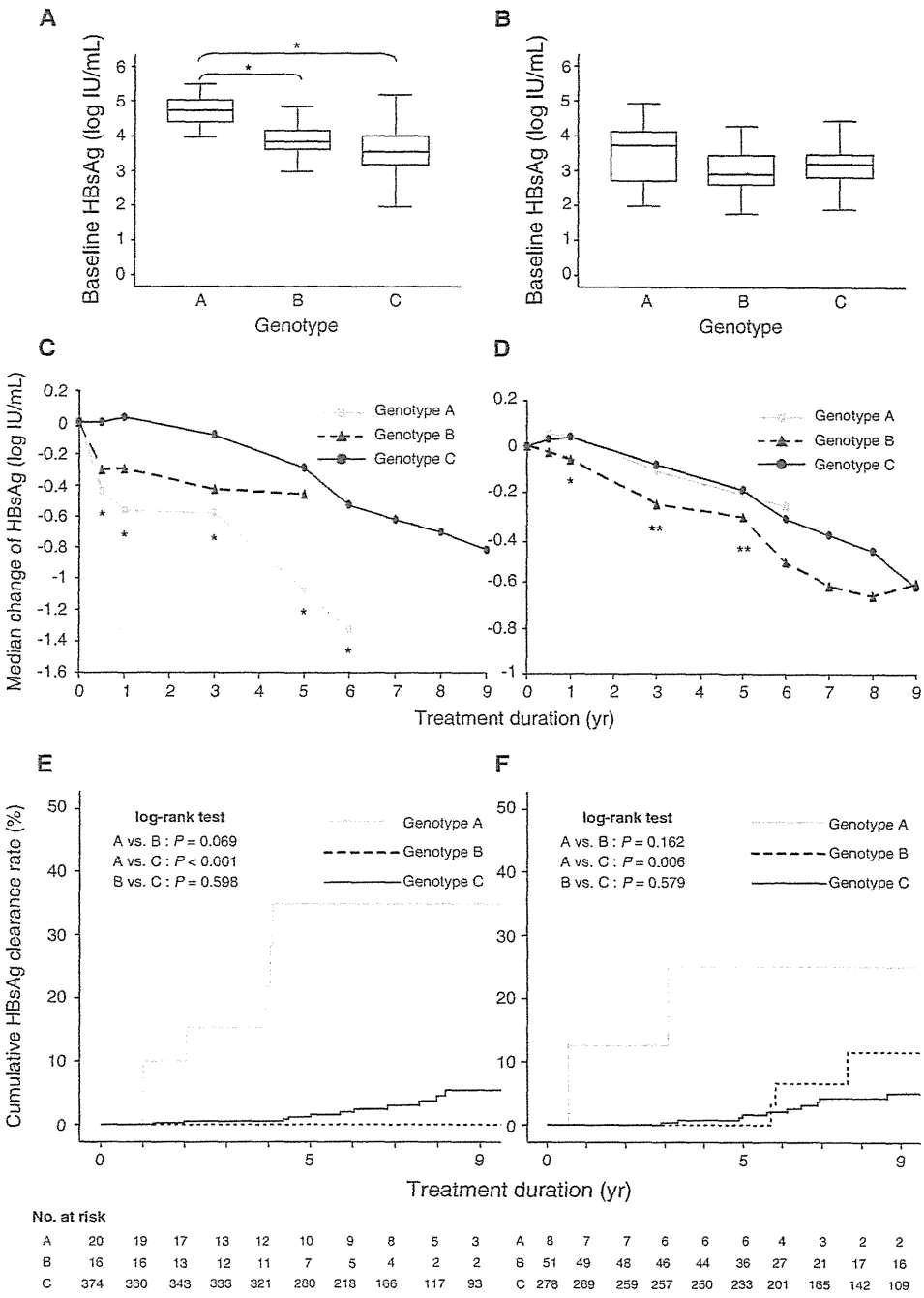

Fig. 2 **a** Box plot of baseline HBsAg levels in patients with different HBV genotypes (HBeAg+ cohort). The *asterisk* (*) indicates a statistical significance of $P < 0.001$, as determined by the Mann–Whitney U test and Bonferroni correction. **b** Box plot of baseline HBsAg levels in patients with different HBV genotypes (HBeAg− cohort). **c** Median change in HBsAg level from baseline in patients with different HBV genotypes (HBeAg+ cohort). A *single asterisk* (*) indicates $P < 0.001$, as determined by the Kruskal–Wallis test. **d** Median change in HBsAg level from baseline in patients with different HBV genotypes (HBeAg− cohort). A *single asterisk* (*) indicates $P < 0.001$ and a *double asterisk* (**) indicates $P < 0.02$, as determined by the Kruskal–Wallis test. **e** Kaplan–Meier life table showing cumulative HBsAg clearance rates in patients with different HBV genotypes (HBeAg+ cohort). Cumulative HBsAg clearance rates were significantly higher among patients with genotype A (log-rank test; A vs. B: $P = 0.069$, A vs. C: $P < 0.001$, B vs. C: $P = 0.598$, after Bonferroni correction). **f** Kaplan–Meier life table showing cumulative HBsAg clearance rates in patients with different HBV genotypes (HBeAg− cohort). Cumulative HBsAg clearance rates were significantly higher among patients with genotype A (log-rank test; A vs. B: $P = 0.169$, A vs. C: $P = 0.006$, B vs. C: $P = 0.579$, after Bonferroni correction)

patients with genotype C (Fig. 2f). Clearance rates were significantly higher in patients with genotype A than in those with genotype C ($P < 0.001$ in the HBeAg+ cohort, $P = 0.006$ in the HBeAg− cohort).

Association between on-treatment response and subsequent HBsAg clearance

We stratified patients into three groups according to the amount of HBsAg decline within the first six months of treatment; this allowed us to evaluate the impact of on-treatment response factors on the clearance of HBsAg. The stratifications were as follows: rapid decline (≥1.0 log IU/mL), intermediate decline (0.5–1.0 log IU/mL), and slow decline or steady (<0.5 log IU/mL). Cumulative HBsAg clearance rates in the HBeAg+ cohort were 11 % at year 3, and 40 % at year 5 in the rapid decline group; 0 % at year 3, 2.2 % at year 5, and 13 % at year 9 in the intermediate decline group; and 0 % at year 3, 0 % at year 5, and 2.9 % at year 9 in the slow decline or steady group (Fig. 3a).

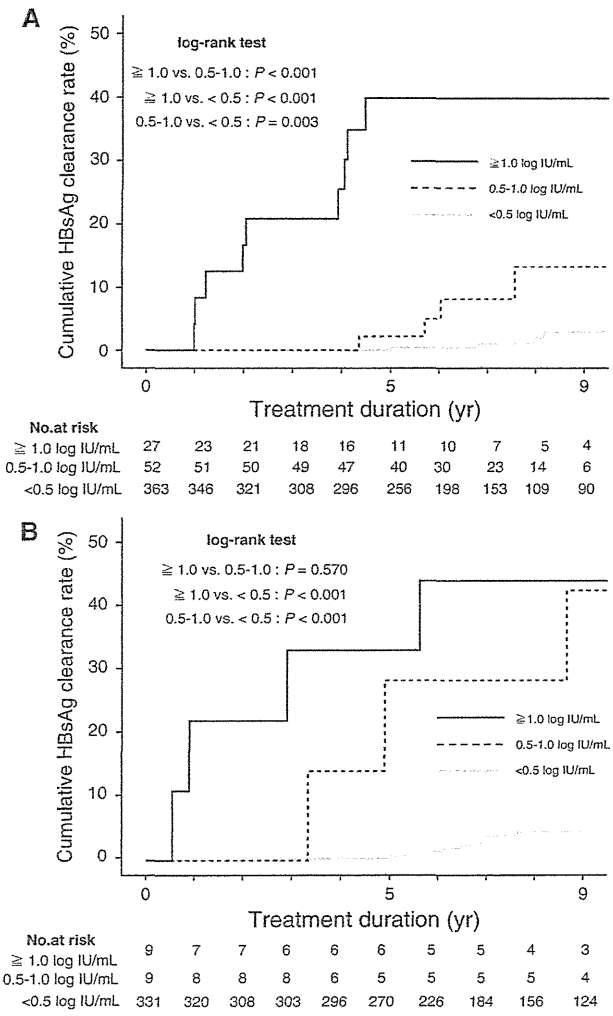

Fig. 3 **a** Kaplan–Meier life table showing cumulative HBsAg clearance rates in patients with varying rates of HBsAg decline within the first six months (HBeAg+ cohort). Clearance rates were highest in the rapid decline group, followed by the intermediate decline group and the slow or steady group (log-rank test; rapid vs. intermediate: $P < 0.001$, rapid vs. slow: $P < 0.001$, intermediate vs. slow: $P = 0.003$, after Bonferroni correction). **b** Kaplan–Meier life table showing cumulative HBsAg clearance rates in patients with varying rates of HBsAg decline within the first six months (HBeAg− cohort). Clearance rates were highest in the rapid decline group, followed by the intermediate decline group and the slow or steady group (log-rank test; rapid vs. intermediate: $P = 0.570$, rapid vs. slow: $P < 0.001$, intermediate vs. slow: $P < 0.001$, after Bonferroni correction)

Cumulative HBsAg clearance rates in the HBeAg− cohort were 33 % at year 5, and 44 % at year 7 in the rapid decline group; 0 % at year 3, 29 % at year 5, and 43 % at year 9 in the intermediate decline group; and 0.3 % at year 3, 0.7 % at year 5, and 4.6 % at year 9 in the slow decline or steady group (Fig. 3b). Clearance rates were highest in the rapid decline group, followed by the intermediate decline group and the slow or steady group in both the HBeAg+ and HBeAg− cohorts. The decline of HBsAg within the first six months was a strong predictor of HBsAg clearance.

Viral breakthrough and subsequent HBsAg clearance

Although VBT was not associated with HBsAg clearance in the multivariate model, as described above, HBsAg clearance was observed in ten patients who experienced VBT (five patients in the HBeAg+ cohort and five in the HBeAg− cohort). All ten patients achieved clearance of HBsAg after VBT occurred. Six of these patients received ADV added on to LAM for VBT, and subsequently achieved clearance of HBsAg (five patients in the HBeAg+ cohort and one in the HBeAg− cohort). The other four patients spontaneously recovered from VBT while continuing to receive LAM monotherapy, and subsequently achieved clearance of HBsAg (one patient in the HBeAg+ cohort and three in the HBeAg− cohort). LAM-resistant mutant strains (M204I/V mutants) were detected in nine patients in whom VBT occurred. HBV DNA negativity continued for the follow-up period after HBsAg clearance in these ten patients. The typical clinical and virological courses of two representative patients who achieved HBsAg clearance after VBT are shown in Fig. 4a, b.

Virological courses after discontinuation of NAs

Sixteen (42.1 %) of 38 patients with HBsAg clearance discontinued NA treatment due to HBsAg clearance. Median interval between HBsAg clearance and discontinuation of NAs was nine months (range 2–29 months). Median follow-up period after discontinuation of NAs was 24 months (range 7–171) in these patients. No relapses of serum HBsAg or HBV DNA were observed during the follow-up period. Serum anti-HBs appeared in 12 (75 %) of the 16 patients who discontinued NAs. Median time to the appearance of anti-HBs after HBsAg clearance was 16 months (range 2–92) in patients who discontinued NAs. Two of 22 patients who continued NAs with HBsAg clearance had the appearance of anti-HBs, and median time to the appearance of anti-HBs after HBsAg clearance was two and seven months in these two patients, respectively.

Discussion

We found that three baseline factors and two on-treatment response factors are associated with HBsAg clearance in patients who begin treatment with LAM and continue with long-term NA therapy. HBV genotype and the decline in HBsAg over the first six months were associated with

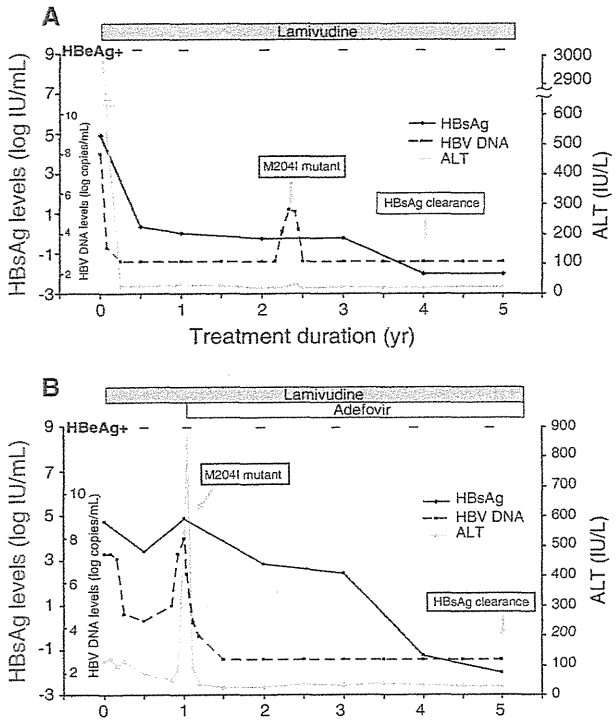

Fig. 4 Case presentation of the typical clinical and virological courses of two representative patients who achieved HBsAg clearance after VBT occurred. **a** Patient 1, a 45-year-old man who was HBeAg+ at baseline and had genotype A. **b** Patient 2, a 38-year-old man who was HBeAg+ at baseline and had genotype A. *VBT* virological breakthrough

HBsAg clearance in both the HBeAg+ and − cohorts, whereas the clearance of HBsAg was associated with previous IFN therapy and the clearance of HBeAg over the first six months only in the HBeAg+ cohort, and baseline HBsAg levels only in the HBeAg− cohort.

HBV genotype was recently reported to influence declines in and the clearance of HBsAg among patients who underwent PEG-IFN therapy [31]. In one study where negativity for serum HBV DNA and seroconversion of HBeAg represented the study end point, genotype was not found to influence response to NA therapy [31]. However, other reports have indicated that genotype does impact on declines in and the clearance of HBsAg [20, 29]. Heathcote et al. [20] reported that 20 HBeAg+ patients (8 %) who were treated with tenofovir achieved HBsAg clearance in three years. Twelve (60 %) of 20 patients were infected with genotype A and the others with genotype D. In this study, cumulative HBsAg clearance rates were 15 % at year 3 in HBeAg+ patients with genotype A. This result seems to be similar regardless of the antiviral potential. Previous studies with more ethnically diverse study populations than ours found that HBsAg clearance rates were highest in patients with genotype A. The similarity between those results and ours implies that the HBV genotype is more influential than ethnicity on HBsAg clearance during NA therapy. Of 28 genotype A patients in our population, the majority (79 %) did not have a family history of infection. Recent work has shown that sexual transmission of acute HBV genotype A infections is increasing in Japan, resulting in chronic HBV infection, especially in young adult patients [32, 33]. Cumulatively, these findings imply that HBsAg clearance is more likely in genotype A patients because they have been infected with HBV for a shorter period of time. Furthermore, Hou et al. [34] demonstrated that genotype A responded better than other HBV genotypes to IFN therapy. They revealed that a lower number of amino acid substitutions at baseline were associated with a better response to IFN therapy, and that this variable was linked with HBV genotype A, which had the lowest number of amino acid substitutions in the core gene among genotypes B, C, or D. Although amino acid substitutions in the core gene were not analyzed in this study, the relation between the core gene and treatment responses of NAs is necessary to be investigated in the future.

Although Gish et al. [19] reported that previous IFN therapy is not associated with HBsAg clearance in patients who are HBeAg+, the opposite was true in our HBeAg+ cohort. These contradictory findings may result from the fact that their patients received NA therapy over a much shorter time period (median duration 23 vs. 75 months, a 3.2-fold difference). We believe that there are two main reasons why HBsAg clearance rates were higher in patients who had previously received IFN therapy: the influence of AST/ALT flares after IFN therapy and changes in host immune response to HBV as a result of the immune-modulating activity of IFN. It has previously been shown that in patients with high baseline ALT levels, HBV DNA and HBeAg are likely to rapidly decrease during NA therapy [35, 36]. In this study, HBsAg clearance was likely to occur in patients who had high ALT levels at baseline, and in patients with previous IFN therapy (Table 2) in the HBeAg+ cohort. High virological responses have been reported in response to robust ALT flares induced by IFN therapy [37, 38]. Moreover, Wursthorn et al. [29] recently indicated that the antiviral potential of NAs and antiviral T cell reactivity are associated with HBsAg clearance in response to telbivudine treatment. These findings may be also associated with the achievement of HBsAg clearance after VBT occurs. Taken together, these results imply that both direct antiviral potential and host immune response are needed to achieve HBsAg clearance, especially in HBeAg+ patients.

We found that the initial HBsAg reduction was a strong predictor of subsequent HBsAg clearance during NA therapy, which supports a similar previous finding [29]. HBsAg reduction over the initial six months is important

for predicting the subsequent HBsAg kinetics in both HBeAg+ and HBeAg− patients. The novel finding in this study was that HBeAg− individuals achieved HBsAg clearance. We found that the median duration to HBsAg clearance was longer in patients with HBeAg− than in those who were HBeAg+ in this study (6.0 vs. 4.4 years). Manesis et al. [28] used modeling to determine that HBeAg− patients receiving LAM treatment would likely require >10 years to achieve HBsAg loss. Furthermore, baseline HBsAg titers were <730 IU/mL in 60 % (12/20) of HBeAg− patients who achieved HBsAg clearance. The only baseline predictive factor of HBsAg clearance was baseline HBsAg levels in HBeAg− patients, except for genotype. There was no difference in HBsAg clearance rates in HBeAg− patients with high- and low-baseline HBV DNA or ALT levels. We hypothesize that HBsAg clearance in these patients may result from long treatment duration and low HBsAg titers.

Our study was limited by the fact that it was a hospital-based retrospective analysis, which means there may be some bias associated with patient type and treatment selection. We were unable to compare HBsAg clearance rates obtained in our study with those of controls untreated with NA. Because all subjects in the study received LAM as an initial NA, and then received rescue therapy when drug-resistant mutations emerged, NA therapy regimens were not uniform across all patients, and there were variations in both treatment dose and duration of previous IFN therapy. We were not able to collect immunological data on our subjects. Finally, our results need to be validated by further studies investigating a large study population receiving long-term ETV or tenofovir with high antiviral potential and a high genetic barrier.

Despite these drawbacks, we were able to determine several factors associated with HBsAg clearance, including HBV genotype and a decline in HBsAg over the initial six months of treatment (HBeAg+ and − cohorts); previous IFN therapy and clearance of HBeAg over the initial six months of treatment (HBeAg+ cohort only); and HBsAg levels (HBeAg− cohort only). It seems that both direct antiviral potential and host immune response are needed to achieve HBsAg clearance by NA therapy. Future studies are needed to validate these findings and to develop treatment regimens for HBsAg clearance in patients with chronic hepatitis B.

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Conflict of interest Dr. Kumada reports having received investigator, lecture, and consulting fees from Bristol–Myers Squibb, Dainippon Sumitomo Pharma Co., MSD K.K., and Toray Co. Dr. Ikeda reports having received investigator, lecture, and consulting fees from Dainippon Sumitomo Pharma Co. No other potential conflicts of interest relevant to this article were reported.

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Prediction of Treatment Efficacy and Telaprevir-Resistant Variants after Triple Therapy in Patients Infected with Hepatitis C Virus Genotype 1

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It is often difficult to predict the response to telaprevir-pegylated interferon (PEG-IFN)-ribavirin triple therapy and the appearance of telaprevir-resistant variants. The present study determined the predictive factors of a sustained virological response (SVR) to 12- or 24-week triple therapy (T12PR12 or T12PR24, respectively) in 194 Japanese patients infected with hepatitis C virus genotype 1b (HCV-1b). The study also evaluated whether ultradeep sequencing technology can predict at baseline the emergence of resistant variants after the start of therapy. Analysis of the data of the entire group indicated that an SVR was achieved in 78% of the patients. Multivariate analysis identified *IL28B* rs8099917 (genotype TT), the substitution of amino acid (aa) 70 (Arg70), response to prior treatment (naive or relapse), PEG-IFN dose (≥1.3 μg/kg of body weight), and treatment regimen (T12PR24) as significant determinants of SVR. Among patients of the T12PR24 group, 92% with genotype TT achieved an SVR, irrespective of a substitution at aa 70. In patients with the non-TT genotype, an SVR was achieved in 76% of those with Arg70, while only 14% of patients with the non-TT genotype, Gln70(His70), and nonresponse to ribavirin combination therapy achieved an SVR. Ultradeep sequencing was conducted for 17 patients who did not achieve an SVR to determine the emergence of resistant variants during therapy. *De novo* resistant variants were detected in 16 of 17 patients (94%), regardless of the variant frequencies detected at baseline. In conclusion, the results indicate that the response to triple therapy can be predicted by the combination of host, viral, and treatment factors and that it is difficult to predict at baseline the telaprevir-resistant variants that emerge during triple therapy, even with the use of ultradeep sequencing.

New strategies have been introduced recently for the treatment of chronic hepatitis C virus (HCV) infection based on the inhibition of protease in the nonstructural NS3/NS4 proteins of the HCV polyprotein. Of the new agents currently available, telaprevir (VX-950) is used for the treatment of chronic HCV infection (1). Three studies (PROVE1, PROVE2, and a Japanese study) showed that a 24-week regimen of triple therapy (consisting of telaprevir, pegylated interferon [PEG-IFN], and ribavirin) for 12 weeks followed by dual therapy (PEG-IFN and ribavirin) for 12 weeks (also called the T12PR24 regimen) achieved sustained virological response (SVR) (defined as negative HCV RNA lasting >24 weeks after withdrawal of treatment) rates of 61%, 69%, and 73%, respectively, for the three studies, in patients infected with HCV genotype 1 (HCV-1) (2–4). However, a recent study (PROVE3) showed lower SVR rates following the T12PR24 regimen (39%) in HCV-1-infected nonresponders to previous PEG-IFN-ribavirin therapy, and who did not achieve HCV RNA negativity during or at the end of the initial triple therapy (5).

Telaprevir-based therapy is reported to induce resistant variants in HCV (6, 7). Recent reports have described the advantages of ultradeep sequencing technology, including faster processing and large-scale sequencing, in addition to providing a better understanding of the dynamics of variants in HCV quasispecies (8–11). However, it is not clear at this stage whether such technology is useful for the prediction of the emergence of telaprevir-resistant variants during or after the administration of triple therapy.

Based on the above background, there is a need to determine the predictive factors of non-SVR to triple therapy with telaprevir-PEG-IFN-ribavirin before the use of this treatment in order to avoid the appearance of telaprevir-resistant variants. The aim of this study was to determine the predictive factors of SVR to triple therapy and the emergence of telaprevir-resistant variants during such therapy (using ultradeep sequencing technology) in patients infected with HCV genotype 1b.

MATERIALS AND METHODS

Study population. From May 2008 through April 2013, 332 consecutive patients infected with HCV were selected for triple therapy with telaprevir (MP-424 or Telavic; Mitsubishi Tanabe Pharma, Osaka, Japan), PEG-IFN-α-2b (PEG-Intron; MSD, Tokyo, Japan), and ribavirin (Rebetol; MSD, Tokyo) at the Department of Hepatology, Toranomon Hospital (located in metropolitan Tokyo). Subsequently, 194 of these patients received the triple therapy based on the following inclusion criteria: (i) diagnosis of chronic hepatitis C, (ii) HCV genotype 1b confirmed by sequence analysis, (iii) HCV RNA levels of ≥5.0 log IU/ml determined by the cobas TaqMan HCV test (Roche Diagnostics, Tokyo), (iv) age at study entry of 20 to 65 years, (v) follow-up duration of ≥24 weeks after the completion of triple therapy, (vi) no history of treatment with NS3/NS4A protease inhibitors, (vii) lack of decompensated liver cirrhosis and hepatocellular carcinoma (HCC), (viii) negativity for hepatitis B surface anti-

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TABLE 1 Profile and laboratory data at commencement of telaprevir, pegylated interferon, and ribavirin triple therapy in patients infected with HCV genotype 1b

Study or patient characteristics	Data
Demographic data	
No. of patients	194
Sex (no.)	
Male	117
Female	77
Age (median [range]) (yr)	56 (21–65)
Body mass index (median [range]) (kg/m^2)	22.7 (16.0–36.7)
Blood plasma levels (median [range])	
Viremia (log IU/ml)	6.7 (5.0–7.8)
Aspartate aminotransferase (IU/liter)	36 (15–118)
Alanine aminotransferase (IU/liter)	41 (12–175)
Albumin (g/dl)	3.9 (2.9–4.6)
Total bilirubin (mg/dl)	0.8 (0.2–2.0)
Gamma-glutamyl transpeptidase (IU/liter)	34 (3–240)
Creatinine (g/dl)	0.7 (0.4–1.1)
Leukocyte count (cells/mm^3)	4,800 (2,000–8,400)
Hemoglobin (g/dl)	14.4 (12.1–17.4)
Platelet count ($\times 10^4/mm^3$)	17.5 (8.9–33.8)
Alpha-fetoprotein (μg/liter)	4 (2–104)
Total cholesterol (mg/dl)	174 (112–301)
High-density lipoprotein cholesterol (mg/dl)	48 (20–117)
Low-density lipoprotein cholesterol (mg/dl)	97 (41–216)
Triglycerides (mg/dl)	97 (36–336)
Uric acid (mg/dl)	5.7 (2.0–8.6)
Fasting plasma glucose (mg/dl)	93 (64–169)
Treatment dose	
PEG-IFN-α-2b (median [range]) (μg/kg)	1.5 (0.9–1.7)
Ribavirin (median [range]) (mg/kg)	11.0 (4.3–15.8)
Telaprevir (median [range]) (mg/kg)	31.8 (14.5–59.2)
Telaprevir (no.)	
1,500 mg/day	74
2,250 mg/day	120
Treatment regimen (no.)	
T12PR12 group	20
T12PR24 group	174
Response to prior treatment (no.)	
Treatment naive/relapse to prior treatment	71
Relapse after prior treatment	78
No response to prior treatment	44
IFN monotherapy	10
IFN-ribavirin dual therapy	34
Unknown	1
Amino acid substitutions in HCV genotype 1b (no.)	
Core aa 70	
Arginine	128
Glutamine (histidine)	65
ND[a]	1
Core aa 91	
Leucine	104
Methionine	89
ND	1
ISDR of NS5A	
Wild type	155
Non-wild type	17
ND	22
IRRDR of NS5A	
≤5	144
≥6	38
ND	1
V3 of NS5A	
≤2	49
≥3	144
ND	1
IL28B genotype (no.)	
rs8099917 genotype	
TT	139
Non-TT	53
ND	2
ITPA genotype (no.)	
rs112735 genotype	
CC	147
Non-CC	47
Telaprevir-resistant variants by direct sequencing (no.)[b]	
V36	1
T54	6
R155	0
A156	1
V170	0

[a] ND, not determined.

[b] Telaprevir-resistant variants, detected by direct sequencing, included V36A/C/M/L/G, T54A/S, R155K/T/I/M/G/L/S/Q, A156V/T/S/I/G, and V170A.

gen (HBsAg), (ix) no evidence of HIV infection, (x) negative history of autoimmune hepatitis, alcohol liver disease, hemochromatosis, and chronic liver disease other than chronic hepatitis C, (xi) negative history of depression, schizophrenia or suicide attempts, angina pectoris, cardiac insufficiency, myocardial infarction, severe arrhythmia, uncontrolled hypertension, uncontrolled diabetes, chronic renal dysfunction, cerebrovascular disorders, thyroidal dysfunction that is uncontrollable by medical treatment, chronic pulmonary disease, allergy to medication, or anaphylaxis at baseline; pregnant or breastfeeding women or those open to becoming pregnant during the study and men with pregnant partners were excluded. The study protocol was in compliance with the Good Clinical Practice Guidelines and the 1975 Declaration of Helsinki and was approved by the institutional review board of Toranomon Hospital. Each patient received ample information about the goals and potential side effects of the treatment and their right to withdraw from the study. Each provided a signed consent form before participating in the trial.

The efficacy of treatment was evaluated by absence of HCV RNA at 24 weeks after the completion of therapy (i.e., SVR), as measured by the cobas TaqMan HCV test (Roche Diagnostics). Furthermore, failure to achieve an SVR was classified as nonresponse (if HCV RNA was detected during or at the end of treatment) or relapse (reelevation of viral load after the end of treatment, even when HCV RNA was negative at the end of treatment).

Twenty patients (10%) were assigned to a 12-week regimen of triple therapy (the T12PR12 group) and were randomly subdivided into two groups treated with either 1,500 mg/day or 2,250 mg/day of telaprevir to evaluate the treatment efficacy during 12 weeks of treatment. Sixty patients (31%) were allocated to a 24-week regimen of the same triple therapy described above followed by dual therapy of PEG-IFN and ribavirin for another 12 weeks (the T12PR24 group) to evaluate treatment efficacy according to the response to prior treatment. All subjects in the T12PR24

group were treated with telaprevir at 2,250 mg/day. Another group of 114 patients (59%) were treated as described above for the T12PR24 group, except for telaprevir dose, and were subdivided into two groups treated with either 1,500 mg/day or 2,250 mg/day of telaprevir, as prescribed by the attending physician. Table 1 summarizes the profiles and laboratory data of the entire group of 194 patients at the commencement of treatment. They included 117 males and 77 females, aged 23 to 65 years (median, 56 years). At the start of treatment, telaprevir was administered at a median dose of 31.8 mg/kg of body weight (range, 14.5 to 59.2 mg/kg) daily. Especially, 120 patients (62%) were treated with telaprevir at a dose of 2,250 mg/day, while the other 74 patients (38%) were treated with telaprevir at a dose of 1,500 mg/day. PEG-IFN-α-2b was injected subcutaneously at a median dose of 1.5 μg/kg (range, 0.9 to 1.7 μg/kg) once a week. Ribavirin was administered at a median dose of 11.0 mg/kg (range, 4.3 to 15.8 mg/kg) daily. Each drug was discontinued or its dose reduced, as required upon judgment of the attending physician, in response to a fall in hemoglobin level, leukocyte count, neutrophil count, or platelet count, or the appearance of side effects. Triple therapy was discontinued when the leukocyte count decreased to $<1{,}000/mm^3$, neutrophil count to $<500/mm^3$, or platelet count to $<5.0 \times 10^4/mm^3$, or when hemoglobin decreased to <8.5 g/dl.

Follow-up. Clinical and laboratory assessments were performed at least once every month before, during, and after treatment. Especially, they were performed every week in the initial 12 weeks of treatment. Adverse effects were monitored clinically by careful interviews and a medical examination at least once every month. Compliance with treatment was evaluated by a questionnaire.

Measurement of HCV RNA. The antiviral effects of triple therapy on HCV were assessed by measuring blood plasma HCV RNA levels. In this study, HCV RNA levels during treatment were evaluated at least once every month before, during, and after therapy. HCV RNA concentrations were determined using the cobas TaqMan HCV test (Roche Diagnostics). The linear dynamic range of the assay was 1.2 to 7.8 log IU/ml, and undetectable levels were defined as negative samples.

Determination of *IL28B* and *ITPA* genotypes. *IL28B* rs8099917 and *ITPA* rs112735 genotypes have been reported as predictors of treatment efficacy and side effects to PEG-IFN-ribavirin dual therapy, and they were genotyped by the Invader assay, TaqMan assay, or direct sequencing, as described previously (12–16).

Detection of amino acid substitutions in core and NS5A regions of HCV-1b. With the use of HCV-J (accession no. D90208) as a reference (17), the sequence of amino acids (aa) 1 to 191 in the core protein of HCV-1b was determined and compared with the consensus sequence constructed in a previous study to detect substitutions at aa 70 of arginine (Arg70) or glutamine/histidine (Gln70[/His70]) and aa 91 of leucine (Leu91) or methionine (Met91) (18). The sequence of aa 2209 to 2248 in the NS5A of HCV-1b (the interferon sensitivity determining region [ISDR]) reported by Enomoto and coworkers (19) was determined, and the numbers of aa substitutions in the ISDR were defined as wild-type (≤1) or non-wild-type (≥2) compared against HCV-J. Furthermore, the sequence of aa 2334 to 2379 in the NS5A of HCV-1b (IFN-ribavirin resistance-determining region [IRRDR]) reported by El-Shamy and coworkers (20), including the sequence of aa 2356 to 2379 referred to as variable region 3 (V3), was determined and compared with the consensus sequence constructed in a previous study. The numbers of aa substitutions in the IRRDR and V3 were divided into two groups for analysis (numbers of aa substitutions in the IRRDR of ≤5 and ≥6, and those in V3 of ≤2 and ≥3). In the present study, aa substitutions of the core region and NS5A-ISDR-IRRDR-V3 of HCV-1b were analyzed by direct sequencing.

Assessment of telaprevir-resistant variants. The genome sequence of the N-terminal 609 nucleotides (203 amino acids) in the NS3 region of HCV isolates from the patients was examined. HCV RNA was extracted from 100 μl of serum, and the nucleotide sequences were determined by direct sequencing and deep sequencing. The primers used to amplify the NS3 region were NS3-F1 (5′-ACA CCG CGG CGT GTG GGG ACA T-3′; nucleotides 3295 to 3316) and NS3-AS2 (5′-GCT CTT GCC GCT GCC AGT GGG A-3′; nucleotides 4040 to 4019) as the first (outer) primer pair and NS3-F3 (5′-CAG GGG TGG CGG CTC CTT-3′; nucleotides 3390 to 3407) and NS3-AS2 (sequence above) as the second (inner) primer pair (21). Thirty-five cycles of first and second amplifications were performed as follows: denaturation for 30 s at 95°C, annealing of primers for 1 min at 63°C, extension for 1 min at 72°C, and final extension at 72°C for 7 min. The PCR-amplified DNA was purified after agarose gel electrophoresis and then used for direct sequencing and ultradeep sequencing.

All patients were examined for telaprevir-resistant variants by direct sequencing before the start of triple therapy. Furthermore, patients who did not achieve an SVR were analyzed by ultradeep sequencing, at baseline and at the time of reelevation of viral loads. Telaprevir-resistant variants included V36A/C/M/L/G, T54A/S, R155K/T/I/M/G/L/S/Q, A156V/T/S/I/G, and V170A (22, 23).

Direct sequencing was analyzed by the dye-terminator method. Dideoxynucleotide termination sequencing was performed with the BigDye deoxy terminator v1.1 cycle sequencing kit (Life Technologies, Carlsbad, CA) (21). Sequence data were deposited in GenBank. Ultradeep sequencing was performed using the Ion personal genome machine (PGM) sequencer (Life Technologies). An Ion Torrent adapter-ligated library was prepared using an Ion Xpress Plus fragment library kit (Life Technologies). Briefly, 100 ng of fragmented genomic DNA was ligated to the Ion Torrent adapters P1 and A. The adapter-ligated products were nick translated and PCR amplified for a total of 8 cycles. Subsequently, the library was purified using AMPure beads (Beckman Coulter, Brea, CA), and the concentration was determined using the StepOnePlus real-time PCR (Life Technologies) and Ion Library quantitation kit, according to the instructions provided by the manufacturer. Emulsion PCR was performed using Ion OneTouch (Life Technologies) in conjunction with Ion OneTouch 200 template kit v2 (Life Technologies). Enrichment for templated Ion Sphere particles (ISPs) was performed using the Ion OneTouch enrichment system (Life Technologies), according to the instructions provided by the manufacturer. Templated ISPs were loaded onto an Ion 314 chip and subsequently sequenced using 130 sequencing cycles according to the Ion PGM 200 sequencing kit user guide. Total output read length per run is >10 Mb (0.5 m-TAG, 200-base read) (24). The results were analyzed with the CLC Genomics Workbench software (CLC bio, Aarhus, Denmark) (25).

We also included a control experiment to validate the error rates in ultradeep sequencing of the viral genome. In this study, the amplification products of the second-round PCR were ligated with plasmid and transformed in *Escherichia coli* in a cloning kit (TA Cloning; Invitrogen, Carlsbad, CA). A plasmid-derived NS3 sequence was used as the template by the control experiment. The fold coverage values evaluated per position for aa 36, aa 54, aa 155, aa 156, and aa 170 in the NS3 region were 359,379×, 473,716×, 106,435×, 105,979×, and 49,058×, respectively. Thus, using the control experiment based on a plasmid encoding an HCV NS3 sequence, amino acid mutations were defined as amino acid substitutions at a frequency of >0.2% of the total coverage. This frequency ruled out putative errors caused by the ultradeep sequence platform used in this study (26).

Statistical analysis. Nonparametric tests (chi-square test and Fisher's exact probability test) were used to compare the characteristics of the groups. Univariate and multivariate logistic regression analyses were used to determine those factors that significantly contributed to SVRs. The odds ratios (OR) and 95% confidence intervals (CI) were also calculated. All P values of <0.05 by the two-tailed test were considered significant. Variables that achieved statistical significance ($P < 0.05$) on univariate analysis were entered into multiple logistic regression analysis to identify significant independent predictive factors. The potential pretreatment factors associated with SVR included sex, age, body mass index, levels of viremia, aspartate aminotransferase, alanine aminotransferase, albumin, total bilirubin, gamma-glutamyl transpeptidase (GGT), and creatinine, leukocyte count, hemoglobin level, platelet count, alpha-fetoprotein level, total cholesterol, high-density lipoprotein cholesterol, low-density lipo-

protein cholesterol, triglycerides, uric acid, fasting blood plasma glucose, PEG-IFN dose/kg body weight, ribavirin dose/kg body weight, telaprevir dose/kg body weight, telaprevir dose/day, kind of treatment regimen, response to prior treatment, amino acid substitutions in the core region and NS5A-ISDR-IRRDR, *IL28B* genotype, *ITPA* genotype, and telaprevir-resistant variants. Statistical analyses were performed using the SPSS software (SPSS, Inc., Chicago, IL). Sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) were also calculated to determine the reliability of the predictors of response to therapy.

RESULTS

Virological response to therapy. Analysis of the entire group showed that 78% (151 of 194 patients) achieved an SVR. According to the treatment regimen, an SVR was achieved by 45% (9 of 20 patients) and 82% (142 of 174 patients) of the T12PR12 group and T12PR24 groups, respectively. Taking into consideration the response to prior treatment, in the 173 patients of the T12PR24 group, an SVR was achieved in 89% (54 of 61 patients), 89% (66 of 74 patients), and 55% (21 of 38 patients) of treatment-naive patients, patients who showed relapse after prior treatment, and nonresponders to prior treatment, respectively. Furthermore, SVRs were achieved by 100% (8 of 8 patients) and 43% (13 of 30 patients) of the nonresponders to prior IFN monotherapy and IFN-ribavirin dual therapy, respectively.

Predictors of SVR. Univariate analysis of the data of the entire group identified seven parameters that correlated significantly with SVR: *IL28B* rs8099917 (genotype TT) ($P < 0.001$), substitution of aa 70 (Arg70) ($P = 0.001$), response to prior treatment (naive or relapse) ($P < 0.001$), PEG-IFN dose (≥1.3 μg/kg) ($P = 0.004$), treatment regimen (T12PR24 group) ($P = 0.001$), platelet count (≥15.0 × 10^4/mm^3) ($P = 0.013$), and GGT (<50 IU/liter) ($P = 0.001$). Multivariate analysis that included the above variables identified 5 parameters that independently influenced SVR: *IL28B* rs8099917 (genotype TT) (OR, 9.52; $P < 0.001$), substitution of aa 70 (Arg70) (OR, 2.67; $P = 0.038$), response to prior treatment (naive or relapse) (OR, 3.80; $P = 0.007$), PEG-IFN dose (≥1.3 μg/kg) (OR, 35.5; $P < 0.001$), and treatment regimen (T12PR24 group) (OR, 12.8; $P < 0.001$) (Table 2).

TABLE 2 Multivariate analysis of factors associated with sustained virological response to telaprevir, pegylated interferon, and ribavirin triple therapy in patients infected with HCV genotype 1b

SVR-influencing factor	OR (95% CI)[a]	*P*
IL28B rs8099917 genotype		
Non-TT	1	
TT	9.52 (3.36–27.0)	<0.001
Substitution of aa 70		
Gln70 (His70)	1	
Arg70	2.67 (1.05–6.76)	0.038
Response to prior treatment		
Nonresponse	1	
Naive or relapse	3.80 (1.44–10.1)	0.007
PEG-IFN-α−2b dose (μg/kg of body weight)		
<1.3	1	
≥1.3	35.5 (6.37–198)	<0.001
Treatment regimen		
T12PR12 group	1	
T12PR24 group	12.8 (3.44–48.1)	<0.001

[a] OR, odds ratio; CI, confidence interval.

Host, viral, and treatment factors for prediction of non-SVR. Using data of the 172 patients of the T12PR24 group, we evaluated the ability to predict non-SVR by host factor (*IL28B* rs8099917 genotype), viral factor (substitution of aa 70), and treatment factor (response to prior treatment). With the combination of the rs8099917 non-TT genotype, Gln70 (His70), and nonresponse to ribavirin combination therapy, the sensitivity, specificity, PPV, and NPV for non-SVR were 38% (12 of 32 patients), 99% (138 of 140 patients), 86% (12 of 14 patients), and 87% (138 of 158 patients), respectively. These results indicate that using the combination of the above three predictors has high specificity, PPV, and NPV for the prediction of non-SVR.

The SVR rates using the combination of rs8099917 genotype, substitution of aa 70, and response to prior treatment are shown in Fig. 1. In 126 patients with the rs8099917 TT genotype, the degree of SVR was not significantly different between Arg70 (91% [86 of 95 patients]) and Gln70 (His70) (97% [29 of 30 patients]). In contrast, in 46 patients with the rs8099917 non-TT genotype, a significantly higher proportion of patients with Arg70 (76% [16 of 21 patients]) achieved an SVR than did patients with Gln70 (His70) (32% [8 of 25 patients]) ($P = 0.004$). Furthermore, in 25 patients with the rs8099917 non-TT genotype and Gln70 (His70), a lower proportion of nonresponders to ribavirin combination therapy (14% [2 of 14 patients]) tended to achieve an SVR than did other patients (55%[6 of 11 patients]) ($P = 0.081$). These results highlight three properties of triple therapy: (i) a high efficacy of triple therapy was seen in patients with the TT genotype who achieved an SVR at 92%, irrespective of substitution of aa 70, (ii) among patients with the non-TT genotype, 76% of those with Arg70 achieved an SVR, and (iii) only 14% of the patients with the three factors of the rs8099917 non-TT genotype, Gln70 (His70), and nonresponders to ribavirin combination therapy achieved an SVR.

Evolution of telaprevir-resistant variants over time detected by ultradeep sequencing. Between May 2008 and the end of September 2009, 17 patients (4 treatment-naive patients, 3 who had a relapse after prior ribavirin combination therapy, and 10 nonresponders to ribavirin combination therapy) did not achieve SVR with triple therapy, and they were analyzed for telaprevir-resistant variants by ultradeep sequencing at baseline and at the time of reelevation of viral load.

In 6 of 17 patients (35%), telaprevir-resistant variants were detected at baseline by ultradeep sequencing. In 4 of these 6 patients, a very low frequency of variants at baseline (0.2% of 32,413× coverage for V36A, 0.2% of 27,915× coverage for V36A, 0.2% of 26,230× coverage for T54A, and 0.4% of 29,881× coverage for V170A) were replaced after treatment by *de novo* high-frequency variants (97.2% of 36,757× coverage for V36C, 27.7% of 5,032× coverage for T54A, 50.2% of 15,487× coverage for A156S, and 99.6% of 14,757× coverage for A156T), respectively. In one of the 6 patients, very high-frequency variants of T54S (99.9% of 33,830× coverage) at baseline persisted during treatment as very high-frequency variants of T54S (99.7% of 26,348× coverage), and *de novo* very high-frequency variants of R155K (96.1% of 20,630× coverage) also emerged during treatment. In another patient, variants of T54A increased from very low frequency at baseline

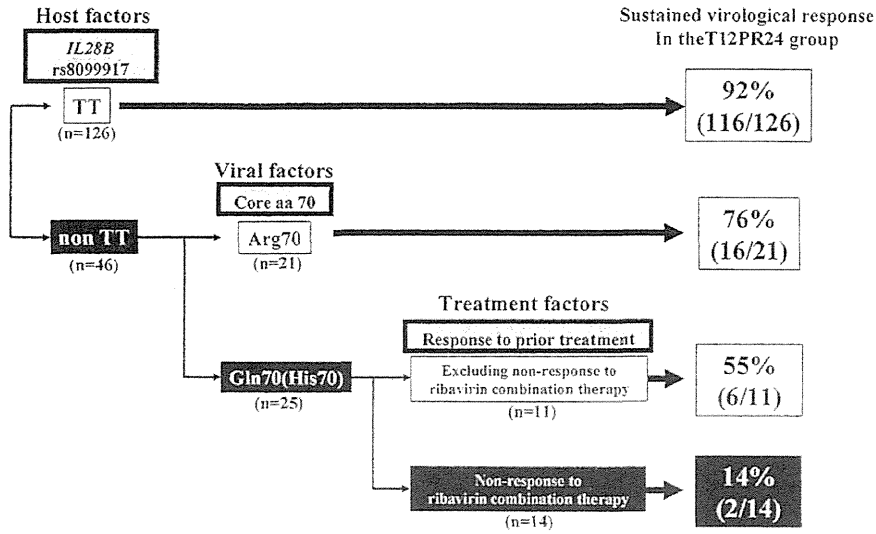

FIG 1 Prediction of sustained virological response (SVR) by the combination of *IL28B* rs8099917 genotype, substitution of aa 70, and response to prior treatment. In the T12PR24 group, treatment efficacy was high in patients with the TT genotype who achieved an SVR (92%), irrespective of the substitution of aa 70. In patients with the non-TT genotype, those with Arg70 achieved a high SVR (76%). Patients with the non-TT genotype, Gln70 (His70), and nonresponse to ribavirin combination therapy achieved the lowest frequency of an SVR (14%).

(0.2% of 53,127× coverage) to high frequency during treatment (99.9% of 45,240× coverage).

In the other 11 of 17 patients (65%), telaprevir-resistant variants were not detected by ultradeep sequencing at baseline, but *de novo* resistant variants were detected according to treatment (4 patients with V36A/C/M [median 41.5% of median 27,769× coverage], 8 with T54A/S [median 40.2% of median 27,067× coverage], 3 with R155K/Q [median 0.3% of median 17,847× coverage], and 8 with A156S/T [median 2.1% of median 18,150× coverage]).

Thus, in 16 of 17 patients (94%), *de novo* resistant variants were detected according to treatment. In other words, using ultradeep sequencing, the present study detected the emergence of *de novo* telaprevir-resistant variants regardless of variant frequencies at baseline, and the emergence of variants after the start of treatment could not be predicted at baseline.

DISCUSSION

Along with resulting in a high SVR, triple therapy is expensive and associated with serious side effects. Furthermore, employing ultradeep sequencing, the present study demonstrated the emergence of *de novo* telaprevir-resistant variants regardless of variant frequencies at baseline, and that the emergence of variants after the start of triple therapy could not be predicted at baseline. Hence, patients who failed to achieve an SVR with triple therapy need to be identified beforehand to avoid unnecessary side effects, high costs, and the emergence of telaprevir-resistant variants. Host genetic factors (e.g., *IL28B* genotype), and viral factors (e.g., amino acid substitutions in the core-NS5A region) have often been used as pretreatment predictors of poor virological response to PEG-IFN-ribavirin dual therapy (12, 14, 16, 18, 20) and telaprevir-PEG-IFN-ribavirin triple therapy (27, 28). The present study identified that the treatment efficacy of triple therapy could be predicted by the combination of host (*IL28B* rs8099917 genotype), viral (substitution of aa 70), and treatment (response to prior treatment, PEG-IFN dose, and T12PR24 regimen) factors. Especially, the use of the combination of rs8099917 non-TT genotype, Gln70 (His70), and nonresponse to ribavirin combination therapy had high specificity, PPV, and NPV for the prediction of non-SVR in the T12PR24 group. Unfortunately, the lowest frequency of an SVR (14%) was in patients who possessed the above three factors (namely, the treatment-resistant group). Previous studies showed that IFN monotherapy reduced the risk of HCC (29–31). Furthermore, analysis of the Hepatitis C Antiviral Long-Term Treatment against Cirrhosis (HALT-C) cohort recently showed that long-term PEG-IFN monotherapy reduced the incidence of HCC among patients with cirrhosis who did not achieve an SVR after previous IFN treatment, with or without ribavirin (32). Thus, the present study suggests that the treatment-resistant group should be selected for IFN monotherapy to overcome the problem of telaprevir-resistant variants, and to reduce the risk of hepatocarcinogenesis.

Interestingly, ultradeep sequencing identified telaprevir-resistant variants at baseline in 5 patients (2 patients with V36A [0.2%], 2 with T54A [0.2%], and t1 with V170A [0.4%]) at a very low frequency, but the frequency of resistant variants did not increase over time, except for one patient with T54A in whom it increased from 0.2% at baseline to 99.9% during treatment. This finding may be due to one or more reasons. One reason is probably related to the high susceptibility of telaprevir-resistant variants to IFN. A previous study indicated that mice infected with a resistant strain (A156F [99.9%]) developed only low-level viremia, and the virus was successfully eliminated with IFN therapy (9). Furthermore, this finding probably suggests that a small number of mutant-type viral RNAs may be incomplete or defective, since a large proportion of viral genomes are thought to be defective due to the high replication and mutation rates of the virus (33). Further studies should be performed to evaluate the significance of the presence of low-frequency variants detected by ultradeep sequencing.

A recent study using the human hepatocyte chimeric mouse model and deep sequencing reported that the rapid emergence of *de novo* telaprevir-resistant HCV quasispecies was induced by mu-

tation of the wild-type strain of HCV *in vivo* (9). In the present study, ultradeep sequencing did not detect any telaprevir-resistant variants at baseline in 11 patients, although *de novo* resistant variants emerged in all 11 patients over time. The present clinical results based on patients who did not achieve an SVR provide evidence in support of a *de novo* emergence of telaprevir resistance that is induced by viral mutation.

The results of the present study should be interpreted with caution, since the study was performed in a small number of Japanese patients infected with HCV-1b. Any generalization of the results should await confirmation by a multicenter randomized trial based on a larger number of patients, including patients of other races and those infected with HCV-1a. Furthermore, the other limitation of the present study is that the existence of very low-frequency telaprevir-resistant variants was not investigated long after the cessation of therapy by ultradeep sequencing. Further large-scale studies using ultradeep sequencing should be performed to investigate the effects of telaprevir-resistant variants on the response to treatment using new drugs, including direct-acting antiviral agents.

In conclusion, this study, which is based on Japanese patients infected with HCV genotype 1b, indicated that the efficacy of triple therapy could be predicted by the combination of host, viral, and treatment factors. However, the present results show that it might be difficult to predict at baseline the emergence of telaprevir-resistant variants during triple therapy, even with the use of ultradeep sequencing. Further large-scale prospective studies are needed to investigate the pretreatment predictors of treatment efficacy and the emergence of telaprevir-resistant variants after triple therapy, and to develop more effective therapeutic regimens in patients infected with HCV genotype 1.

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Protocols

Comparative quantitative analysis of hepatitis C mutations at amino acids 70 and 91 in the core region by the Q-Invader assay

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A B S T R A C T

Hepatitis C virus (HCV) is a major worldwide public health problem, and mutations at amino acids 70 and 91 in the genotype 1b core region predict the effectiveness of combination therapy with peginterferon and ribavirin. An assay based on the Q-Invader technology was developed to determine the relative ratios of the mutant to wild-type virus with high sensitivity. The assay detected a minor type plasmid that constituted only 1% of a mixture of plasmids containing wild-type and mutant sequences. The calculated ratios agreed with those of the template DNA. A total of 123 serum samples of HCV in Japan were examined with the Q-Invader assay. The Q-Invader assay detected all of the mutations that were detected by direct sequencing and even some mutants that direct sequencing could not. PCR with mutant specific primers confirmed those mutations found by the Q-Invader assay and not by direct sequencing. The Q-Invader assay, thus, is a useful tool for detecting mutations at positions 70 and 91 in the HCV-1b core region.

Contents

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Table 1
Primer and Invader probe with the Q-Invader assay.

Target		Sequence (5′–3′)
Core 70	F-primer	CCTCGTGGAAGGCGACAACCTAT
	R-primer	GGCCADGGRTACCCRGGCTG
	p1 probe	CGCGCCGAGGCGRMRAKCYTTGG
	p2 probe	ACGGACGCGGAGTGRMRAKCYTTGG
	io probe	GCCCAGGHYCTRCCCTCGKBNA
core 91	F-primer	CCTGGGCTCAGCCYGGGTA
	R-primer	CGGGGTGACAGGAGCCATC
	p1 probe	CGCGCCGAGGYTRGGRTGGRCAG
	p2 probe	ACGGACGCGGAGATRGGRTGGRCAGGAT
	io probe	TTGGCCCCTCTAYGGCAAYKAGGGYT

F-primer, forward primer; R-primer, reverse primer; p1 probe, primary probe (FAM); p2 probe, primary probe (RED); io probe, Invader oligo.
Underlined sequence represents the 5′ flap of probe. Amino-blocked 3′ end of all primary probes. Boldfaced sequences denote the cleavage site of primary probes.

Table 2
Comparison of the number of mutant by the Q-Invader assay with those of sequencing.

Core 70	Sequencing		
	Wild	Mixed	Mutant
Q-Invader (Mutant%)			
Less than 1%	43		
1–99%	12	21	4
More than 99%			43
Core 91	**Sequencing**		
	Wild	Mixed	Mutant
Q-Invader (Mutant%)			
Less than 1%	55		
1–99%	9	9	4
More than 99%			46

1. Introduction

Hepatitis C virus (HCV) affects approximately 170 million people and is a major worldwide public health problem. It is responsible for chronic liver disease and increases the risk for severe diseases, such as cirrhosis and hepatocellular carcinoma (HCC) (Seeff, 2002). Interferon (IFN) therapy in chronic hepatitis C infection reduces the risk of developing HCC and liver-related death by clearing the virus. Since 2004, combination therapy with peginterferon (PEG-IFN) and ribavirin (RBV) has been the standard treatment (Fried et al., 2002). Recently, a triple therapy of peginterferon, ribavirin and a direct-acting antiviral (DAA), such as the protease inhibitor boceprevir or telaprevir, was approved and has been routinely used since 2011. However, for some patients, a sustained virological response does not last long. In Japan, the most common HCV genotype is genotype 1b (about 70%), but no virological response was found in 26.3% of patients infected with genotype 1b (Akuta et al., 2006).

In the present study, two key factors were related to the lack of a virological response in patients with the HCV genotype 1b: host polymorphisms at the neighboring IL28B gene (Ge et al., 2009; Tanaka et al., 2009; Thomas et al., 2009) and mutations in the HCV-1b core region (Akuta et al., 2005, 2006). The HCV-1b mutations (*e.g.*, arginine to glutamine or histidine at position 70 and/or from leucine to methionine at position 91) were significantly more common in virological non-responders, and the rate of decline in the HCV load during combination therapy in patients with mutant variants was less than those of patients with wild-type virus (Akuta et al., 2005). With the triple therapy, the proportions of sustained virological response were predicted by the prevalence of mutations at position 70 and the polymorphisms at the neighboring IL28B gene (Akuta et al., 2010). In addition, the prevalence of mutations at position 70 was increased by positive selection during combination therapy (Kurbanov et al., 2010). Therefore, for HCV therapy, monitoring the ratios of mutant variant to wild-type at position 70/91 may be as important as monitoring the HCV load. However, measuring the ratios of one point mutation with high sensitivity is difficult because only one quantitative method is available (Nakamoto et al., 2009).

This report describes a novel approach that uses the real-time PCR monitoring Invader reaction (Q-Invader assay) in a comparative quantitative assay for mutations at positions 70/91 in HCV-1b core region. The Invader technology has high specificity for detecting single-nucleotide differences in genomic DNA or PCR products (Lyamichev et al., 2000), and a highly sensitive quantification assay can be attained by adding real-time PCR (Tadokoro et al., 2009, 2010). To demonstrate its sensitivity and effectiveness, the method was used to detect mutations in clinical samples, and the results were compared to those from sequencing (Akuta et al., 2005) and PCR with mutation-specific primers (Okamoto et al., 2007).

2. Materials and methods

2.1. Source of patients

Serum samples were obtained from 123 patients infected with HCV-1b at the Toranomon Hospital (Kanagawa, Japan). The study was conducted in accord with the ethical principles of the Declaration of Helsinki and was approved by the Toranomon Hospital Ethical Committee. Written informed consent was obtained from each patient.

2.2. Design of Invader probes

The primary probe and Invader oligo to detect mutations (*i.e.*, R70H/Q and L91M in the core region) were designed with the Invader technology creator (TWT, Madison, WI, USA) (Fig. 1 and Table 1). Variations in neighboring regions were confirmed by analyzing 55 individual sequences of the HCV core region from a database at the National Center for Biotechnology Information (NCBI) (see Table 1).

2.3. Extraction HCV RNA and cDNA synthesis

HCV RNA was extracted from 200 μl of serum and eluted in 10 μl RNAse/DNAse-free water with the PureLink Viral RNA/DNA Mini Kit (Life Technologies, Carlsbad, CA, USA). cDNA was synthesized with random primers in 20-μl reactions with a commercial kit (SuperScript III cDNA Synthesis Kit, Life Technologies).

2.4. The Q-Invader assay

Comparative quantitative analysis of mutant and wild-type virus was completed by the Q-Invader assay. Two fluorescence signals (carboxyfluorescein or FAM for wild-type; REDmond RED or RED for mutants) could be detected in a single reaction with a Universal General Purpose Reagent (TWT), including Cleavase and FRET mix with two common fluorescence probes. Template c-DNA was added to a 15-μl reaction mixture containing 500 nM primers for amplification HCV-1b core region, 300 nM of each primary probe, 700 nM Invader oligo, 2 U AmpliTaq gold (Life Technologies), Universal General Purpose Reagent (TWT) and FRET mix (Table 1). The reaction mixture was preheated in a 384-PCR plate (Roche, Basel, Switzerland) at 95 °C for 20 min, and a two-step PCR was carried out for 40 cycles (95 °C for 15 s, 65 °C for 60 s) in a LightCycler 480 (Roche) (Tadokoro et al., 2009). Fluorescence values of FAM

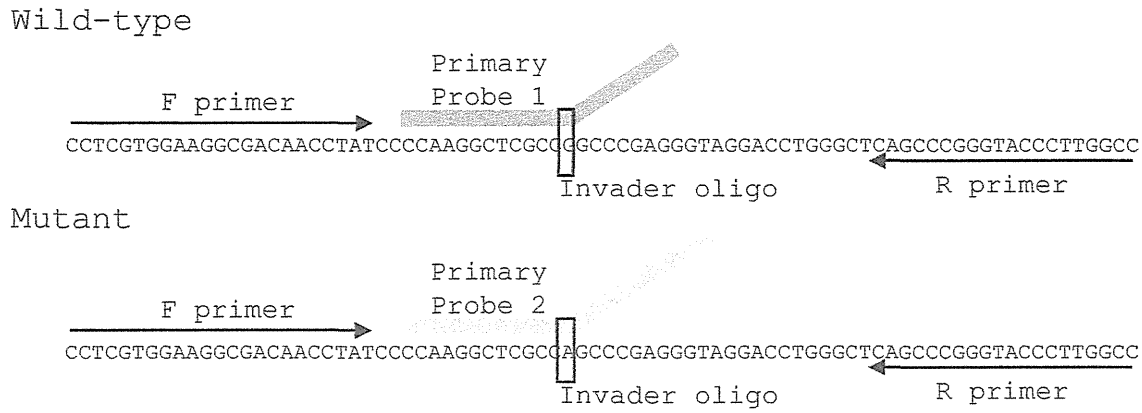

Fig. 1. Cleavage point for the Q-Invader assay at position 70 in the HCV-1b core region. White boxes represent cleavage points in PCR amplicon. Reference sequence: HCV-J.

(wavelength/bandwidth: excitation, 465 nm; emission, 510 nm) and RED (excitation, 533 nm; emission, 610 nm) were measured at end of the incubation/extension step at 65 °C for each cycle and by standard real-time PCR. By analyzing the results, a crossing point (Cp) can be obtained by a fit point method (Luu-The et al., 2005) in the LightCycler 480 software.

2.5. Sequencing

The sequence at position 70/91 in clinical samples was determined as described (Akuta et al., 2005). The amplified products were sequenced by the dideoxy method with the BigDye Terminator v1.1 Cycle Sequencing Kit (Life Technologies) in a model 3130 fluorescent DNA sequencer (Life Technologies).

2.6. Manufacture of control plasmid DNA by cloning clinical HCV sequence

HCV RNA, wild-type and that with mutations at positions 70/91, was isolated from clinical samples and amplified by. PCR amplicons were cloned into the pCRII-TOPO vector (Life Technologies) and sequenced (Tadokoro et al., 2009).

2.7. Mutation analysis by wild-type/mutant-specific primers

The mutations of positions 70/91 in patient c-DNA were determined by PCR with wild-type/mutant specific primer (Okamoto et al., 2007). The PCR amplicon was confirmed by

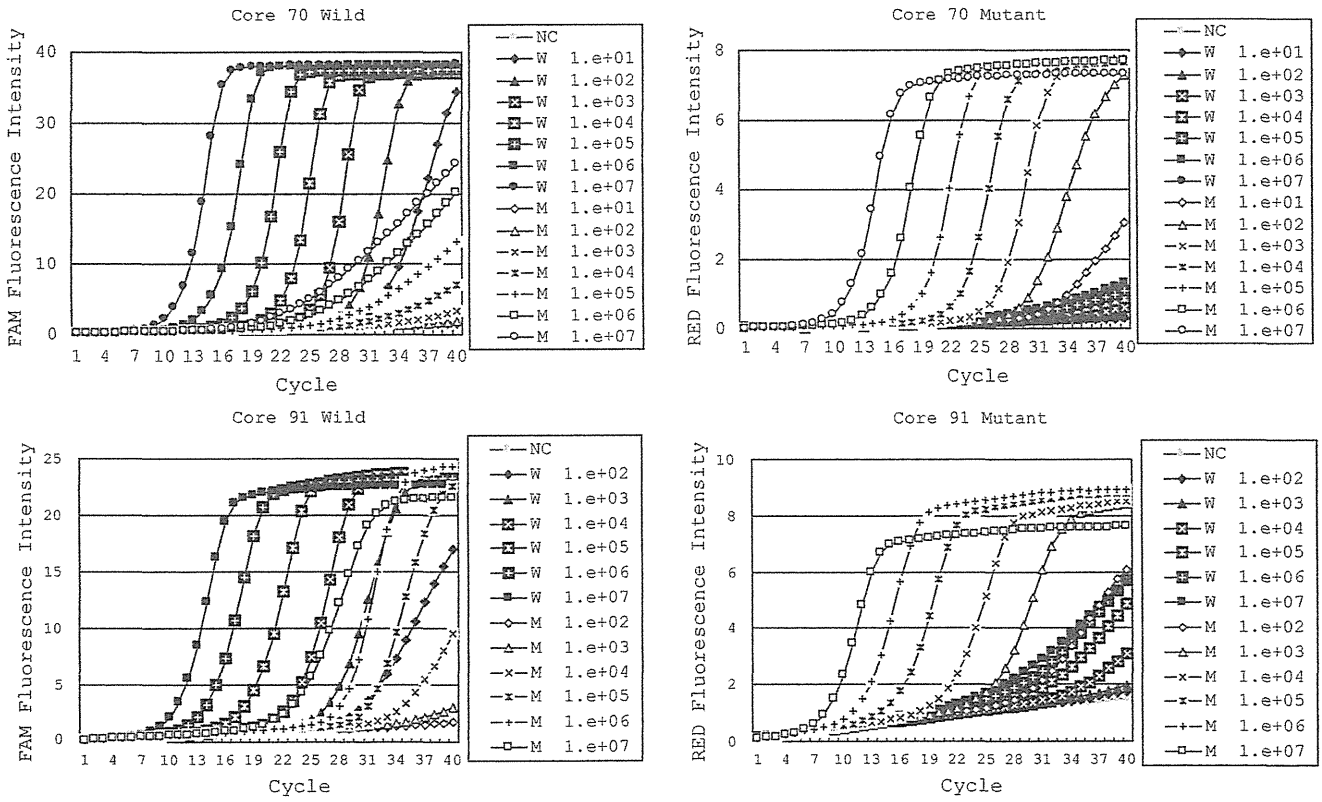

Fig. 2. Amplification plots generated by the Q-Invader assay. Fluorescence intensity was plotted against the number of cycles for a 10^{-7} dilution of plasmid (W: wild-type plasmid DNA, M: mutant plasmid DNA).

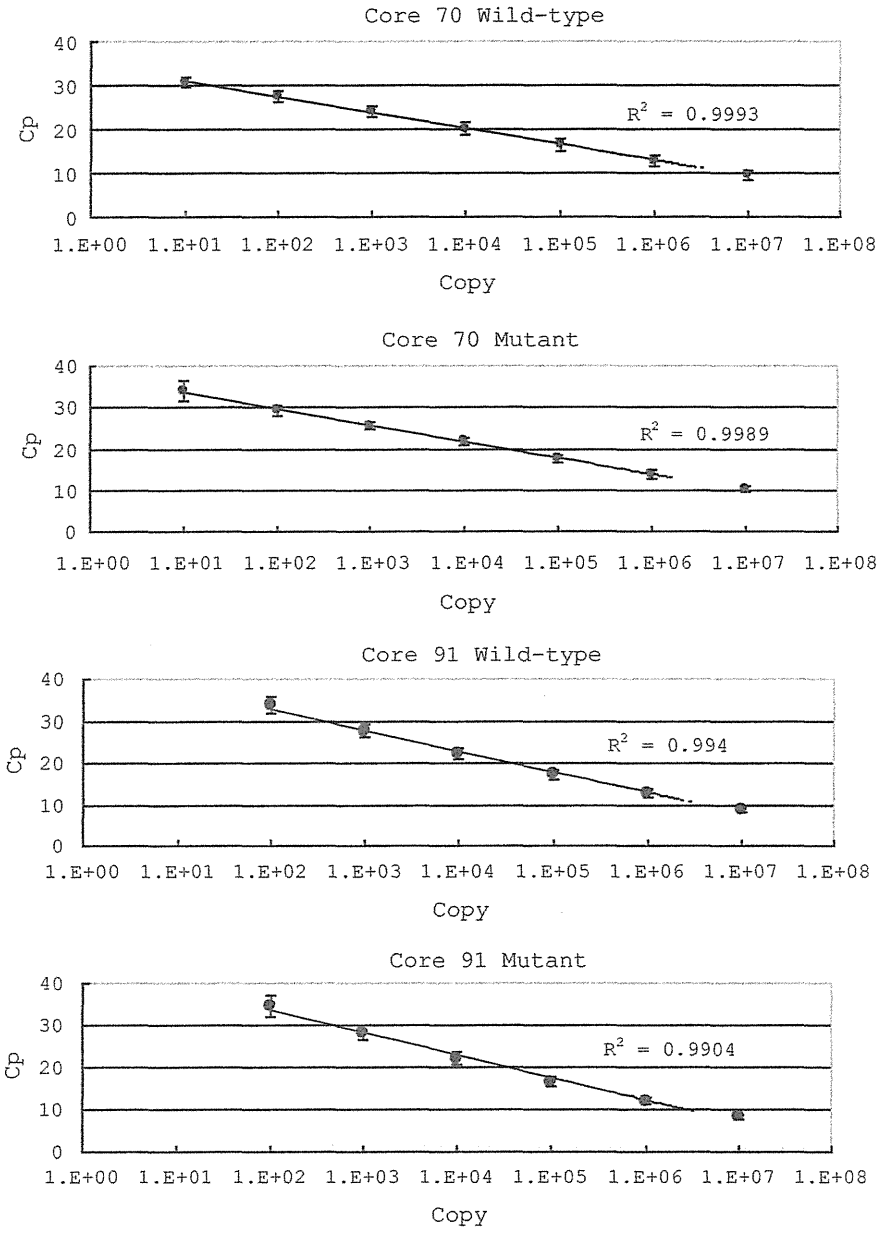

Fig. 3. Standard curves generated by the Q-Invader assay. The Cp was calculated by the fit point method with a 10^{-7} dilution of plasmid.

electrophoresis with a MultiNA microchip electrophoresis system (Shimadzu, Kyoto, Japan).

2.8. Detection sensitivity and assay variation for the Q-Invader assay

Detection sensitivity and assay variation for the Q-Invader assay were examined with single or mixed plasmids for template DNA. The detection limits of the Q-Invader assay in each measurement were determined with a 10^{-7} dilution of plasmid. Mixtures of plasmids were prepared in various ratios and determined in the Q-Invader assay for comparative quantitative analysis. A fivefold measurement was performed three times each to examine assay variation.

3. Results

3.1. Detection threshold and assay variation

Measurements of the mutant and wild-type virus at position 70 were both effective between 10^1 and 10^7 copies. The detection thresholds of both measurements were 10^1 copies of HCV c-DNA. Measurements at position 91 (mutant and wild-type) were effective between 10^2 and 10^7 copies, and the detection threshold was 10^2 copies (Figs. 2 and 3).

3.2. Comparative quantitative analysis for calculating relative ratios

Comparative quantitative analysis was used to determine the relative ratios in competitive infections by mutant variant and

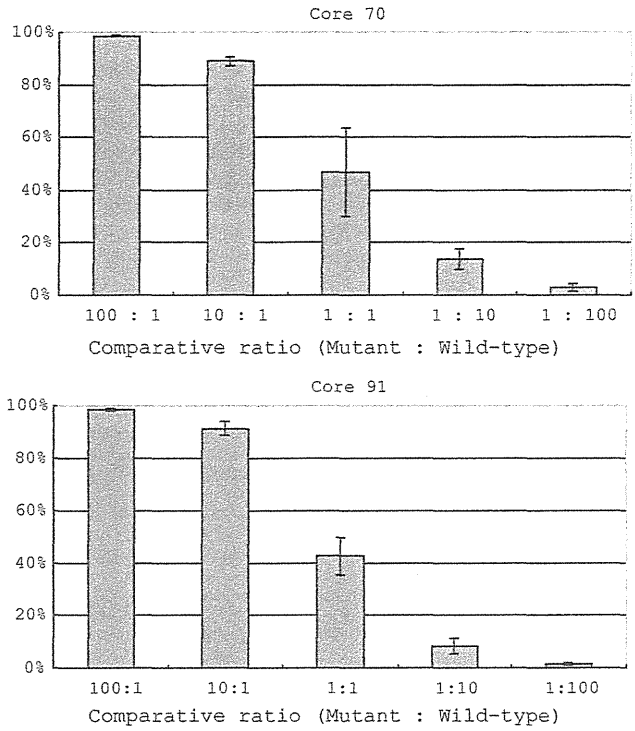

Fig. 4. Detection threshold in the Q-Invader assay with plasmid DNA at ratios of 100:1 to 1:100. At position 70, the minimum number of minor types was 10 copies. Position 91 was 100 copies.

wild-type virus. Copy numbers of mutant and wild-type virus were calculated separately by fit point analysis with each standard plasmid. To determine the relative ratios, the number of mutants was divided by those of total (mutant + wild-type). When the mutant and wild-type plasmids were mixed in various ratios for template DNA, the minor plasmid (mutant or wild-type) in template DNA could be detected down to 1:100 (Fig. 4), and the calculated relative ratios agreed with those of template DNA (Fig. 5).

3.3. Determination of relative ratios in clinical samples

Sequences from the HCV core region of 123 serum samples were confirmed by direct sequencing. The Q-Invader assay was used to determine the relative ratios of mutants at positions 70/91. The relative ratios identified three categories to compare by direct sequencing: less than 1% (wild-type), 1–99% (mixed) and more than 99% (mutant). Mixed types were found in 37 samples (30.1%) at position 70, and only 43.2% (16 of 37) of the cases were detected by the Q-Invader assay. At position 91, 22 samples (17.9%) were found to be of the mixed type, and only 59.1% (13 of 22) of the cases were detected by Q-Invader assay (Table 2).

3.4. Confirming mixed type by PCR with specific primer

To confirm the mixed type decided by the Q-Invader assay, but not by direct sequencing, PCR with wild-type/mutant specific primer was performed for each four samples. At position 70, a minor mutant variant was detected in samples 99 and 118 (4% and 5%). In samples 80 and 18, mutant variant held most (98% and 99%). By PCR with specific primer, both wild-type and mutant were detected in all samples. A similar result was obtained at position 91 (Table 3).

4. Discussion

Accurate, rapid determination of the relative ratios of mutations at positions 70/91 in the HCV-1b core region is important to insure

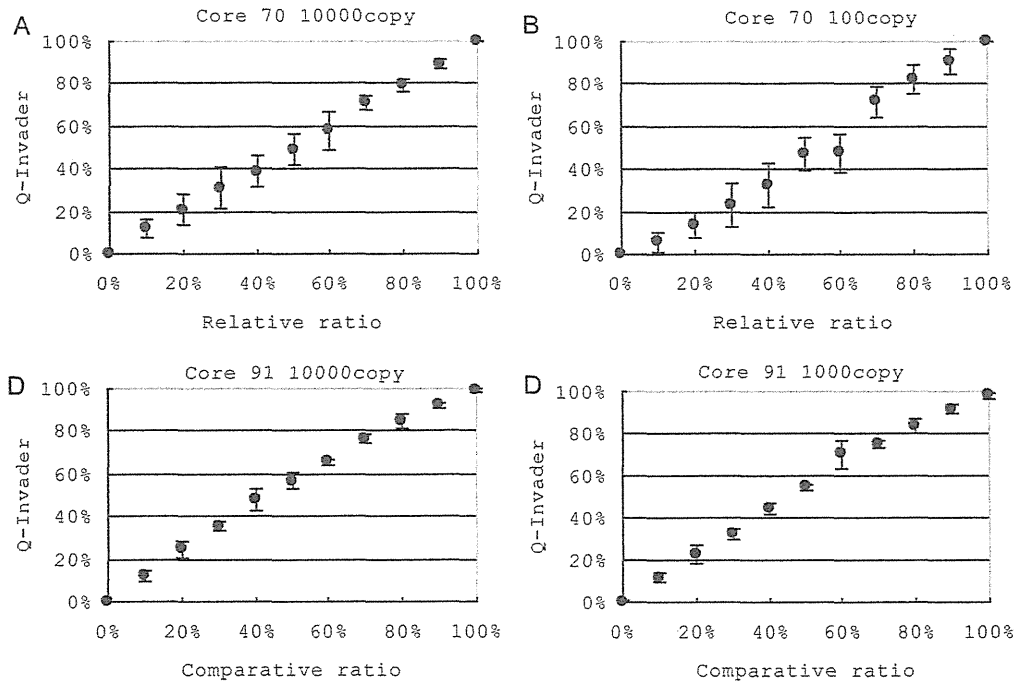

Fig. 5. Assay variation in the Q-Invader assay with 0–100% mixed plasmid DNA. Plasmid DNA was mixed to 0, 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100% with the mutation. At position 70, total numbers of plasmid DNA copies were 10,000 (A) and 100 (B). Position 91 was also 10,000 (C) and 1000 (D) copies.

Table 3
Confirmation for mixed type by PCR with specific primers.

Core 70	Q-Invader mutant (%)	Sequencing	PCR with specific primers	
			Wild	Mutant
Sample 099	4%	Wild	+	+
Sample 118	5%	Wild	+	+
Sample 080	98%	Mutant	+	+
Sample 018	99%	Mutant	+	+
Core 91	Q-Invader mutant (%)	Sequencing	PCR with specific primers	
			Wild	Mutant
Sample 088	2%	Wild	+	+
Sample 067	2%	Wild	+	+
Sample 090	98%	Mutant	+	+
Sample 071	98%	Mutant	+	+

the effectiveness of the combination therapy with peginterferon and ribavirin. A highly sensitive method with comparative quantitative analysis was developed in this study. The Q-Invader assay was examined for detection sensitivity and accuracy and compared with direct sequencing and PCR with type-specific primers.

Multiple reaction systems with two florescence probes were examined for detection of wild-type/mutant sequences at two positions (70 and 91) in the HCV-1b core region. The detection sensitivities at position 70, both wild-type and mutant, were 10 copies of template DNA, and those at position 91 were 100 copies (Fig. 3). In examinations of mixtures of plasmids with wild-type and mutant sequences in various ratios, the Q-Invader assay could be determined down to 1% of the minor type (Fig. 4), and the calculated relative ratios agreed with those determined by use of template DNA. The relative ratios were not influenced by total number of DNA templates (Fig. 5).

To validate the Q-Invader assay, 123 clinical samples from patients were analyzed for mutations at positions 70/91 in HCV-1b core region by direct sequencing and the Q-Invader assay. All of mutations found by direct sequencing were also detected by the Q-Invader assay. Moreover, The Q-Invader assay could detect wild-type/mutant that slightly included in sample. At position 70, both wild-type and mutant were detected in 37 samples by the Q-Invader assay and 56.8% (21 of 37) of the cases could detect only either one by direct sequencing. To confirm the existence of the minor type detected by the Q-Invader assay, the PCR with specific primer was performed (Table 3).

The relevance of amino acid mutations at positions 70/91 in HCV-1b core region to the effectiveness of combination or triple therapy was reported in many studies (Akuta et al., 2005, 2010; Nakamoto et al., 2010). In addition, amino acid mutations at the positions 70/91 were resistant to interferon *in vitro* (Funaoka et al., 2011). The expression levels of IL-6, which upregulates SOCS3, in cells transfected with the core mutant were significantly higher than with wild type. These mechanisms may explain the clinical resistance of amino acid mutations at positions 70/91 for interferon therapy. Furthermore, the amino acid mutations at position 70 in the HCV-1b core region are significant as an independent predictor of HCC in virological non-responders (Seko et al., 2013).

In studies of interferon therapy and HCC development, the importance of the amino acid mutations at positions 70/91 is increasing. In addition, the relative ratios of mutants varied in individual patients (Okamoto et al., 2007) and changed between therapies (Kozuka et al., 2012; Kurbanov et al., 2010). Therefore, monitoring the relative ratios of mutant variants should contribute to new knowledge and efficacy prediction for HCV therapy. A high-quality quantitative system is required to push forward a study. The Q-Invader assay, which is more high sensitive method than existing method, would provide detailed dynamic change of relative ratios.

In summary, the Q-Invader assay had a high sensitivity for calculating the relative ratios of mutant variants at positions 70 and 91 in HCV-1b core region. The mutant variants were detected down to 1% of the total, and mixed template DNAs in various ratios were quantified accurately by the Q-Invader assay. The Q-Invader assay will be useful for patients with HCV in clinical setting.

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Effect of Type 2 Diabetes on Risk for Malignancies Includes Hepatocellular Carcinoma in Chronic Hepatitis C

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The aim of this retrospective cohort study was to assess the cumulative development incidence and predictive factors for malignancies after the termination of interferon (IFN) therapy in Japanese patients for hepatitis C virus (HCV). A total of 4,302 HCV-positive patients treated with IFN were enrolled. The mean observation period was 8.1 years. The primary outcome was the first onset of malignancies. Evaluation was performed using the Kaplan-Meier method and Cox proportional hazard analysis. A total of 606 patients developed malignancies: 393 developed hepatocellular carcinoma (HCC) and 213 developed malignancies other than HCC. The cumulative development rate of HCC was 4.3% at 5 years, 10.5% at 10 years, and 19.7% at 15 years. HCC occurred significantly ($P < 0.05$) when the following characteristics were present: advanced histological staging, sustained virological response not achieved, male sex, advanced age of ≥50 years, total alcohol intake of ≥200 kg, and presence of type 2 diabetes (T2DM). T2DM caused a 1.73-fold enhancement in HCC development. In patients with T2DM, HCC decreased when patients had a mean hemoglobin A1c (HbA1c) level of <7.0% during follow-up (hazard ratio, 0.56; 95% confidence interval, 0.33-0.89; $P = 0.015$). The cumulative development rate of malignancy other than HCC was 2.4% at 5 years, 5.1% at 10 years, and 9.8% at 15 years. Malignancies other than HCC occurred significantly when patients were of advanced age of ≤50 years, smoking index (package per day × year) was ≥ 20, and T2DM was present. T2DM caused a 1.70-fold enhancement in the development of malignancies other than HCC. *Conclusion:* T2DM causes an approximately 1.7-fold enhancement in the development of HCC and malignancies other than HCC in HCV-positive patients treated with IFN. In T2DM patients, maintaining a mean HbA1c level of <7.0% reduces the development of HCC. (HEPATOLOGY 2013;57:964-973)

Hepatitis C virus (HCV) is one of the more common causes of chronic liver disease worldwide. Chronic hepatitis C is an insidiously progressive form of liver disease that relentlessly but silently progresses to cirrhosis in 20%-50% of cases over a period of 10-30 years.[1,2] In addition, HCV is a major risk factor for hepatocellular carcinoma (HCC).[3-7]

On the other hand, the prevalence of patients with type 2 diabetes mellitus (T2DM) is increasing in many nations, including Japan.[8] Thus, the management of T2DM patients who are chronically infected with HCV is one of the most important issues confronted by physicians. Few studies have reported relationships between T2DM and total malignancies, including HCC in HCV patients. In addition, it is not clear whether the stringent control of T2DM is necessary for protecting the development of malignancies in HCV patients. This issue needs to be confirmed via long-term follow-up of a large cohort of patients at high risk of developing malignancy.

Abbreviations: CH, chronic hepatitis; CI, confidence interval; HbA1c, hemoglobin A1c; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; HR, hazard ratio; IFN, interferon; LC, liver cirrhosis; SVR, sustained virological response; T2DM, type 2 diabetes mellitus; TAI, total alcohol intake.

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