurrence in patients with hepatocellular carcinoma (HCC) who have undergone hepatic resection. We investigated the clinical usefulness of K19 positivity in patients who had received curative radiofrequency ablation (RFA). **Methods:** We retrospectively evaluated the clinicopathological features, including imaging and K19 expression, in 246 patients with HCC who were within the Milan criteria and had received curative RFA. Using a two-step insertion method, tumor biopsies were obtained just prior to RFA and were evaluated histologically. **Results:** Tumor seeding due to liver biopsy and RFA was not observed. Ten patients (4.1%) had K19-positive HCC. Imaging findings were similar between K19-positive and -negative HCC ($p = 0.187$). Nine out of 10 patients (90%) who had K19-positive HCC had

recurrence of HCC after RFA, and intrahepatic recurrences were observed within 12 months in 6 out of 10 (60.0%). K19 positivity was a significant risk factor for recurrence ($p < 0.0001$) and early recurrence (<1 year after RFA; $p = 0.012$). K19 expression ($p = 0.016$) was an independent risk factor for tumor status exceeding the Milan criteria after RFA. **Conclusion:** Expression of K19 is related to high recurrence of HCC after curative RFA.

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Introduction

Radiofrequency ablation (RFA) is regarded as an important treatment modality for hepatocellular carcinoma (HCC) [1–4], and its efficacy, especially for tumors <2 cm in diameter, is better than that of ethanol and nearly comparable to that of surgical resection [5]. In addition, RFA

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is considered to be a bridge to liver transplantation because the prolonged waiting time for cadaveric livers leads to dropouts from the waiting list [6]. Tumor recurrence after curative RFA has been a problem, as it is after hepatic resection. Tumor size (>3 cm in diameter) [7], time after treatment (>1 year) [7], the number of HCC nodules [8] and hepatitis C virus (HCV) infection [8] have been reported to be risk factors for intrahepatic tumor recurrence after curative RFA. Moreover, primary technical failure is reported to be a risk factor for tumor progression beyond the Milan criteria after RFA [9].

Keratin (K) 19, which is considered to be a biliary/hepatic progenitor cell marker [10], has attracted attention as a useful predictive marker for detecting the more aggressive HCCs after curative resection, because tumors with K19 expression have a poorer prognosis [11, 12] and higher rates of recurrence [13, 14] and lymph node metastasis [12] than K19-negative HCC. In these previous studies, surgical specimens were investigated and K19 positivity was defined as expression in >5% of tumor cells [11–14].

As a result, one would expect that K19 expression might be a useful predictive marker for detecting HCC with a worse outcome after RFA, especially regarding tumor recurrence. To the best of our knowledge, the correlation between clinicopathological features and K19 expression has not been investigated in HCC patients treated by RFA. Therefore, we performed a clinicopathological study on 246 HCC cases treated with RFA and investigated the relationship between the K19 expression and recurrence and prognosis after treatment.

Methods

Patients

Between April 1999 and February 2010, 1,284 patients were admitted to the Musashino Red Cross Hospital for the first treatment of HCC. A total of 684 patients were treated with RFA as the initial therapy for HCC. Ablation therapy was chosen either because the patients were considered not to be suitable for resection ($n = 323$), when considering impairment of liver function, number and distribution of the tumors as well as cardiopulmonary dysfunction, or because they preferred ablation and provided informed consent ($n = 361$), despite surgery also being feasible. From the outset, 172 patients were excluded because RFA was performed without tumor biopsy. Therefore, 512 consecutive patients, on whom tumor biopsies had been performed before RFA, were included and we evaluated these specimens retrospectively. The result of retrospective analysis was that there were 57 patients with no residual samples, 119 patients with no tumorous lesion and 9 patients with no definitive histological diagnosis because of a small and/or fragmented specimen. The remaining specimens

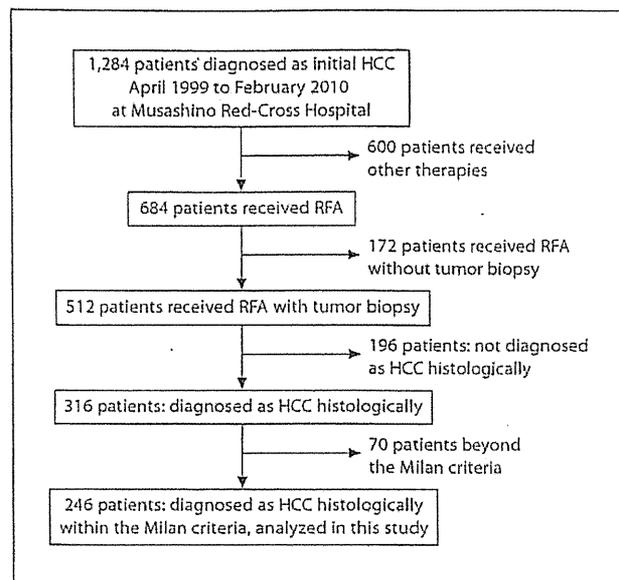


Fig. 1. Flow chart summarizing the patient selection for the study.

were diagnosed as HCC in 316 patients, as dysplastic nodule in 6 patients, as adenocarcinoma in 4 patients and as neuroendocrine tumor in 1 patient. Seventy patients were excluded, because their states of HCC were beyond the Milan criteria (≤ 3 cm and up to 3 nodules, or ≤ 5 cm and a single nodule). Therefore, 246 consecutive patients, on whom tumor biopsies had been performed before RFA and diagnosed as HCC retrospectively, were included in the study (fig. 1). The inclusion criteria for receiving RFA were as follows: total bilirubin concentration < 3.0 mg/dl, platelet count $> 3 \times 10^5/\text{mm}^3$, prothrombin activity $> 50\%$ (approximately equal to an international normalized ratio of 1.5) and Child-Pugh score < 8 points. Ascites were controlled by administration of diuretics before RFA. Patients with macroscopic vascular invasion or extrahepatic metastases were excluded. The criteria of the International Union against Cancer were used for TNM classification [15]. Written informed consent was obtained from all patients, and the study was approved by the ethics committee at Musashino Red Cross Hospital, in accordance with the Declaration of Helsinki.

Diagnosis of HCC

All the patients were diagnosed as having HCC on the basis of tumor markers and a combination of typical imaging findings on ultrasonography (US) and dynamic computed tomography (CT), according to the American Association for the Study of Liver Diseases and the Japan Society of Hepatology guidelines [1, 16]. When patients had 2 or 3 HCC nodules, a needle biopsy was taken from the main nodule. The histological diagnosis of HCC was based on the World Health Organization criteria [17].

For the evaluation of vascularity and Kupffer cell activity of the target nodule, CT during arteriography (CTHA) and CT dur-