undetectable time point are plotted in Figure 2(a), which shows a very strong and a significant correlation ($r^2 = 0.67$, $P < 0.0005$).

The validity of the prediction formula was investigated in the validation group. Analysis was possible in 32 patients, as the other patients were excluded from the analysis due to the following reasons: therapy was discontinued before viral clearance in eight patients, PEG-IFN dosage was reduced before viral clearance in nine patients and viral clearance was achieved before day 14 in two patients. There were six cases of NVR, and incomplete blood collections from 13 patients on day 7 and/or 14. A strong and a significant correlation was demonstrated between the undetectable time points that were predicted using this formula and the actual undetectable time points (Fig. 2c, $r = 0.53$, $P = 0.005$).

Although only one case was predicted to achieve a rapid virological response (undetectable viral load at week 4)[13] in the model validation group, the actual undetectable time point of this patient was week 8 (Fig. 2d). In contrast, all nine cases who were predicted to achieve a complete early virological response (undetected viral load until week 12),[13] the actual undetectable time points of these patients were within week 12. Because the prediction formula was derived by the least squares method, half of the patients, who were predicted not to achieve the complete early virological response, actually achieved it.

DISCUSSION

NUMEROUS STUDIES HAVE documented that the undetectable time point is related to therapeutic responses, and its usefulness in predicting therapeutic efficacy is clear.[9–13] In the present study, we were able to derive a formula for predicting the undetectable time point for patients with HCV genotype 1b and high serum viral loads during PEG-IFN-α-2b/ribavirin combination therapy. Though the various parameters for the HCV dynamics were investigated, the change in viral load from day 7 to 14 was the only parameter that was useful for predicting the undetectable time point.

The standard length of PEG-IFN/ribavirin combination therapy is 48 weeks for patients with HCV genotype 1b and high serum viral loads; however, a 72-week administration is recommended to improve therapeutic response.[3,13,18] Therefore, when undetectable time points are predicted as from weeks 13–24 by our formula, the SVR rates could be improved by continuing the IFN therapy for longer periods. By prediction of the undetectable time point early during the treatment using our formula, the physician can make early modification and optimization of currently ongoing therapy.

Another important issue of PEG-IFN/ribavirin treatment is adherence to treatment. Because dose reductions may delay the time until serum viral clearance, patients in whom the dosage of IFN and ribavirin was reduced during therapy were excluded in the present study. However, there are many patients in whom the dosage of drugs has to be reduced during therapy for a wide variety of clinical reasons. If reducing dosage before the predicted undetectable time point, administration of IFN for longer periods should be considered.

In conclusion, we created a formula for predicting the undetectable time point in patients treated with PEG-IFN-α-2b/ribavirin combination therapy. Viral eradication is the ultimate objective of IFN-based therapy, but many patients failed to achieve viral eradication for some reason. Because our prediction formula for the undetectable time point was made with a small population, it is necessary to correct it by further analysis with a larger population. However, an early viral response reflects efficacy of the therapy, and the estimation of an undetectable time point by our formula would be useful for determining the optimal duration of treatment in the early period of the therapy for each chronic hepatitis C patient.

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Original Article

Cancer preventive effect of pegylated interferon α-2b plus ribavirin in a real-life clinical setting in Japan: PERFECT interim analysis

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Aim: This study was conducted to clarify the incidence of hepatocellular carcinoma (HCC) and the factors contributing to its occurrence by following chronic hepatitis C patients who received pegylated interferon (PEG-IFN) α-2b plus ribavirin (RBV) combination therapy.

Methods: Patients who received PEG-IFN α-2b and RBV combination therapy with no history of HCC or HCC within 3 months after the start of treatment were observed for the onset of HCC at 67 centers.

Results: Sustained virological response (SVR) was observed in 999 (53.5%) of 1865 patients eligible for analysis. During the observation period (median duration: 4 years and 3 months), HCC developed in 59 patients (3.1%). A significant difference was observed in the 5-year cumulative incidence of HCC between SVR and non-SVR patients (1.1% vs. 7.1%). Factors contributing to HCC selected in multivariate analysis were therapeutic efficacy, sex, age, alanine aminotransferase (ALT) level at 24 weeks after the end of treatment, and platelet count. Non-SVR patients with ALT improvement after the end of treatment had a significantly lower 5-year cumulative incidence of HCC than those without (3.4% vs. 11.0%). HCC developed in 10 patients who achieved SVR, and multivariate analysis indicated that ALT level at 24 weeks after the end of treatment was the only significant factor contributing to HCC.

Conclusion: Several known risk factors for HCC contributed to HCC in patients who received PEG-IFN α-2b and RBV combination therapy, and ALT abnormality after the end of treatment contributes to the onset of HCC in both non-SVR and SVR patients.

Key words: alanine aminotransferase, chronic hepatitis C virus, hepatocellullar carcinoma, pegylated inteferon, ribavirin

Abbreviations: AFP, alpha fetoprotein; ALT, alanine aminotransferase; BR, biochemical response; CHC, chronic hepatitis C; HCC, hepatocellular carcinoma; IFN, interferon; LVR, late virological response; NR, no response; NVR, non-virological response; PEG-IFN, pegylated interferon; ; RBV, ribavirin; SVR, sustained virological response; TR, transient response.

INTRODUCTION

THE INCREASE IN the incidence of hepatocellular carcinoma (HCC) in Japan peaked in 2004 and is now in a declining trend.[1] The HCC mortality rate, however, is still particularly high among developed countries,[2] and even now nearly 35 000 people die

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annually from HCC. In Japan, about 70% of patients diagnosed with HCC are positive for hepatitis C virus antibody.[3] The hepatitis C virus infection rate[2] and incidence of HCC both increase with the age of the patient,[4] and curing chronic hepatitis C (CHC) to reduce HCC and deaths due to HCC is a pressing issue.

With the discovery of interferon (IFN), CHC became a curable disease, and with the addition of ribavirin (RBV), therapeutic outcomes have improved dramatically. Currently, about 50%[5–8] of patients with HCV genotype 1b and high virus load and more than 80%[9] of genotype 2 patients achieve sustained virologic response (SVR), and the SVR rate is reported to improve further with long-term treatment[10,11] and with combination therapy plus a statin.[12]

The efficacy achieved with these IFN therapies is also reported to lead to the inhibition of the onset of HCC and deaths due to HCC[13–15], but only a few reports are available of long-term observation of patients receiving PEG-IFN α plus RBV combination therapy.

We therefore examined the HCC preventive effect of combination therapy in 1865 patients who received PEG-IFN α-2b and RBV.

METHODS

Patients and treatment

PERFECT (THE PEG-IFN and Ribavirin, Find Evidence of Chronic Hepatitis C Therapy in Tokyo) Study Group, consisting of 67 centers in Tokyo and Yamanashi Prefecture, conducted a retrospective study to investigate the efficacy and safety of PEG-IFN α-2b plus RBV in CHC patients in a real-life clinical setting. The participating centers, targeted patients, and the treatment method have already been reported[10] and are summarized below.

Patients seen from December 2004 who completed PEG-IFN α-2b plus RBV combination therapy by September 2007 were registered regardless of genotype, history of IFN treatment, or alanine aminotransferase (ALT) levels. Excluded from this study were pregnant or possibly pregnant and lactating women, and patients with severe heart disease, chronic kidney failure or creatinine clearance of ≤50 mL/min, current or history of severe psychiatric disorder, and autoimmune hepatitis. Doses of PEG-IFN α-2b and RBV and dose adjustment followed the Japanese package insert. The duration of treatment was 48 weeks, the standard of care for patients with genotype 1 and high virus load. In patients with late viral response (LVR) who did not achieve viral negativity by week 12, treatment could be extended up to 72 weeks. Patients other than those with genotype 1 and high virus load were treated for 24 weeks.

Included in this analysis were the patients registered in the PERFECT Study who had no history of HCC and for whom SVR/non-SVR status could be confirmed. The patients who developed HCC within 3 months of the start of treatment were excluded from analysis to rule out the possibility of inclusion of patients with HCC already present at the start of treatment.

The start of the follow-up period was defined as the first day of PEG-IFN α-2b and RBV treatment. The patients were monitored for the onset of HCC by routine follow-up methods practiced by each center. The diagnosis of HCC was based on the presence of typical hypervascular characteristics on angiography in addition to the findings on computed tomography and ultrasonography. Microscopic examination of fine-needle biopsy specimens was performed in patients whose angiograms did not demonstrate a typical image of HCC.

This multicenter study was approved by the institutional review board of each participating center. The study protocol was carried out according to the ethical guidelines of the 1975 Declaration of Helsinki, and informed consent was obtained from each patient.

Statistical analysis

All statistical analyses were performed using SAS, version 9.13 (SAS Institute, Cary, NC, USA). Intergroup comparison of background variables was performed by Fisher's exact test and Mann–Whitney *U*-test.

The cumulative incidence of HCC was calculated by the Kaplan–Meier method, and intergroup comparison was conducted using the log-rank test. The determination of the factors contributing to HCC was conducted by Cox proportional hazards regression model using a stepwise procedure, incorporating the factors exhibiting $P < 0.2$ by the log-rank test and excluding factors with more than 30% of values missing. The determination of factors associated with biochemical response (BR) was conducted by a stepwise procedure using the results of logistic univariate analysis ($P < 0.2$) in logistic multivariate analysis.

All tests were two-sided, with a significance level set at $P < 0.05$.

RESULTS

Study population

A TOTAL OF 1865 subjects, consisting of 999 SVR patients (SVR rate 53.5%) and 866 non-SVR patients, were eligible for analysis. Of the non-SVR patients, 441 had transient response (TR) defined as viral negativity achieved during treatment (relapse: 408, virus breakthrough: 33), 400 patients had non-virological response (NVR) defined as viral negativity not being achieved, and the change in viral load during treatment was not known for 25 patients.

The duration of observation ranged from 3 months to 5 years and 8 months, with a median of 4 years and 3 months.

During the observation period, HCC developed in 59 patients (3.1%). Between patients who developed HCC and those who did not, significant differences in background factors were detected in age ($P < 0.0001$), hepatic fibrosis ($P = 0.0002$), virological efficacy ($P < 0.0001$), ALT levels ($P = 0.0089$), ALT level at 24 weeks after the end of treatment (≤40 vs. >40 IU/L) ($P < 0.0001$), platelet count ($P = 0.0001$), serum albumin ($P = 0.0062$), and alpha fetoprotein (AFP) ($P < 0.0001$) (Table 1).

Virological efficacy and incidence of HCC

The 5-year cumulative incidence of HCC by the Kaplan–Meier method was 1.1% in SVR patients and 7.1% in non-SVR patients, a difference that was significant ($P < 0.001$) (Fig. 1). No significant difference was observed in the incidence of HCC between TR and NVR patients among non-SVR patients, but the difference between TR and SVR patients was significant ($P < 0.0001$) (Fig. 2). This trend was also observed regardless of gender, with no significant difference in the incidence of HCC observed between TR and NVR in either male or female patients and a significant difference observed between TR and SVR in both male patients ($P = 0.0007$) and female ($P = 0.0065$) patients.

Factors contributing to HCC

The factors contributing to HCC selected in the multivariate analysis were therapeutic efficacy (SVR vs. NVR), sex, age (<60 vs. ≥60 years), ALT level at 24 weeks after the end of treatment (≤40 vs. >40 IU/L), and platelet count (<10 vs. ≥10 × 10 000/mm³) (Table 2).

Biochemical response and incidence of HCC in non-SVR patients

Since ALT levels at 24 weeks after the end of treatment was selected as one factor contributing to HCC, the changes in ALT levels and onset of HCC were examined in 514 non-SVR patients with a pretreatment ALT level of more than 40 IU/L whose ALT level at 24 weeks after the end of treatment was obtained. Of these 514

Table 1 Patient background by onset of hepatocellular carcinoma (HCC) (1865 patients)

Factor	With onset of HCC ($n = 59$)	Without onset of HCC ($n = 1806$)	*P*-value
Gender (male/female)	40/19	1014/792	0.0832
Age	62 (44–74)	56 (17–77)	<0.0001
Diabetes (yes/no/unknown)	6/33/20	100/1040/666	0.1539
Hypertension (yes/no/unknown)	4/6/49	116/569/1121	0.0763
Alcohol abuse (yes/no/unknown)	11/16/32	195/493/1118	0.1930
Fibrosis (0/1/2/3/4/unknown)	0/12/13/15/4/15	57/573/355/205/56/560	0.0002
Genotype (1/2/3/unknown)	52/5/0/2	1421/365/2/18	0.0876
Effect of IFN (SVR/non-SVR)	10/49	989/817	<0.0001
Body mass index (kg/m²)	22.6 (14.2–34.0)	22.9 (14.9–41.2)	0.8546
ALT (IU/L)	79 (24–343)	60 (8–984)	0.0089
ALT at 24 weeks after end of treatment (IU/L) (≤40/>40/unknown)	16/30/13	1105/352/349	<0.0001
Platelet count (×10 000/mm³)	13.3 (4.3–22.2)	16.3 (3.6–213.3)	0.0001
Serum albumin (g/dL)	3.9 (2.9–4.7)	4.1 (2.8–5.9)	0.0062
AFP (ng/mL)	13 (2.2–327.9)	5 (0–875)	<0.0001

Median (minimum – maximum).
AFP, alpha fetoprotein; ALT, alanine aminotransferase; IFN, interferon; SVR, sustained virological response.

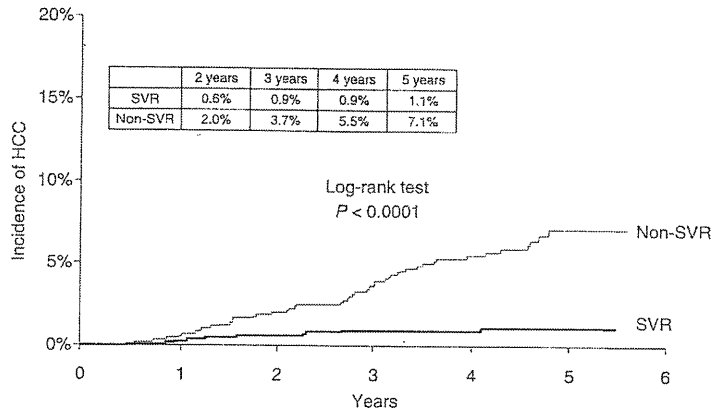


Figure 1 Onset of hepatocellular carcinoma (HCC) by therapeutic efficacy (1865 patients) (sustained virological response [SVR] vs. non-virological response [NVR]). The cumulative incidence of HCC was calculated by the Kaplan–Meier method. The difference between SVR and non-SVR was examined using the log-rank test.

patients, ALT level at 24 weeks after the end of treatment was reduced to less or equal to 40 IU/L (biochemical response: BR) in 234 patients, and the remaining 280 patients had values of more than 40 IU/L (non-BR). There were significant differences between BR and non-BR patients in the background factors of pretreatment ALT level, age, hepatic fibrosis, platelet count, AFP, and treatment duration. Selected as the factors contributing to BR in non-SVR patients in the multivariate analysis were TR, long treatment duration, and high platelet count before the start of treatment (Table 3).

The 5-year cumulative incidence of HCC was 3.4% in BR patients and 11.0% in non-BR patients, and the difference in incidence was significant ($P = 0.0012$) (Fig. 3). The 5-year cumulative incidence of HCC in male patients was 3.6% in BR patients and 13.9% in non-BR patients, and the difference was significant ($P = 0.0012$). In female patients, however, it was 3.5% in BR patients and 7.6% in non-BR patients, and although the incidence of HCC was lower in BR patients, the difference was not significant ($P = 0.0706$).

Incidence of HCC in patients with normal pretreatment ALT levels

When the incidence of HCC was compared between SVR (288) and non-SVR (214) patients among 502 patients with pretreatment ALT levels less or equal to 40 IU/L, the 5-year cumulative incidence of HCC was 0% in SVR patients and 4.8% in non-SVR patients, indicating a significant difference ($P = 0.0005$) between the groups

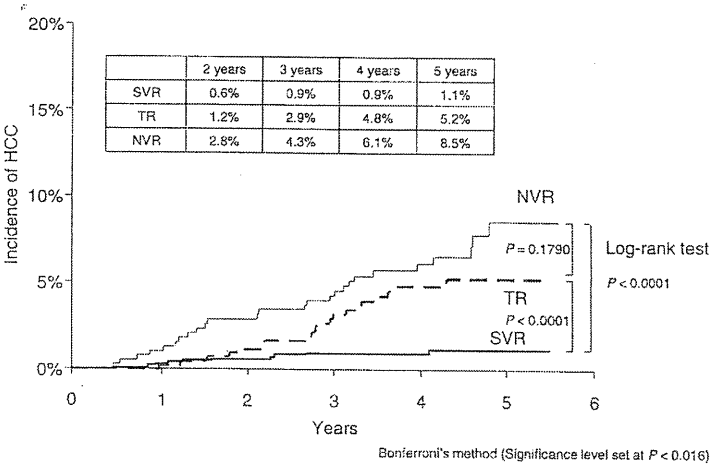


Figure 2 Onset of hepatocellular carcinoma (HCC) by therapeutic efficacy (sustained virological response [SVR] vs. transient response [TR] vs. non-virological response [NVR]). The cumulative incidence of HCC was calculated by the Kaplan–Meier method. The difference between each group was examined using the log-rank test (Bonferroni's Method, significance level set at $P < 0.016$).

Table 2 Factors contributing to hepatocellular carcinoma (all patients) Cox regression analysis (multivariate)

		Hazard ratio	95% confidence interval	*P*-value
Therapeutic efficacy	SVR	1		
	TR	2.055	0.709–5.955	0.1845
	NVR	2.985	1.036–8.601	0.0428
Sex	Male	1		
	Female	0.486	0.243–0.969	0.0405
Age	<60	1		
	≥60	2.005	1.035–3.883	0.0391
ALT at 24 weeks after end of treatment (IU/L)	≤40	1		
	>40	3.940	1.754–8.850	0.0009
Platelet count (×10 000/mm^3)	<10	1		
	≥10	0363	0.169–0.779	0.0093
Serum albumin (g/dL)	<4	1		
	≥4	0.594	0.310–1.140	0.1175

Factors examined: Of the 15 factors exhibiting $P < 0.2$ by log-rank test (therapeutic efficacy [1: SVR, 2: TR, 3: NVR], genotype [1: 1, 2: 2 or 3], sex [1: male, 2: female], age [1: <60, 2: ≥60], pre ALT [1: ≤40, 2: >40], +24 w ALT [1: ≤40, 2: >40], pre PLT [1: <10, 2: ≥10], pre ALB [1 <4, 2: ≥4], pre AFP [1: <20, 2: ≥20], grade [1: A0–1, 2: A2–3], stage [1: F0–1, 2: F2–4], hypertension [1: absent, 2: present], diabetes [1: absent, 2: present], heavy drinking [1: absent, 2: present], and treatment duration [1: ≤48 W, 2: >48 W]), nine factors were examined. Excluded were factors for which approximately 30% of values were missing (AFP, grade, stage, diabetes, hypertension, and heavy drinking).
AFP, alpha fetoprotein; ALB, albumin; ALT, alanine aminotransferase; NVR, non-virological response; PLT, platelet count; SVR, sustained virological response; TR, transient response.

(Fig. 4). This tendency is also observed with the 280 patients having pretreatment ALT levels of less or equal to 30 IU/L.

Onset of HCC in SVR patients

Hepatocellular carcinoma developed in 10 patients who achieved SVR. Multivariate analysis indicated that in SVR patients, the ALT level at 24 weeks after the end of treatment was the only significant factor contributing to HCC ($P = 0.0007$) (Table 4). In SVR patients with an ALT level of more than 40 IU/L at 24 weeks after the end of treatment, the 5-year cumulative incidence of HCC was 5.6% while the incidence in patients with an ALT level of less or equal to 40 IU/L was 0.7%, indicating a significant difference ($P = 0.0004$) between the groups (Fig. 5).

DISCUSSION

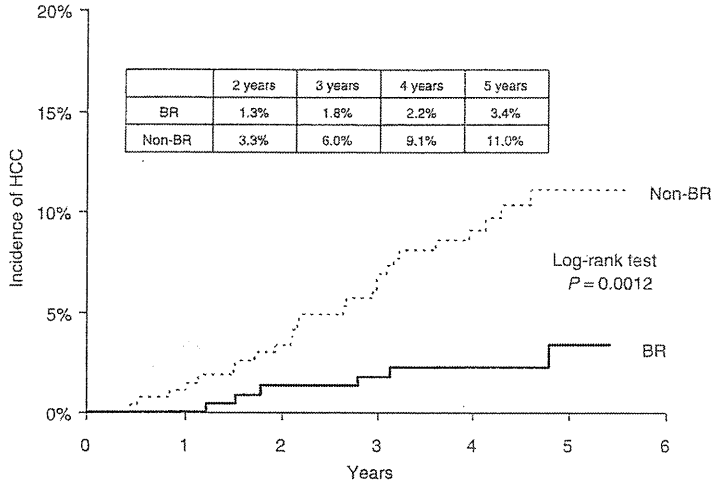
THIS STUDY INDICATED that the risk factors for HCC after PEG-IFN α-2b plus RBV combination therapy are NVR, male sex, older age, low platelet count, and an ALT level of more than 40 IU/L at 24 weeks after the end of treatment.

Kurokawa *et al.*[16] tracked 403 patients receiving PEG-IFN α-2b plus RBV combination therapy for a median

Table 3 Factors contributing to biochemical response in non-sustained virological response patients Logistic regression analysis (multivariate)

		Odds ratio	95% confidence interval	*P*-value
Virological response	NVR	1	1.480–3.203	0.0001
	TR	2.177		
Treatment duration	per week	1	1.000–1.022	0.0424
		1.011		
Platelet count	per 10 000/mm^3	1	1.018–1.099	0.0043
		1.058		

Factors examined were those exhibiting $P < 0.2$ by log-rank test: Genotype, virological response (TR/NVR), treatment duration, pre platelet count, diabetes, stage, and alanine aminotransferase (ALT).

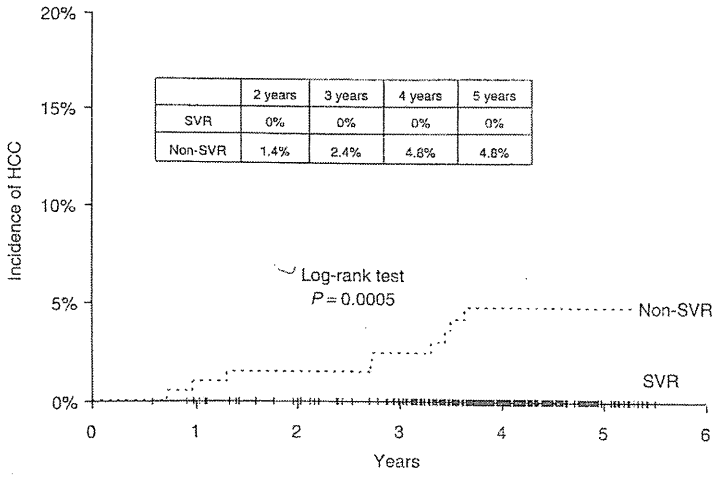


	2 years	3 years	4 years	5 years
BR	1.3%	1.8%	2.2%	3.4%
Non-BR	3.3%	6.0%	9.1%	11.0%

Figure 3 Alanine aminotransferase (ALT) normalization and hepatocellular carcinoma (HCC) in non-virological response [NVR] patients. The cumulative incidence of HCC was calculated by the Kaplan–Meier method. Log-rank test was used to study the difference between biochemical response (BR) and non-BR.

duration of 36.5 months and reported that in multivariate analysis, virological efficacy (SVR vs. non-SVR), age, and hepatic fibrosis were selected as the factors contributing to HCC. Arase *et al.*[15] tracked 500 patients 60 years of age and older receiving IFN alone or in combination with RBV for an average duration of 7.4 years and also reported that the factors contributing to HCC are virological efficacy (SVR vs. non-SVR), age, and hepatic fibrosis. In our study, hepatic fibrosis was not tested with multivariate analysis because more than 30% of values were missing, but it was selected as a significant factor in the univariate analysis. Platelet count was selected in multivariate analysis, and the results in our study are therefore considered to be generally consistent with these reports.

The results of the present study indicated no significant difference between TR and NVR in non-SVR in stratified cumulative incidence of HCC, and although there was a significant difference between SVR and both TR and NVR, TR was not significant against SVR in multivariate analysis, and NVR was the only significant factor. Kurokawa *et al.*[16] reported the same results by



	2 years	3 years	4 years	5 years
SVR	0%	0%	0%	0%
Non-SVR	1.4%	2.4%	4.8%	4.8%

Figure 4 Therapeutic efficacy and hepatocellular carcinoma (HCC) in patients with pretreatment alanine aminotransferase (ALT) of ≤40. The cumulative incidence of HCC was calculated by the Kaplan–Meier method. Log-rank test was used to study the difference between sustained virological response (SVR) and non-virological response (NVR).

Table 4 Factors contributing to hepatocellular carcinoma (sustained virological response [SVR] patients) Cox regression analysis (multivariate)

		Hazard ratio	95% confidence interval	*P*-value
ALT at 24 weeks after end of treatment (IU/L)	≤40	1		
	>40	16.054	3.235–79.681	$P = 0.0007$
Serum albumin (g/dL)	<4	1		
	≥4	0.196	0.036–1.073	$P = 0.0603$

Factors examined: Of the 10 factors exhibiting $P < 0.2$ by log-rank test (Genotype [1: 1, 2: 2 or 3], age [1: <60, 2: ≥60], pre ALT [1: ≤40, 2: >40], +24 w ALT [1: ≤40, 2: >40], pre PLT [1: <10, 2: ≥10], pre ALB [1: <4, 2: ≥4], pre AFP [1: <20, 2: ≥20], grade [1: A0–1, 2: A2–3], stage [1: F0–1, 2: F2–4], and diabetes [1: absent, 2: present]), 5 factors were examined. Excluded were pre ALT, with which HCC did not occur in the ≤40 group, and AFP, grade, stage, and diabetes, the factors for which approximately 30% of values were missing.
ALB, albumin; ALT, alanine aminotransferase; PLT, platelet count;

comparing cumulative incidences of HCC among SVR, TR and NVR (the results of multivariate analysis are not known). On the other hand, Morgan *et al.*,[19] in their follow-up study of the HALT-C Trial, reported that there was no difference between TR and NVR in the incidence of HCC or death related to hepatic disease/liver transplantation, but when all hepatic-related outcomes were examined, a significantly superior inhibition was observed with TR compared to NVR. Our results also demonstrate that although the difference is not significant, the cumulative incidence of HCC is lower in TR patients than in NVR patients, especially in male patients (5-year cumulative incidence of HCC: 6.0% vs. 10.7%). It is therefore necessary to continue to observe this for an extended number of years.

Our results study indicated that in non-SVR patients, whether or not ALT level is normalized after treatment is a greater contributing factor for the onset of HCC than virological response. Normalization of ALT has already been reported to contribute to the inhibition of the onset of HCC even under HCV-positive conditions,[13,20] and this was found to apply also to non-SVR patients receiving PEG-IFN α plus RBV combination therapy.

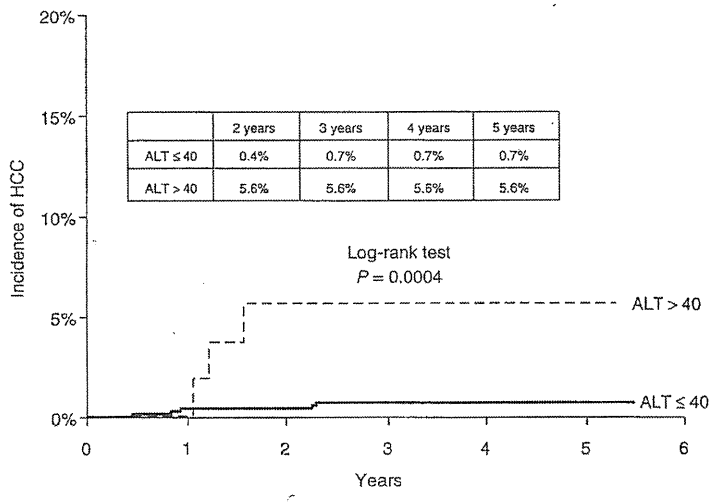


Figure 5 Alanine aminotransferase (ALT) levels at 24 weeks after end of treatment and hepatocellular carcinoma (HCC) in patients with sustained virological response (SVR). The cumulative incidence of HCC was calculated by the Kaplan–Meier method. Log-rank test was used to study the difference between SVR patients with an ALT level of more than 40 IU/L at 24 weeks after the end of treatment and those with an ALT level of less or equal to 40 IU/L.

Our investigation also indicated that abnormal ALT levels also contribute to the onset of HCC in SVR patients. In multivariate analysis, the only contributing factor to the development of HCC in SVR patients was ALT levels at 24 weeks after the end of treatment. However, the onset of HCC is also observed in patients who achieve ALT normalization after treatment, and it is therefore difficult to conclude that ALT is the only risk factor for the onset of HCC in SVR patients. The potential involvement of hepatic fibrosis as well as hepatic steatosis, which persists after viral clearance[21] and small amounts of virus remaining in the liver[22] have also been suggested as risk factors for the onset of HCC in SVR patients. Further detailed investigation is therefore necessary. Nevertheless, regardless of whether or not SVR is achieved, it is clear that abnormal ALT is a factor affecting the onset of HCC. Careful monitoring of changes in ALT and instituting measures to normalize ALT are therefore important regardless of whether or not SVR is achieved.

With the administration of PEG-IFN α plus RBV combination therapy tailored for individual patients and the addition of direct-acting antivirals to current combination therapy, the therapeutic outcomes for CHC will continue to further improve, and the number of patients who develop hepatic cirrhosis and HCC from hepatitis C can be expected to decrease in the future. HCC can occur even in patients achieving SVR, and even if SVR is not achieved, as long as the possibility to inhibit the onset of HCC remains, there will be a need for various treatment innovations to achieve the prevention of HCC, the ultimate goal of treatment of CHC.

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APPENDIX I

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ORIGINAL ARTICLE

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

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Abstract
Background and aims Protease inhibitor (PI)-resistant hepatitis C virus (HCV) variants may be present in substantial numbers in PI-untreated patients according to recent reports. However, influence of these viruses in the clinical course of chronic hepatitis C has not been well characterized.
Methods The dominant HCV nonstructural 3 (NS3) amino acid sequences were determined in 261 HCV genotype 1b-infected Japanese patients before pegylated interferon plus ribavirin (PEG-IFN/RBV) therapy, and investigated the patients' clinical characteristics as well as treatment responses including sustained virological response (SVR) rate. HCV-NS3 sequences were also determined in 39 non-SVR patients after completion of the therapy.
Results Four single mutations (T54S, Q80K, I153V, and D168E) known to confer PI resistance were found in 35 of 261 patients (13.4%), and double mutations (I153V plus T54S/D168E) were found in 6 patients (2.3%). Responses to PEG-IFN/RBV therapy did not differ between patients with and without PI-resistance mutations (mutation group, SVR 48%; wild-type group, SVR 40%; $P = 0.38$). On the other hand, two mutations appeared in two non-SVR patients after PEG-IFN/RBV therapy (I153V and E168D, 5.1%).
Conclusions PI-resistance-associated NS3 mutations exist in a substantial proportion of untreated HCV-1b-infected patients. The impact of these mutations in the treatment of PIs is unclear, but clinicians should pay attention to avoid further development of PI resistance.

Keywords HCV · Protease inhibitor · Naturally occurring viral resistance mutations

Introduction

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

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

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

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

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

Methods

Patients

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

Amplification and sequencing of full-length HCV genomes

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

Sequence alignment and analysis

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

Determination of PI resistance mutations

Multiple viral NS3 mutations were observed in amino acid positions reported to confer PI resistance among 261 patients: V36, Q41, F43, T54, V55, Q80, R109, I153, R155, A156, D168, V170, and M175. NS3 amino acid mutations with proven PI resistance in previously published studies (Table 1) were designated as resistance proven mutations (e.g., V36M/A). Mutations in the PI-resistance site not known to confer drug resistance were designated resistance unproven mutations (e.g., V36I). Patients were allocated to two groups according to the presence of PI-resistance

mutations (including resistance unproven mutations), and clinical characteristics including HCV RNA levels and responses to PEG-IFN/RBV therapy were compared. To assess the influence of PEG-IFN/RBV therapy on NS3 mutational status, posttreatment HCV-NS3 sequences in 39 of 58 non-SVR patients were also examined.

Statistical analysis

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

Results

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

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

Clinical characteristics of patients with PI-resistance mutations

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

Table 1 Prevalence of PI-resistance-associated NS3 mutations

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

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

Double mutations found were as follows: V36I and I153V × 1, T54S and I153V × 4, I153V and D168E × 2

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

Unimpaired in vivo fitness of viral strains with resistance mutations

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

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

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

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

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

Discussion

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

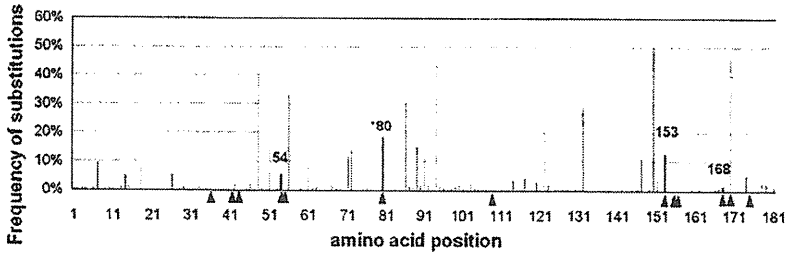

Fig. 1 Frequency of polymorphic mutations for each of the 181 NS3 protease amino acid residues in 261 patients. *Arrowheads* indicate the sites reported to confer PI resistance. *Dark bars* denote the amino acid variations at the resistant sites in this study. **80*, we detected one resistant mutation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

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

Characteristics	Mutation type ($N = 47$)	Wild-type ($N = 214$)	P value
Patients' characteristics			
Age, median (range)	59 (46–72)	57 (19–77)	0.17
Male, no. (%)	26 (55)	112 (52)	0.70
BMI, median (range)	23.2 (15.5–31.9)	22.8 (16.1–31.9)	0.41
ALT IU/ml	81.3 ± 72.6[a]	74.8 ± 51.9	0.93
Serum albumin g/dl	4.00 ± 0.37	4.01 ± 0.36	0.81
Platelet count × $10^4/\mu l$	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
IRRDR mutation >3 no. (%)	25/38 (66)	107/190 (56)	0.34

[a] Mean ± SD

[b] Number/total number (%)

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

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

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

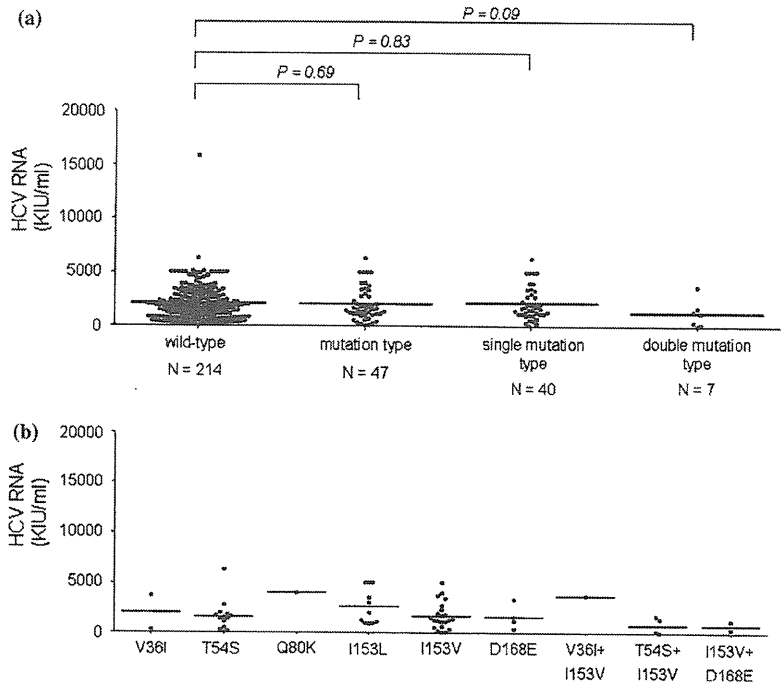

Fig. 2 In vivo fitness of HCV with PI-resistance-associated NS3 mutations. HCV RNA levels were compared between patients with and without NS3 PI-resistance-associated mutations (a) and between patients with each resistance mutation (b). The estimated P value (Mann–Whitney U test) indicates no significant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

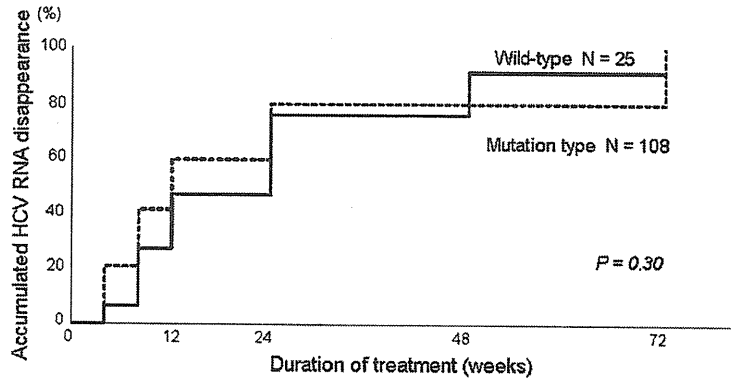

Fig. 3 Comparison of virologic response to PEG-IFN/RBV therapy between HCV-infected patients with and without PI-resistance-associated NS3 mutations. Time-dependent HCV clearance rate analysis was based on serum HCV RNA positivity during PEG-IFN/RBV therapy for HCV isolates with resistance mutations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

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

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

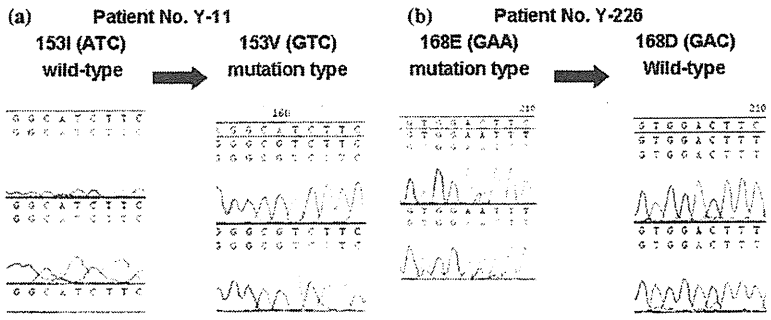

Fig. 4 Appearance of PI-resistance-associated NS3 mutations during the PEG-IFN/RBV therapy. Chromatograms show part of the HCV NS3 sequence demonstrating PI-resistance mutations in two patients receiving therapy. **a** Site 153 isoleucine (Ile) (ATC) changed to valine (Val) (GTC), **b** Site 168 glutamic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

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

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

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

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

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

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