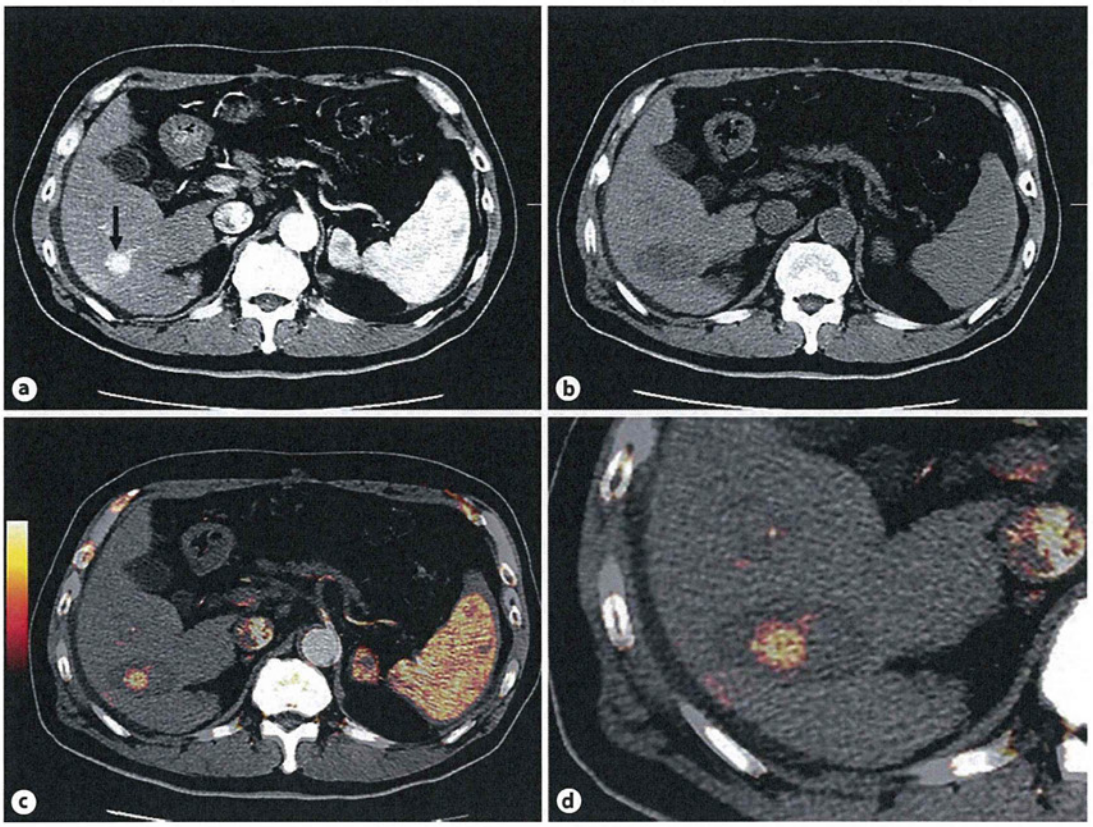

Fig. 4. CT fusion imaging system for the evaluation of treatment effects of RFA in a 68-year-old man with hypervascular HCC. **a** Arterial phase of pre-RFA dynamic CT. **b** Portal phase of post-RFA dynamic CT. **c** Fusion image of pre- and post-RFA CT. **d** Fusion image of pre- and post-RFA CT (enlarged). The hypervascular HCC in segment VI (arrow) was treated with RFA. Compared with side-by-side interpretation of pre- and post-RFA CT, the positional relationship becomes clear and it becomes possible to evaluate the ablative margin accurately on CT fusion images.

tumor on the reference images. One example is demonstrated in figure 3. The tumor on hepatobiliary phase of Gd-EOB-DTPA-enhanced MRI, used as a reference, was considered to be covered by the low echoic area on the post-vascular phase of Sonazoid-enhanced US performed after RFA, which becomes quite comprehensive using the GPS function. Since the volume data of US, as well as those of CT and MRI, can also be used as a reference, US images of pre- and post-RFA can be compared.

Although inherent limitations still remain in terms of the accuracy, due to difficulty in the fusion technology of volume data of US, the US fusion imaging system is useful for the assessment of the treatment effects of RFA by virtue of its convenience, minimal invasiveness, and real-time characteristics.

CT Fusion Imaging System for the Evaluation of Treatment Effects of RFA

For the evaluation of treatment effects of RFA using dynamic CT, pre- and post-RFA CT have been conventionally compared in a side-by-side manner. However, since it is quite difficult to comprehend the locational relationship of the tumor and ablation zone graphically in this side-by-side interpretation, the assessment tends to be subjective and inaccurate.

Recently, to overcome these problems, fusion images of pre- and post-RFA CT have been utilized for judging the curative effects of RFA [16–19]. Figure 4 shows the case of an HCC patient who underwent RFA and the treatment effects were assessed using a CT fusion imaging system. CT fusion images are created with Advantage

Workstation VolumeShare 4 (GE Healthcare Japan). After automatic alignment of pre- and post-RFA CT using the rigid registration method, manual registration was added by referencing to intrahepatic structures such as blood vessels, cysts, or the iodized oil from previous treatments, and hepatic contours around the tumor. Since pretreatment tumor and the ablation zone are overlaid, it becomes easy to grasp the positional relation of the tumor and the ablation zone visually, resulting in more accurate evaluation of the treatment effects of RFA.

At Ikeda Municipal Hospital, a CT fusion imaging system was introduced for the evaluation of treatment effects of RFA in 2011. Now, the creation of CT fusion images of RFA are performed as routine daily work by radiological technicians, and these images can be seen on patients' charts for use in deciding whether to administer additional RFA. The application of a CT fusion imaging system to the evaluation of treatment effects of RFA is just getting started, and it is hoped to be widely used hereafter.

Conclusion

The present state of the multimodality fusion imaging system and its usefulness in the diagnosis and treatment of HCC were outlined in this review. Since US fusion imaging systems have been introduced into clinical practice, it has become possible to perform percutaneous loco-regional treatment for tumors which are difficult to detect on conventional US, but detectable on other imaging modalities, particularly on the hepatobiliary phase of Gd-EOB-DTPA-enhanced MRI. As another multimodality fusion imaging system, CT fusion imaging technology provides more accurate evaluation of the treatment effects of RFA than the conventional side-by-side assessment.

Imaging diagnosis of HCC has been progressing remarkably in recent years. The important thing is to make the best use of the advanced imaging modalities, combining the strong points of each modality complementarily. In particular, multimodality fusion imaging seems to play an important role in the diagnosis and treatment of HCC.

Disclosure Statement

The authors declare that no financial or other conflict of interest exists in relation to the content of the article.

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RESEARCH ARTICLE Open Access

Significance of a reduction in HCV RNA levels at 4 and 12 weeks in patients infected with HCV genotype 1b for the prediction of the outcome of combination therapy with peginterferon and ribavirin

Hidenori Toyoda[1], Takashi Kumada[1*], Noritomo Shimada[2], Koichi Takaguchi[3], Tatsuya Ide[4], Michio Sata[4], Hiroyuki Ginba[5], Kazuhiro Matsuyama[5] and Namiki Izumi[6]

Abstract

Background: The importance of the reduction in hepatitis C virus (HCV) RNA levels 4 and 12 weeks after starting peginterferon (PEG-IFN) and ribavirin combination therapy has been reported to predict a sustained virologic response (SVR) in patients infected with HCV genotype 1. We conducted a multicenter study to validate this importance along with baseline predictive factors in this patient subpopulation.

Methods: A total of 516 patients with HCV genotype 1 and pretreatment HCV RNA levels ≥5.0 $\log_{10}$ IU/mL who completed response-guided therapy according to the AASLD guidelines were enrolled. The reduction in serum HCV RNA levels 4 and 12 weeks after starting therapy was measured using real-time PCR, and its value in predicting the likelihood of SVR was evaluated.

Results: The area under the receiver operating characteristics (ROC) curve was 0.852 for 4-week reduction and 0.826 for 12-week reduction of HCV RNA levels, respectively. When the cut-off is fixed at a 2.8-$\log_{10}$ reduction at 4 weeks and a 4.9-$\log_{10}$ reduction at 12 weeks on the basis of ROC analysis, the sensitivity and specificity for SVR were 80.9% and 77.9% at 4 weeks and were 89.0% and 67.2% at 12 weeks, respectively. These variables were independent factors associated with SVR in multivariate analysis. Among 99 patients who showed a delayed virologic response and completed 72-week extended regimen, the area under ROC curve was low: 0.516 for 4-week reduction and 0.482 for 12-week reduction of HCV RNA levels, respectively.

Conclusions: The reduction in HCV RNA levels 4 and 12 weeks after starting combination therapy is a strong independent predictor for SVR overall. These variables were not useful for predicting SVR in patients who showed a slow virologic response and experienced 72-week extended regimen.

Keywords: Chronic hepatitis C, Peginterferon, Ribavirin, Reduction in HCV RNA levels, Four and twelve weeks, Baseline factors, Response-guided therapy, Extended treatment

* Correspondence: hosp3@omh.ogaki.gifu.jp
[1]Department of Gastroenterology, Ogaki Municipal Hospital, Ogaki, Japan
Full list of author information is available at the end of the article

Background

Many investigators have sought to identify factors that can predict the treatment outcome of peginterferon (PEG-IFN) and ribavirin combination therapy in patients infected with HCV genotype 1. Previous studies reported baseline host and viral factors that are associated with the treatment outcomes. The genetic polymorphisms near the *IL28B* gene (rs12979860 or rs8099917) reportedly constitute a host factor that is strongly associated with treatment outcome [1-5], and studies from Japan have reported that amino acid substitutions at residue 70 of the HCV core region and residues 2209–2248 of the NS5A region of HCV (i.e., interferon sensitivity-determining region, ISDR) are viral factors associated with treatment outcome in patients infected with HCV genotype 1 [6-10]. In addition to the baseline predictive factors, the response to HCV during therapy, i.e., the changes in serum HCV RNA levels after initiation of therapy, has also been shown to be an important predictor of treatment outcome [11-14]. Especially, the disappearance or the reduction in serum HCV RNA levels at 4 and 12 weeks after starting therapy have been reported to be important, therefore, rapid virologic response (RVR) or early virologic response (EVR) defined at 4 and 12 weeks after starting therapy, respectively, is a pivotal criteria in predicting treatment response [11-23].

There are adverse effects associated with PEG-IFN and ribavirin antiviral therapy, and the treatment course is costly. For these reasons, it is important to predict the likelihood that a patient will achieve SVR during early stages of therapy with high reliability, in order to prevent unnecessary treatment. This will become increasingly important with the emergence of new antiviral drugs against HCV [24-28]. In the present study, we conducted a multicenter cohort study to examine whether the reduction in HCV RNA levels 4 and 12 weeks after starting PEG-IFN and ribavirin combination therapy, along with baseline predictive factors, has any value in predicting SVR.

Methods

Patients, treatments, and evaluation of responses

The inclusion criteria for this multicentre study were (i) infection with HCV genotype 1 without co-infection with hepatitis B virus or human immunodeficiency virus; (ii) pretreatment HCV RNA levels ≥5.0 $\log_{10}$ IU/mL, based on a quantitative real-time PCR-based method (COBAS AmpliPrep / COBAS TaqMan HCV Test; Roche Molecular Systems: Pleasanton, CA, US.; lower limit of quantification, 1.6 $\log_{10}$ IU/ mL: lower limit of detection, 1.2 $\log_{10}$ IU/ mL) [29,30]; (iii) standard PEG-IFN and ribavirin therapy according to the American Association for the Study of the Liver Diseases (AASLD) guidelines [31] started between December 2004 and January 2010; (iv) completed treatment regimen of 48- or 72-week duration with virologic outcomes available for evaluation; and (v) 100% medication adherence for both PEG-IFN and ribavirin during the initial 4 weeks of therapy and 80% or more throughout the treatment period. With regard to inclusion criterion (i), this study did not include any patients infected with HCV genotype 1a because this genotype is usually not found in the Japanese general population. With regard to criterion (ii), we focused on patients with pretreatment HCV RNA level ≥5.0 $\log_{10}$ IU/mL because the use of ribavirin along with PEG-IFN is not allowed by Japanese National Medical Insurance System for patients with pretreatment HCV RNA levels <5.0 $\log_{10}$ IU/mL. With regard to criterion (iv), the treatment duration was determined based on the response-guided therapy according to AASLD guidelines. Patients in whom serum HCV RNA disappeared until 12 weeks after starting therapy (complete EVR) underwent 48-week treatment regimen. Patients in whom serum HCV RNA disappeared after 12 weeks but until 24 weeks after starting therapy (delayed virologic response) underwent 72-week extended treatment regimen. Patients whose treatment was discontinued due to the presence of serum HCV RNA at 24 weeks of therapy (partial responders or null responders as per the AASLD guidelines), or due to viral breakthrough were also included in the study.

A total of 808 patients underwent the combination therapy with PEG-IFN and ribavirin between December 2004 and January 2010 in one of the following five Liver Centers: Musashino Red Cross Hospital, Kurume University Hospital, Ogaki Municipal Hospital, Shinmatsudo Central General Hospital, and Kagawa Prefectural Central Hospital. For 126 patients, the treatment regimen consisted of weekly PEG-IFN alpha-2a (Pegasys, Chugai Pharmaceutical, Tokyo, Japan) and daily ribavirin (Copegus, Chugai Pharmaceutical). The other 682 patients were treated with weekly PEG-IFN alpha-2b (Pegintron, MSD Co., Tokyo, Japan) and daily ribavirin (Rebetol, MSD Co.). We excluded patients who had been treated with PEG-IFN alpha-2a and ribavirin in order to avoid the influence of PEG-IFN subtype on the association between viral dynamics and treatment outcome. In 682 patients who received PEG-IFN alpha-2b, 516 patients fulfilled the eligibility criteria and were included for analysis (Figure 1). The doses of PEG-IFN alpfa-2b and ribavirin were adjusted based on the patient's body weight. Patients ≤ 45 kg were given 60 μg of PEG-IFN alpha-2b weekly, those > 45 kg and ≤ 60 kg were given 80 μg, those > 60 kg and ≤ 75 kg were given 100 μg, those > 75 kg and ≤ 90 kg were given 120 μg, and those > 90 kg were given 150 μg. Patients ≤ 60 kg were given 600 mg of ribavirin daily, those > 60 kg and ≤ 80 kg were given 800 mg, and those > 80 kg were given 1000 mg per

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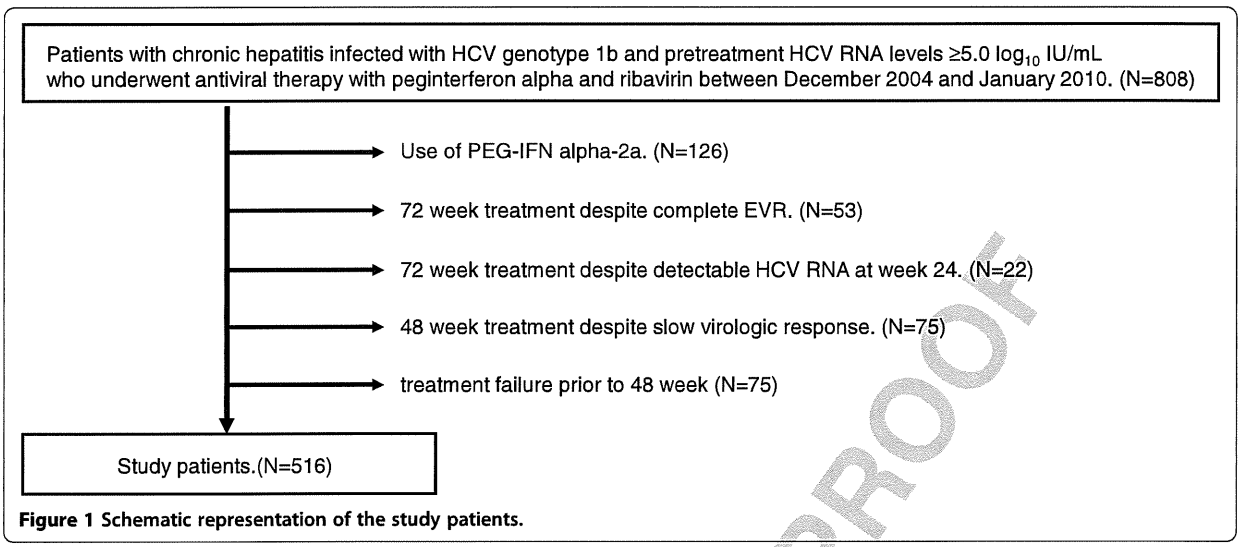

Figure 1 Schematic representation of the study patients.

day. Dose modifications of PEG-IFN or ribavirin were based on the manufacturer's recommendations.

SVR was defined as undetectable serum HCV RNA 24 weeks after the end of therapy. A patient was considered to have relapsed when serum HCV RNA levels became detectable between the end of treatment and 24 weeks after completion of therapy, although serum HCV RNA levels were undetectable at the end of therapy. A non-response was defined as detectable serum HCV RNA at 24 weeks after initiation of therapy (i.e., null response or partial non-response according to the AASLD guidelines). RVR was defined as undetectable serum HCV RNA 4 weeks after starting therapy. EVR was defined as the disappearance or a decrease in serum HCV RNA levels by at least 2 $\log_{10}$ at 12 weeks after starting therapy. Patients were considered to have a complete EVR if the serum HCV RNA levels were undetectable 12 weeks after starting therapy and a partial EVR if the serum HCV RNA levels were detectable but had decreased by at least 2 $\log_{10}$ at 12 weeks of therapy. A non-EVR was defined as a lack of a decrease of HCV RNA by more than 2 $\log_{10}$ at 12 weeks when compared to pretreatment levels. Patients were considered to have a delayed virologic response if serum HCV RNA levels became undetectable after 12 weeks but until 24 weeks on treatment.

The study protocol was in compliance with the Helsinki Declaration and was approved by the ethics committee of each participating institution, i.e., the ethics committee of Musashino Red Cross Hospital, the ethics committee of Kurume University Hospital, the ethics committee of Ogaki Municipal Hospital, the ethics committee of Shinmatsudo Central General Hospital, and the ethics committee of Kagawa Prefectural Central Hospital. Prior to initiating the study, written informed consent was obtained from each patient to use their clinical and laboratory data and to analyze stored serum samples.

Measurements of serum HCV RNA levels, amino acid substitution at residue 70 in the HCV core, amino acid sequence of HCV NS5A-ISDR, and genetic polymorphisms near the IL28B gene

After a patient gave informed consent, serum samples were obtained during the patient's regular hospital visits, just prior to beginning treatment, and every 4 weeks during the treatment period and the 24-week follow-up period after treatment. Serum samples were stored at −80°C until they were analyzed. HCV RNA levels were measured using a quantitative real-time PCR-based method (COBAS AmpliPrep/ COBAS TaqMan HCV Test) [29,30]. The reduction in HCV RNA 4 and 12 weeks after initiation of therapy was calculated. When calculating the decrease in serum HCV RNA, HCV RNA level was defined as 0 when HCV RNA was undetectable.

Amino acid 70 of the HCV core region and the amino acid sequence of ISDR region (residues 2209–2248 of the NS5A region) were analyzed by direct nucleotide sequencing of each region as previously reported [6,7]. The following PCR primer pairs were used for direct sequencing of the HCV core region:

5′-GCCATAGTGGTCTGCGGAAC-3′ (outer, sense primer),
5′-GGAGCAGTCCTTCGTGACATG-3′ (outer, antisense primer),
5′-GCTAGCCGAGTAGTGTT-3′ (inner, sense primer), and
5′-GGAGCAGTCCTTCGTGACATG-3′ (inner, antisense primer).

The following PCR primers were used for direct sequencing of ISDR:

5′-TTCCACTACGTGACGGGCAT-3′ (outer, sense primer),
5′-CCCGTCCATGTGTAGGACAT-3′ (outer, antisense primer),
5′-GGGTCACAGCTCCCTGTGAGCC-3′ (inner, sense primer), and
5′-GAGGGTTGTAATCCGGGCGTGC-3′ (inner, antisense primer).

When evaluating ISDR, HCV was defined as wild-type when there were 0 or 1 amino acid substitutions in residues 2209–2248 as compared with the HCV-J strain [32], and as non-wild-type when there was more than 1 substitutions.

Genotyping of rs 8099917 polymorphisms near the *IL28B* gene was performed using the TaqMan SNP assay (Applied Biosystems, Carlsbad, CA) according to the manufacturer's guidelines. A pre-designed and functionally tested probe was used for rs8099917 (C__11710096_10, Applied Biosystems). Genetic polymorphism of rs8099917 reportedly corresponds to rs12979860 in more than 99% of individuals of Japanese ethnicity [33]. The TT genotype of rs8099917 corresponds to the CC genotype of rs12979860, the GG genotype of rs8099917 corresponds to the TT genotype of rs12979860, and the TG heterozygous genotype of rs8099917 corresponds to the CT of rs12979860.

Statistical analyses

Quantitative values are reported as medians and ranges. Differences in percentages between groups were analyzed with the chi-square test. Differences in mean quantitative values were analyzed by the Mann–Whitney U test. The receiver-operating characteristics (ROC) analyses were performed to determine the cut-offs of the reduction in HCV RNA levels at 4 and 12 weeks after starting therapy to evaluate the sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and accuracy for predicting SVR. Univariate and multivariate analyses using a logistic regression model were performed to identify factors that predict SVR. The factors that are potentially associated with SVR were included in the analyses, i.e., age, sex, body mass index (BMI), serum alanine aminotransferase activity, serum gamma-glutamyl transpeptidase level, total-cholesterol levels, neutrophil count, hemoglobin, platelet count, grade of activity and fibrosis of the liver, pretreatment HCV RNA levels, reduction in HCV RNA levels 4 and 12 weeks after starting therapy, amino acid substitution at residue 70 in the HCV core (arginine vs. glutamine or histidine), amino acid mutations in ISDR (non-wild-type vs. wild-type), and genetic polymorphisms near the *IL28B* gene (rs8099917, genotype TT vs. genotype TG or GG). Data analyses were performed using StatFlex statistical software, version 6 (Artech Co., Ltd., Osaka, Japan). All p values were two-tailed, and $p < 0.05$ was considered statistically significant.

Results

Patient characteristics and treatment outcome

The characteristics of the patients are shown in Table 1. Genotyping of rs8099917 near the *IL28B* gene was performed in 396 patients. Amino acid substitutions at residue 70 in the HCV core region were measured in 361 patients. Amino acid sequences in the ISDR were evaluated in 416 patients. Among 516 patients who were included in the analysis, treatment was completed at 48 weeks in 268 patients who underwent the standard regimen because they showed complete EVR. Treatment was extended from 48 weeks to 72 weeks in 99 patients who yielded delayed virologic response. Treatment was discontinued until 48 weeks in 149 patients because

Table 1 Characteristics of study patients

Age (years), median (range)	60.0 (20.0–80.0)
Sex (male/female) (%)	245 (47.5)/ 271 (52.5)
Body weight (kg), median (range)	58.0 (36.35–107.6)
BMI, median (range)	22.7 (15.8–37.0)
Prior treatment for HCV (no/yes) (%)	359 (69.6)/ 157 (30.4)
Initial dose of PEG-IFN (μg), median (range)	80.0 (40.0–150.0)
Initial dose of ribavirin (mg), median (range)	600 (400–1000)
Pretreatment HCV RNA levels (log^{10} IU/mL), median (range)	6.1 (5.0–7.7)
Platelet count ($\times 10^3$/μL)	161 (43–352)
Hemoglobin (g/dL)	13.9 (9.7–17.9)
Neutrophil count (/μL)	2489 (578–7480)
Alanine aminotransferase (IU/L)	47 (10–485)
LDL-cholesterol (mg/dL)	99 (25–226)
Total-cholesterol (mg/dL)	171 (29–325)
γ-glutamyl transpeptidase (IU/L)	34.5 (7.0–579)
Alfa fetoprotein (ng/mL)	5.0 (0.8–584)
Fibrosis score (F1/F2/F3/F4) (%)	208(45.9)/139(30.7)/69(15.2)/37(8.2)
Activity score (A1/A2/A3/A4) (%)	258(56.1)/178(38.7)/24(5.2)/0(0)
Genetic polymorphisms of rs8099917 (TT/GG or TG) (%)	288 (72.7)/ 108(27.3)
Amino acid at residue 70 of HCV core (arginine/glutamine or histidine) (%)	242 (67.0)/ 119 (33.0)
Amino acid sequence of ISDR (non-wild-type/wild-type) (%)	110 (26.4)/ 306 (73.6)

BMI, body mass index; *HCV*, hepatitis C virus; *PEG-IFN*, peginterferon; *ISDR*, interferon sensitivity-determining region.
(N = 516).

serum HCV RNA remained positive 24 weeks after starting therapy (partial response or null response), or because patients experienced viral breakthrough during therapy.

As a final outcome, 272 patients (52.7%) achieved SVR, 90 patients (17.5%) relapsed, and 128 patients (24.8%) had a non-response (48 patients with partial response and 80 patients with null-response). Viral breakthrough was observed in 26 patients (5.0%). The rate of SVR was 79.9% (214 of 268 patients) among patients with complete EVR in whom treatment was completed at 48 weeks and 58.6% (58 of 99 patients) among patients with delayed virologic response who underwent the extended 72-week regimen.

Baseline factors affecting SVR in all patients who underwent response-guided therapy according to AASLD guidelines

In all patients who underwent treatment according to the AASLD guidelines, the rate of SVR was significantly higher in patients with the TT genotype of rs8099917 near the *IL28B* gene (179 of 288 patients [62.3%] with TT genotype vs. 15 of 108 patients [13.9%] with TG/GG genotype, $p < 0.0001$). In addition, SVR rate was significantly higher in patients with HCV with arginine at residue 70 in the HCV core region (145 of 242 patients [59.9%] with arginine vs. 34 of 119 patients [28.6%] with glutamine or histidine, $p < 0.0001$). SVR was significantly higher in patients with HCV with non-wild type ISDR (75 of 110 patients [68.2%] with non-wild-type ISDR vs. 139 of 306 patients [45.4%] with wild-type ISDR, $p < 0.0001$). SVR was significantly higher in patients with pretreatment HCV RNA levels <6.0 $\log_{10}$ IU/mL (127 of 199 patients [63.8%] with pretreatment HCV levels <6.0 $\log_{10}$ IU/mL vs. 145 of 317 patients [45.7%] with pretreatment HCV RNA levels ≥6.0 $\log_{10}$ IU/mL, $p < 0.0001$).

Association between reduction of serum HCV RNA levels 4 and 12 weeks after starting therapy and SVR in all patients who underwent response-guided therapy according to the AASLD guidelines

The ROC analysis was performed in 516 patients who underwent the response-guided therapy according to the AASLD guidelines in order to evaluate the association between the reduction in serum HCV RNA levels 4 and 12 weeks after starting therapy and SVR (Figure 2). The area under the ROC curve was 0.852 and the best cut-off was calculated as 2.8 $\log_{10}$ IU/mL, when evaluated with the reduction of serum HCV RNA levels 4 weeks after starting therapy. The rate of SVR was significantly higher in patients with greater than 2.8-$\log_{10}$ reduction at 4 weeks (220 of 274 patients [80.3%] with > 2.8-$\log_{10}$ reduction vs. 52 of 242 patients [21.5%] with ≤ 2.8-$\log_{10}$ reduction, $p < 0.0001$). The sensitivity, specificity, PPV, NPV, and accuracy were 80.9%, 77.9%, 80.3%, 78.5%, and 79.5%, respectively, at this cut-off level. When evaluated with the reduction of serum HCV RNA levels 12 weeks after starting therapy, the area under the ROC curve was

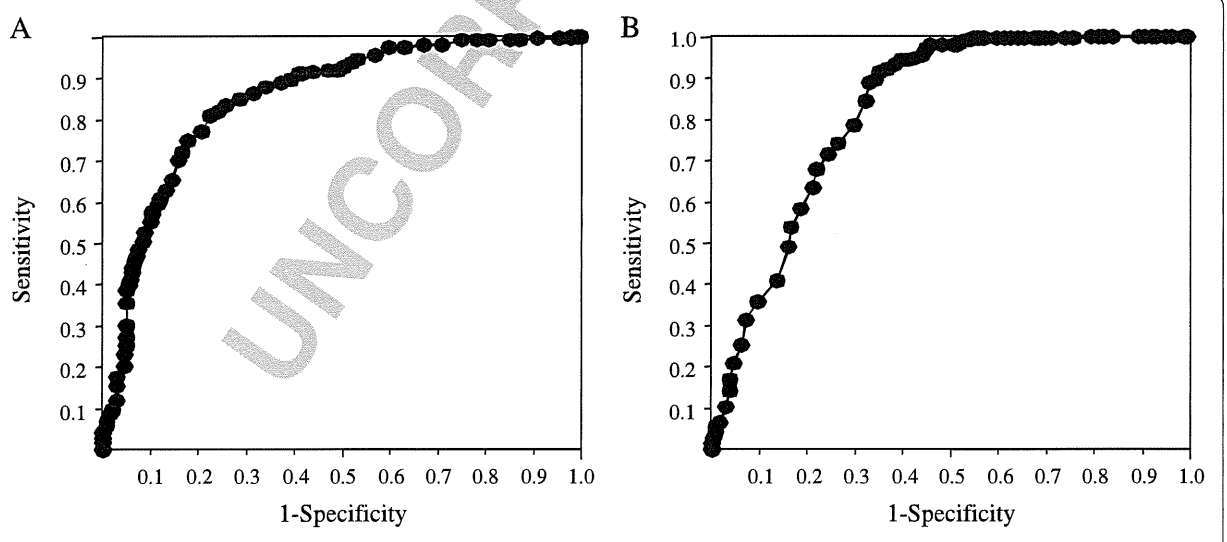

Figure 2 The receiver operating characteristics (ROC) analysis for the prediction of the sustained virologic response to combination therapy with peginterferon alpha-2b and ribavirin according to the reduction in serum HCV RNA levels in all patients who underwent response-guided therapy based on the AASLD guidelines. **A**) According to the reduction in serum HCV RNA levels 4 weeks after starting therapy. The area under the ROC curve was 0.852. **B**) According to the reduction in serum HCV RNA levels 12 weeks after starting therapy. The area under the ROC curve was 0.826.

0.826 and the best cut-off was calculated as 4.9 $\log_{10}$ IU/mL. The rate of SVR was significantly higher in patients with greater than 4.9-$\log_{10}$ reduction at 12 weeks (242 of 321 patients [75.4%] with > 4.9-$\log_{10}$ reduction vs. 30 of 194 patients [15.5%] with ≤ 4.9-$\log_{10}$ reduction, $p <$ 0.0001). The sensitivity, specificity, PPV, NPV, and accuracy were 89.0%, 67.2%, 75.4%, 84.5%, and 78.7%, respectively, at this cut-off level.

A multivariate analysis showed that the reductions in serum HCV RNA levels at 4 and 12 weeks after starting therapy were independent factors associated with SVR, along with pretreatment HCV RNA levels, platelet counts, polymorphisms of rs8099917 near the *IL28B* gene, and amino acid mutations in the HCV NS5A-ISDR (Table 2). T2

Association between reduction of serum HCV RNA levels 4 and 12 weeks after starting therapy and SVR in patients with delayed virologic response who underwent an extended 72-week regimen according to response-guided therapy

The ROC analysis was performed in 99 patients with delayed virologic response who underwent an extended 72-week treatment regimen according to the response-guided therapy of the AASLD guidelines to evaluate the association between reduction in serum HCV RNA levels 4 and 12 weeks after starting therapy and SVR (Figure 3). The area under the ROC curve was 0.516 and the best cut-off was calculated as 2.3 $\log_{10}$ IU/mL, when evaluated with the reduction of serum HCV RNA levels 4 weeks after starting therapy. There was no significant difference in the rate of SVR according to the reduction at 4 weeks (21 of 33 patients [63.6%] with > 2.3-$\log_{10}$ reduction vs. 37 of 66 patients [56.1%] with ≤ 2.3-$\log_{10}$ reduction, $p = 0.6120$). The area under the ROC curve was 0.482 and the best cut-off was calculated as 5.1 $\log_{10}$ IU/mL, when evaluated with the reduction of serum HCV RNA levels 12 weeks after starting therapy. There was no significant difference in the rate of SVR according to the reduction at 12 weeks (24 of 42 patients [57.1%] with > 5.1-$\log_{10}$ reduction vs. 34 of 57 patients [59.6%] with ≤ 5.1-$\log_{10}$ reduction, $p = 0.9634$). F3

Discussion

Several previous studies have reported that patients who achieved RVR, in whom serum HCV RNA levels become undetectable 4 weeks after starting the therapy, had a high likelihood of achieving SVR [15-18]. However, there are relatively few patients infected with treatment-resistant HCV genotype 1 who achieve RVR. A considerable percentage of patients achieve SVR even without RVR. Therefore, RVR has high specificity but low

Table 2 Univariate and multivariate analyses for sustained virologic response to the combination therapy with peginterferon and ribavirin in patients who underwent response guided therapy according to the AASLD guidelines

	Univariate analysis	Multivariate analysis*	Odds ratio (95% confidence interval)
Age (years)	< 0.001	N.S.	
Sex (male/female)	0.005	N.S.	
BMI, median (range)	N.S.		
Prior treatment for HCV (no/yes)	N.S.		
Pretreatment HCV RNA levels ($\log_{10}$ IU/mL), (≤6.0 vs. 6.0<)	0.015	0.013	2.235 (1.189-4.203)
Platelet count (×10^3/μL)	< 0.001	0.011	1.007 (1.002-1.013)
Hemoglobin (g/dL)	0.002	N.S.	
Neutrophil count (/μL)	0.003	N.S.	
Alanine aminotransferase (IU/L)	N.S.		
Total-cholesterol (mg/dL)	0.001	N.S.	
γ-glutamyl transpeptidase (IU/L)	0.014	N.S.	
Fibrosis score (F1 or F2/F3 or F4)	< 0.001	N.S.	
Activity score (A1 or A2/A3 or A4)	0.002	N.S.	
Genetic polymorphisms of rs8099917 (TT/GG or TG)	< 0.001	< 0.001	5.782 (2.298-14.552)
Amino acid at residue 70 of HCV core (arginine/glutamine or histidine)	< 0.001	N.S.	
Amino acid sequence of ISDR (non-wild-type/wild-type)	< 0.001	0.038	2.077 (1.041-4.147)
Reduction of HCV RNA [Pre - 4 week] ($\log_{10}$ IU/mL), (≤2.8 vs. 2.8<)	< 0.001	< 0.001	3.911 (1.935-7.908)
Reduction of HCV RNA [Pre - 12 week] ($\log_{10}$ IU/mL), (≤4.9 vs. 4.9<)	< 0.001	0.013	2.578 (1.220-5.448)

*Multivariate analysis was performed on 314 patients in whom all variables were available. (N = 516).

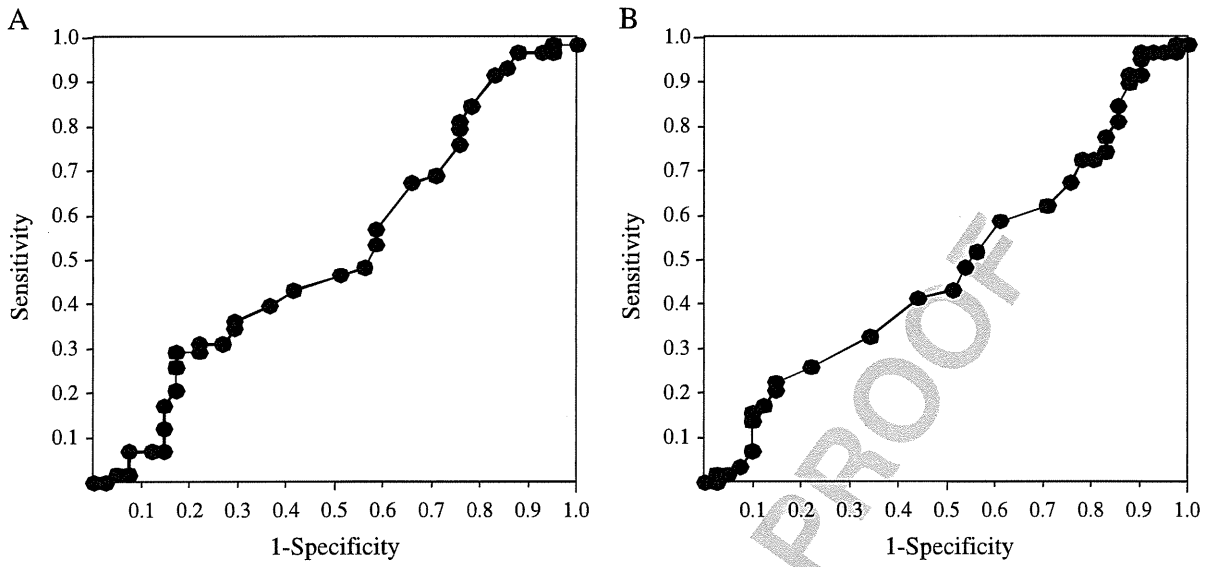

Figure 3 The receiver operating characteristics (ROC) analysis for the prediction of the sustained virologic response to combination therapy with peginterferon alpha-2b and ribavirin according to the reduction in serum HCV RNA levels in patients with delayed virologic response who underwent an extended 72-week regimen according to response-guided therapy. A) According to the reduction in serum HCV RNA levels 4 weeks after starting therapy. The area under the ROC curve was 0.516. **B)** According to the reduction in serum HCV RNA levels 12 weeks after starting therapy. The area under the ROC curve was 0.482.

sensitivity for predicting SVR. Previous studies from Asia evaluated the predictive value of the degree of reduction in serum HCV RNA levels 4 weeks after starting therapy, in addition to RVR [19-21]. However, the number of patients in these studies was small and the analyses were not sufficient to form reliable conclusions.

In the present study, we evaluated the ability of a decrease in serum HCV RNA levels 4 weeks after starting therapy to predict the likelihood of SVR as a final outcome in Japanese patients infected with HCV genotype 1b, based on the data from a large, multi-institution study. The ROC analyses showed that a reduction in serum HCV RNA levels 4 week after starting therapy was strongly associated with SVR, and its predictive value was higher than that of a reduction in serum HCV RNA levels 12 weeks after starting therapy, with higher area under the ROC curve and accuracy. Multivariate analyses including baseline factors that were associated with SVR revealed that the reductions of HCV RNA level at both 4 and 12 weeks after starting therapy were independent factors associated with SVR, and the reduction at 4 weeks had a second strongest impact for SVR, following genetic polymorphisms of rs8099917 near *IL28B* gene.

The important novelty from this study is that the reductions of HCV RNA level 4 and 12 weeks after starting therapy had no predictive value for SVR when focusing on patients who showed delayed virologic response and underwent the extended 72-week treatment regimen according to the response-guided therapy. This was in contrast to the prediction for SVR in all patients who underwent response-guided therapy. The impact of the reduction of HCV RNA level on the prediction of SVR would decline by the selection of patients based on the delayed virologic response. There were also no baseline factors that were associated with SVR in patients who underwent the extended 72-week treatment (data not shown). Prolonged treatment duration may relieve delayed virologic responders from unfavorable conditions. Further studies will be, therefore, needed to identify predictive factors for SVR in patients with delayed virologic response who underwent the 72-week treatment regimen.

There are several limitations to this study. The data were based on Japanese patients infected with HCV genotype 1b. Therefore, these results should be confirmed in patients of other ethnicities and patients infected with HCV genotype 1a. In addition, the value of the reduction in HCV RNA levels 4 and 12 weeks after starting therapy as predictors of SVR should be evaluated in patients who underwent therapy with PEG-IFN alpha 2a and ribavirin to determine the best cut-off levels with that regimen. Statistically, there were many missing data. We performed complete case analysis without the imputation of missing data for multivariate analysis. Although comparison between cases with and without missing data did not show statistically significant differences for cases characteristics, we cannot rule

out that the condition of data missing completely at random does not hold. Furthermore, this resulted in the decrease in the number of patients analyzed in multivariate analysis and might have substantially caused the reduction of statistical power, altering the value of non-significant results. In addition, the study did not perform internal validation. The use of hold-out method or split-group validation was difficult because of the number of study patients. Therefore, the validation in another larger study patients will be required in the future for confirming the results of this study.

Conclusions

A reduction in HCV RNA levels 4 and 12 weeks after starting therapy indicated likelihoods that patients will achieve SVR as a final outcome of combination therapy for HCV infection when patients underwent the response-guided therapy according to the AASLD guidelines. These reductions in serum HCV RNA levels were not predictive for SVR when focusing on patients who showed delayed virologic response and underwent the extended 72-week regimen.

Abbreviations

HCV: Hepatitis C virus; PEG-IFN: Peginterferon; SVR: Sustained virologic response; ROC: Receiver operating characteristics; ISDR: Interferon sensitivity-determining region; RVR: Rapid virologic response; EVR: Early virologic response; AASLD: American Association for the Study of the Liver Diseases; BMI: Body mass index; PPV: Positive predictive value; NPV: Negative predictive value.

Competing interests

The authors declare the following matters.
The authors have not received reimbursements, fees, funding, or salary from an organization that may in any way gain or lose financially from the publication of this manuscript, neither now nor in the future.
The authors have no stocks or shares in an organization that may in any way gain or lose financially from the publication of this manuscript, neither now nor in the future.
The authors are currently applying no patents relating to the content of the manuscript. We have not received reimbursements, fees, funding, or salary from an organization that holds or has applied for patents relating to the content of the manuscript.
The authors do not have any other financial competing interests.
There are no non-financial competing interests to declare in relation to this manuscript.
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Authors' contributions

Study design: HT, TK, NS, KT, TI, MS, HG, KM, and NI. Treatment of patients and data acquisition: HT, TK, NS, KT, TI, MS, and NI. Data analyses: HG and KM. Manuscript preparation: HT. Read and approval of the final manuscript: HT, TK, NS, KT, TI, MS, HG, KM, and NI. All authors read and approved the final manuscript.

Author details

[1]Department of Gastroenterology, Ogaki Municipal Hospital, Ogaki, Japan. [2]Division of Gastroenterology and Hepatology, Shinmatsudo Central General Hospital, Matsudo, Japan. [3]Department of Internal Medicine, Kagawa Prefectural Central Hospital, Takamatsu, Japan. [4]Department of Digestive Disease Information and Research, Kurume University School of Medicine, Kurume, Japan. [5]Department of Life Cycle Management, Roche Diagnostics Japan K.K, Tokyo, Japan. [6]Division of Gastroenterology and Hepatology, Musashino Red Cross Hospital, Musashino, Japan.

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Comparison of LecT-Hepa and FibroScan for assessment of liver fibrosis in hepatitis B virus infected patients with different ALT levels

Dongning Du [a,b,c,1], Xuejuan Zhu [a,1], Atsushi Kuno [b], Atsushi Matsuda [b], Chikayuki Tsuruno [d], Demin Yu [a], Yan Zhang [c,e], Yuzuru Ikehara [b], Yasuhito Tanaka [f], Xinxin Zhang [a,*], Hisashi Narimatsu [b,c,**]

[a] Department of Infectious Diseases, Ruijin Hospital, Shanghai Jiao Tong University School of Medicine, 197, Ruijin Er Road, Shanghai 200025, China
[b] Research Center for Medical Glycoscience (RCMG), National Institute of Advanced Industrial Science and Technology (AIST), 1-1-1 Umezono, Tsukuba, Ibaraki 305-8568, Japan
[c] SCSB (China) - AIST (Japan) Joint Medical Glycomics Laboratory, 800 Dong Chuan Road, Minhang, Shanghai 200240, China
[d] Product Development Div. 2, Sysmex Corporation, 4-4-4 Takatsukadai, Nishi-ku, Kobe, Hyogo 651-2271, Japan
[e] Ministry of Education Key Laboratory of Systems Biomedicine, Shanghai Center for Systems Biomedicine (SCSB), Shanghai Jiao Tong University, 800 Dong Chuan Road, Minhang, Shanghai 200240, China
[f] Department of Virology and Liver Unit, Nagoya City University Graduate School of Medical Sciences, Kawasumi, Mizuho, Nagoya, Aichi 467-8601, Japan

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ABSTRACT

Background: FibroScan is one of the noninvasive techniques based on the transient elastography that can assess the progression of liver fibrosis in chronic hepatitis patients in daily clinical practice. Recently, LecT-Hepa was validated as a serological glycomarker correlating well with the fibrosis stage determined by liver biopsy, and was superior to many other noninvasive biochemical markers and tests. We compared the reliability of LecT-Hepa with that of FibroScan for evaluation of liver fibrosis.
Methods: The effects of increased alanine aminotransferase (ALT) activities on LecT-Hepa and FibroScan were investigated.
Results: The areas under the receiver-operating characteristic curves, sensitivity and specificity for detecting cirrhosis, which is one of the outcomes of fibrosis estimation, were 0.82, 72.5% and 78.2% of LecT-Hepa, 0.85, 87.0% and 74.1% of FibroScan; these did not differ significantly. The count distribution of LecT-Hepa in non-cirrhosis group or cirrhosis group did not differ between the patients grouped according to their ALT levels, whereas that of FibroScan was substantially affected.
Conclusion: LecT-Hepa was confirmed as a reliable noninvasive test for the evaluation of liver fibrosis in hepatitis B virus-infected patients with comparable performance to that of FibroScan and proved to be unaffected by inflammation.

1. Introduction

It is estimated that about 2 billion people worldwide have been infected with the hepatitis B virus (HBV), and >350 million of them have chronic HBV infection [1]. In China, a seroepidemiological survey of HBV infection in 2006 showed that the prevalence of hepatitis B surface antigen positivity was 7.18%. It was estimated that 93 million people were HBV carriers, of whom 30 million were patients with chronic hepatitis B (CHB) [2]. CHB may progress to cirrhosis and hepatocellular carcinoma. An accurate method for monitoring the progression of liver fibrosis is urgently needed for the prognosis and management of chronic liver diseases. Liver biopsy is generally considered as the gold standard for assessing hepatic histology in CHB [3–5]. However, it often has limitations due to its invasiveness, risk of complications, sampling errors, and interobserver variability [6–8]. Many noninvasive methods for replacing or complementing the liver biopsy have been developed in recent years [9–12]. FibroScan (transient elastography) and FibroTest (serological marker test) have been evaluated most frequently; these methods have similar diagnostic accuracies for predicting fibrosis staging from receiver-operating characteristic (ROC) curves [13–16]. FibroTest employs a narrow and complex algorithm for 5 biochemical markers (α2-macroglobulin, apolipoprotein A1, haptoglobin, γ-glutamyl transferase, and bilirubin), which requires extensive and specialized blood analysis [17]. Recently, we developed a novel diagnostic score named LecT-Hepa for convenient and rapid monitoring of liver fibrosis progression. It is based on glyco-alteration (e.g., fucosylation and desialylation) of serum α1-acid glycoprotein

Abbreviations: HBV, hepatitis B virus; CHB, chronic hepatitis B; PLT, platelet count; AGP, α1-acid glycoprotein; LSM, liver stiffness measurement; LC, liver cirrhosis; non-LC, non-cirrhosis; DSA, *Datura stramonium* agglutinin; MAL, *Maackia amurensis* lectin; AOL, *Aspergillus oryzae* lectin.

* Corresponding author.
** Correspondence to: H. Narimatsu, Research Center for Medical Glycoscience (RCMG), National Institute of Advanced Industrial Science and Technology (AIST), 1-1-1 Umezono, Tsukuba, Ibaraki 305-8568, Japan.
E-mail addresses: zxx10375@rjh.com.cn (X. Zhang), h.narimatsu@aist.go.jp (H. Narimatsu).
[1] These authors contributed equally to this paper.

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(AGP), which is assessed using a triplex lectin–antibody immunoassay [18,19]. It has been demonstrated to be well correlated with the fibrosis stage determined by liver biopsy, and verified to be more efficient by comparing with other serological methods (hyaluronic acid, tissue inhibitor of metalloproteases-1, platelet count, APRI, Forns index, Fib-4 index, and Zeng's score) in a multicenter study [20]. Here, to evaluate the reliability of LecT-Hepa for assessing liver fibrosis, we compared the diagnostic performance of LecT-Hepa and FibroScan for distinguishing cirrhosis from non-cirrhosis in a large cohort of HBV-infected Chinese patients with different serum alanine aminotransferase (ALT) levels.

2. Materials and methods

2.1. Patients

A total of 239 patients who had been positive for hepatitis B surface antigen for at least 6 months were enrolled retrospectively from Ruijin Hospital (Shanghai, China) from March 2009 to May 2011. Patients who were coinfected with another hepatitis virus or HIV, or who had excessive alcohol intake (>20 g/d), hepatocellular carcinoma, or other causes of liver diseases were excluded. For all patients, serum biochemical parameters, including the levels of aspartate aminotransferase (AST) and ALT, as well as platelet (PLT), were assessed at the time of the liver stiffness measurement. Normal values for ALT and AST ranged between 10 and 64 IU/l and between 8 and 40 IU/l, respectively, which were determined based on the manufacturer's instructions and adjusted according to the results of validation test by medical laboratory of Ruijin Hospital. Serum samples were collected at the time of the liver stiffness measurement for detection of lectins and stored at −20 °C until analysis. The patients were divided into two groups: liver cirrhosis (LC) group and non-cirrhosis (non-LC) group. The diagnosis of cirrhosis was based on clinical and morphological criteria and ultrasonography according to standard definitions [21]. The institutional ethics committees of Ruijin Hospital of Shanghai Jiao Tong University approved this study, and the informed consent was obtained from all patients.

2.2. Liver stiffness measurement

Liver stiffness was measured by transient elastography using FibroScan (EchoSens, Paris, France). The measurement depth was between 25 mm and 65 mm. For each patient, 10 validated measurements were performed. The success rate was calculated as the number of validated measurements divided by the total number of measurements. The results were expressed in kilopascals. The median value was considered representative of the elastic modulus of the liver. Only procedures with 10 validated measurements and a success rate of at least 60% were considered reliable.

2.3. Automatic acquisition of quantitative glyco-alteration of AGP

Each individual serum (5 μl) that had been stored at −20 °C was diluted 10 fold with phosphate buffered saline containing 0.2% sodium dodecyl sulfate, and then heated at 95 °C for 20 min. AGP in the resulting solution was enriched by immunoprecipitation with biotinylated anti-AGP antibody using an automated protein purification system (ED-01; GP BioSciences Ltd., Tokyo, Japan). Each elution fraction (100 μl) was kept at −80 °C until a sandwich immunoassay was performed. Subsequent to the enrichment, fibrosis-specific glyco-alteration of AGP was quantified using simultaneous lectin–antibody sandwich immunoassays for three lectins: *Datura stramonium* agglutinin (DSA), *Maackia amurensis* lectin (MAL), and *Aspergillus oryzae* lectin (AOL), by a fully automatic chemiluminescence enzyme immunoassay system (HISCL-2000i; Sysmex Co., Kobe, Japan). The criterion formula of LecT-Hepa was as before described [19]:

$$\text{LecT-Hepa} = \text{Log}_{10}[\text{AOL/DSA}] \times 8.6 - [\text{MAL/DSA}].$$

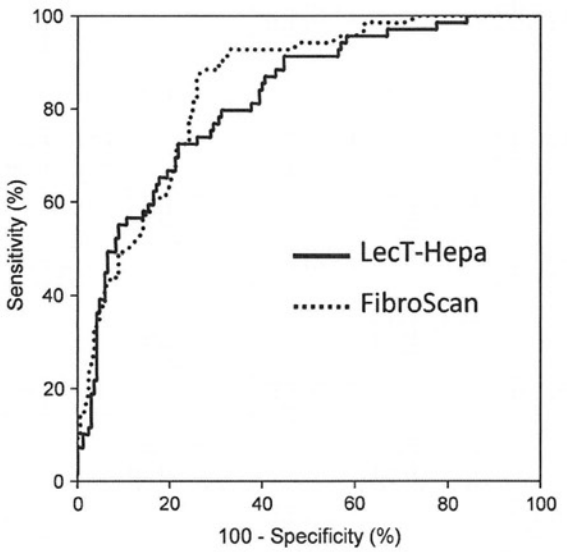
2.4. Statistical analysis

Statistical calculations were performed using software from GraphPad Prism 5 (GraphPad, San Diego, CA). A P value of <0.01 (1%) was considered to be statistically significant. The diagnostic performance of the fibrosis markers and indices were assessed using ROC curves and were then expressed as diagnostic specificity, sensitivity,

Table 1
Clinical characteristics of the patients.

Data	non-LC (n=170)	LC (n=69)	Significance non-LC vs LC
Age (y)	38.5±11.0	47.94±9.0	P<0.0001
Male sex (%)	126 (74.1%)	51 (73.9%)	-
AST (IU/l)	70.5±150.1	88.4±109.8	P=0.0002
ALT (IU/l)	111.6±213.7	88.5±116.1	P=0.1965
PLT (×10^9/l)	167.5±43.9	86.0±48.0	P<0.0001
FibroScan	10.3±8.8	27.0±19.1	P<0.0001
MAL/DSA	10.1±2.0	7.5±2.3	P<0.0001
AOL/DSA	5.1±13.5	24.0±47.6	P<0.0001

Patients were classified as non-LC or LC. LC, liver cirrhosis; non-LC, non-cirrhosis. Quantitative results are expressed as means±standard deviations or n (%).



	FibroScan	LecT-Hepa
AUC	0.85	0.82
(95% CI)	(0.797-0.897)	(0.763-0.877)
Se (%)	87.0	72.5
Sp (%)	74.1	78.2
PPV (%)	57.7	57.5
NPV (%)	93.3	87.5

Fig. 1. Receiver-operating characteristic curves of LecT-Hepa and FibroScan for distinguishing LC from non-LC. AUC, area under the receiver-operating characteristic curve; Se, sensitivity; Sp, specificity; PPV, positive predictive value; NPV, negative predictive value.

positive predictive value (PPV), negative predictive value (NPV) and area under the ROC curve (AUC) values (95% confidence interval [95% CI]).

3. Results

3.1. General characteristics

A total of 239 patients who showed evidence of chronic HBV infection and had undergone liver stiffness measurement were investigated. The mean age was 41.2 ± 11.3 y, and 177 (74%) of them were males. Among the all, 170 (71%) and 69 (29%) patients were diagnosed as non-LC and LC, respectively. Their characteristics are summarized in Table 1. Significant differences were found in Age ($P<0.0001$), AST ($P=0.0002$), PLT ($P<0.0001$), FiborScan ($P<0.0001$), MAL/DSA ($P<0.0001$), and AOL/DSA ($P<0.0001$) between the non-LC group and LC group, whereas ALT ($P=0.1965$) was not significantly different between the two groups.

3.2. Receiver-operating characteristic analysis

The overall diagnosis performances of LecT-Hepa and FibroScan were assessed using ROC curves. Fig. 1 shows the ROC curves for distinguishing LC from non-LC by both methods. The area under the ROC curve (95% CI) was 0.82 (0.763–0.877) for LecT-Hepa and 0.85 (0.797–0.897) for FibroScan. The overall diagnostic accuracies for LecT-Hepa and FibroScan were 77% and 78%, respectively. The obtained values for sensitivity, specificity, PPV, and NPV are shown in the bottom table of Fig. 1. There was no significant difference between both methods.

3.3. Effect of hepatic inflammation on the diagnostic cutoff values

Because the upper limit of the normal value for ALT level was 64 IU/l, the patients were categorized by the normal (≤64 IU/l) and elevated (>64 IU/l) ALT levels. According to this classification, 169 patients (71%) had the normal ALT level and 70 patients (29%) had the elevated ALT level. The proportions of patients with LC in the normal and elevated ALT levels were similar (28% of normal ALT patients and 30% of elevated ALT patients). Distribution of the values obtained by each test is shown in Fig. 2. Medians of these methods increased significantly between the non-LC group and LC group (all $P<0.0001$) in the both ALT levels. LecT-Hepa values in the non-LC group ($P=0.65$) and LC group ($P=0.02$) showed no significant difference between the two ALT categories (Fig. 2A). In contrast, the FibroScan value was obviously increased with the elevation of ALT levels ($P<0.0001$) even in the same diagnostic group (Fig. 2B). Thereby, we could distinguish the LC group in the normal ALT level from non-LC group in the elevated ALT level ($P<0.0001$) by LecT-Hepa, but could not by FibroScan ($P=0.05$). Collectively, the value of FibroScan was greatly affected by the ALT levels, whereas the value for LecT-Hepa was not influenced regardless of the ALT levels.

4. Discussion

This is the first study comparing LecT-Hepa with FibroScan. These results showed the obvious advantage of LecT-Hepa in comparison with FibroScan based on robustness against fluctuation of the ALT levels with a large cohort of HBV-infected Chinese patients at different ALT levels. Thus, the diagnostic performance of LecT-Hepa was the most reliable for monitoring the progression of hepatic fibrosis.

A recent paper showed that the majority of nucleoside-naïve patients with CHB who were treated with entecavir in the long-term cohort achieved substantial histological improvement and regression of fibrosis or cirrhosis [22], suggesting that a noninvasive test for the assessment of liver fibrosis in the treated patients is required during the follow-up. The liver biopsy is limited not only by its invasive nature, but also by its accuracy. A specimen collected in a standard liver biopsy using a short, narrow-gauge needle represents a very small portion of the whole liver mass, resulting in intra- and interobserver variability and sampling errors, which account for 25% of false-negative diagnoses of cirrhosis [23–25]. Therefore, a noninvasive marker that accurately reflects the condition of the whole liver is required.

At present, FibroScan is the most intensively evaluated noninvasive method for the assessment of liver fibrosis. Its diagnostic value is considered to be superior to that of biochemical markers [26]. However, several studies noted that liver stiffness measurements using FibroScan for patients with inflammation and acute liver damage overestimate the actual stage of fibrosis and may reduce the diagnostic accuracy [27,28]. In general, a high ALT level reflects a vigorous immune response

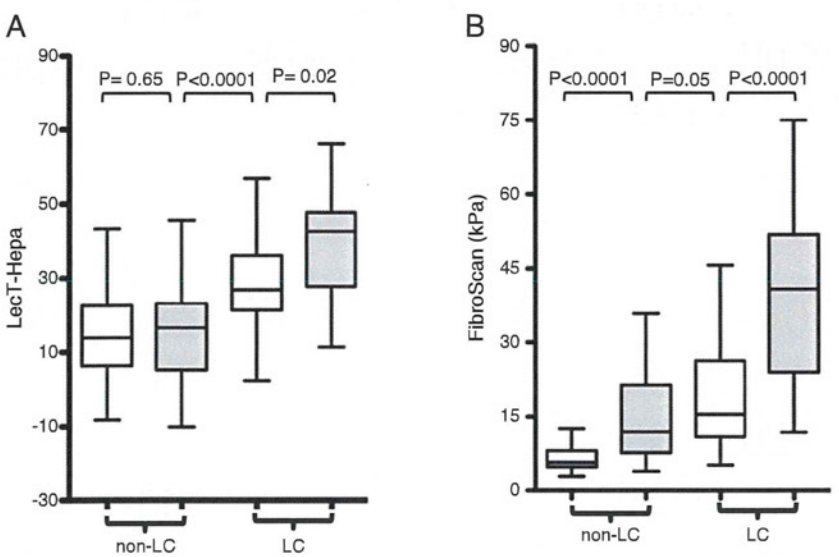


Fig. 2. Distribution of LecT-Hepa (A) and FibroScan (B) values in non-LC and LC patients with different ALT levels. The top and bottom of the whiskers are the 95th and 5th percentiles. The top and bottom of the boxes are the first and third quartiles. The size of the box represents the interquartile range within which 50% of the values are located. The line across the box indicates the median value. LC, liver cirrhosis; non-LC, non-cirrhosis. The open and gray boxes indicate normal (≤64 IU/l) and elevated (>64 IU/l) ALT levels, respectively.

to HBV and histological activity (i.e., necroinflammation). Our study obviously showed that the FibroScan values were substantially affected by ALT fluctuation. These results were also in accordance with the study of Kim et al., in which advanced fibrosis stage (F3-4) or cirrhosis showed a negative correlation with discordance between liver biopsy and FiborScan in assessing liver fibrosis in patients with CHB, and maximal activity grade 3–4 significantly influenced the liver stiffness measurement values in F3 and F4 [28]. In practice, hepatic activation and fibrosis stage should be estimated independently, as should histological diagnoses followed by a biopsy, such as the histological activity index scoring system. Thus, a marker that relies on an analysis of the specific protein content to monitor liver fibrosis should be robust against hepatic inflammation. In this context, we can explain that the reliability of LecT-Hepa is superior to that of FibroScan. LecT-Hepa has been already validated for estimating liver fibrosis using a large amount of serum specimens from patients with well-defined fibrosis stage by biopsy in a multicenter study [21]. This report led us to consider that LecT-Hepa can be a good substitute for liver biopsy. This is the reason we herein focused on the examination into the effect of hepatic inflammation on diagnosis of LC by LecT-Hepa.

In conclusion, we confirmed that LecT-Hepa is unaffected by inflammation. This suggested that LecT-Hepa is the most reliable and effective for the assessment of fibrosis progression in HBV-infected patients whose ALT levels are often fluctuated and thus can be used for routine assessments of liver fibrosis in HBV-infected patients.

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OPEN ACCESS

ORIGINAL ARTICLE

Hepatitis C virus kinetics by administration of pegylated interferon-α in human and chimeric mice carrying human hepatocytes with variants of the *IL28B* gene

Tsunamasa Watanabe,[1] Fuminaka Sugauchi,[2] Yasuhito Tanaka,[1] Kentaro Matsuura,[3] Hiroshi Yatsuhashi,[4] Shuko Murakami,[1] Sayuki Iijima,[1] Etsuko Iio,[3] Masaya Sugiyama,[5] Takashi Shimada,[6] Masakazu Kakuni,[6] Michinori Kohara,[7] Masashi Mizokami[5]

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[1]Department of Virology and Liver Unit, Nagoya City University Graduate School of Medical Sciences, Nagoya, Japan
[2]Department of Gastroenterology, Nagoya City Koseiin Medical Welfare Center, Nagoya, Japan
[3]Department of Gastroenterology and Metabolism, Nagoya City University Graduate School of Medical Sciences, Nagoya, Japan
[4]Department of Therapeutic Research, National Hospital Organization (NHO) Nagasaki Medical Center, Nagasaki, Japan
[5]The Research Center for Hepatitis and Immunology, National Center for Global Health and Medicine, Ichikawa, Japan
[6]PhoenixBio Co. Ltd., Higashi-Hiroshima, Japan
[7]Tokyo Metropolitan Institute of Medical Science, Tokyo, Japan

Correspondence to
Dr Masashi Mizokami, The Research Center for Hepatitis and Immunology, National Center for Global Health and Medicine 1–7–1, Kohnodai, Ichikawa 272–8516, Japan; mmizokami@hospk.ncgm.go.jp

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ABSTRACT

Objective Recent studies have demonstrated that genetic polymorphisms near the *IL28B* gene are associated with the clinical outcome of pegylated interferon α (peg-IFN-α) plus ribavirin therapy for patients with chronic hepatitis C virus (HCV). However, it is unclear whether genetic variations near the *IL28B* gene influence hepatic interferon (IFN)-stimulated gene (ISG) induction or cellular immune responses, lead to the viral reduction during IFN treatment.

Design Changes in HCV-RNA levels before therapy, at day 1 and weeks 1, 2, 4, 8 and 12 after administering peg-IFN-α plus ribavirin were measured in 54 patients infected with HCV genotype 1. Furthermore, we prepared four lines of chimeric mice having four different lots of human hepatocytes containing various single nucleotide polymorphisms (SNP) around the *IL28B* gene. HCV infecting chimeric mice were subcutaneously administered with peg-IFN-α for 2 weeks.

Results There were significant differences in the reduction of HCV-RNA levels after peg-IFN-α plus ribavirin therapy based on the *IL28B* SNP rs8099917 between TT (favourable) and TG/GG (unfavourable) genotypes in patients; the first-phase viral decline slope per day and second-phase slope per week in TT genotype were significantly higher than in TG/GG genotype. On peg-IFN-α administration to chimeric mice, however, no significant difference in the median reduction of HCV-RNA levels and the induction of antiviral ISG was observed between favourable and unfavourable human hepatocyte genotypes.

Conclusions As chimeric mice have the characteristic of immunodeficiency, the response to peg-IFN-α associated with the variation in *IL28B* alleles in chronic HCV patients would be composed of the intact immune system.

Significance of this study

What is already known on this subject?
- Genetic polymorphisms near the *IL28B* gene are associated with a chronic HCV treatment response.
- HCV-infected patients with the *IL28B* homozygous favourable allele had a more rapid decline in HCV kinetics in the first and second phases by peg-IFN-α-based therapy.
- During the acute phase of HCV infection, a strong immune response among patients with the *IL28B* favourable genotype could induce more frequent spontaneous clearance of HCV.

What are the new findings?
- In chronically HCV genotype 1b-infected chimeric mice that have the characteristic of immunodeficiency, no significant difference in the reduction in serum HCV-RNA levels and the induction of antiviral hepatic ISG by the administration of peg-IFN-α was observed between favourable and unfavourable human hepatocyte *IL28B* genotypes.
- By comparison of serum HCV kinetics between human and chimeric mice, the viral decline in both the first and second phases by peg-IFN-α treatment was affected by the variation in *IL28B* genotypes only in chronic hepatitis C patients.

How might it impact on clinical practice in the foreseeable future?
- The immune response according to *IL28B* genetic variants could contribute to the first and second phases of HCV-RNA decline and might be critical for HCV clearance by peg-IFN-α-based therapy.

INTRODUCTION

Hepatitis C is a global health problem that affects a significant portion of the world's population. The WHO estimated that, in 1999, 170 million hepatitis C virus (HCV)-infected patients were present worldwide, with 3–4 million new cases appearing per year.[1]

The standard therapy for hepatitis C still consists of pegylated interferon-α (peg-IFN-α), administered once weekly, plus daily oral ribavirin for 24–48 weeks in countries where protease inhibitors are not available.[2] This combination therapy is quite successful in patients with HCV genotype 2 or 3 infection, leading to a sustained virological response (SVR) in approximately 80–90% of patients treated; however, in patients infected with HCV genotype 1 or 4, only approximately half of all treated individuals achieved a SVR.[3 4]

Table 1 Characteristics of 54 patients infected HCV genotype 1

	IL28B SNP rs8099917		
	TT (n=34)	TG (n=19) + GG (n=1)	p Value
Age (years)	55.6±10.1	54.7±11.3	0.746
Gender (male %)	70	50	0.199
Body mass index (kg/m^2)	24.6±3.1	24.7±3.3	0.870
Viral load at therapy (log IU/ml)	6.0±0.7	5.8±0.8	0.357
SVR rate (%)	50	11	0.012
Serum ALT level (IU/l)	100.3±80.8	79.3±45.0	0.226
Platelet count (×10^4/μl)	17.1±9.0	16.5±5.8	0.771
Fibrosis (F3+4 %)	42	40	0.877

HCV, hepatitis C virus; SNP, single nucleotide polymorphism; SVR, sustained virological response.

Host factors were shown to be associated with the outcome of the therapy, including age, sex, race, liver fibrosis and obesity.[5] Genome-wide association studies have demonstrated that genetic variations in the region near the interleukin-28B (*IL28B*) gene, which encodes interferon (IFN)-λ3, are associated with a chronic HCV treatment response.[6–10] Furthermore, it was demonstrated that genetic variations in the *IL28B* gene region are also associated with spontaneous HCV clearance.[11–12]

Interestingly, a recent report showed the effect of genetic polymorphisms near the *IL28B* gene on the dynamics of HCV during peg-IFN-α plus ribavirin therapy in Caucasian, African American and Hispanic individuals;[13] HCV-infected patients with the *IL28B* homozygous favourable allele had a more rapid decline of HCV in the first phase, which is associated with the inhibition of viral replication as well as the second phase associated with immuno-destruction of viral-infected hepatocytes.[14] However, it is unknown how a direct effect by the *IL28B* genetic variation, such as the induction of IFN-stimulated genes (ISG) or cellular immune responses, would influence the viral kinetics during IFN treatment. Over recent periods, engineered severe combined immunodeficient (SCID) mice transgenic for urokinase-type plasminogen activator (uPA) received human hepatocyte transplants (hereafter referred to as chimeric mice)[15–17] and are suitable for experiments with hepatitis viruses in vivo.[18 19] We have also reported that these chimeric mice carrying human hepatocytes are a robust animal model to evaluate the efficacy of IFN and other anti-HCV agents.[20 21]

The purpose of this study was to reveal the association between genetic variations in the *IL28B* gene region and viral decline during peg-IFN-α treatment in patients with HCV, and to clarify the association between different *IL28B* alleles of human hepatocytes in chimeric mice and the response to peg-IFN-α without immune response. These studies will elucidate whether the immune response by the *IL28B* genetic variation affects the viral kinetics during peg-IFN-α treatment.

MATERIALS AND METHODS

Patients

Fifty-four Japanese patients with chronic HCV genotype 1 infection at Nagasaki Medical Center and Nagoya City University were enrolled in this study (table 1). Patients received peg-IFN-α2a (180 μg) or 2b (1.5 μg/kg) subcutaneously every week and were administered a weight-adjusted dose of ribavirin (600 mg for <60 kg, 800 mg for 60–80 kg, and 1000 mg for >80 kg daily), which is the recommended dosage in Japan. Patients with other hepatitis virus infection or HIV coinfection were not included in the study. The study protocol conformed to the ethics guidelines of the 1975 Declaration of Helsinki as reflected by earlier approval by the institutions' human research committees.

Laboratory tests

Blood samples were obtained before therapy, as well as on day 1 and at weeks 1, 2, 4, 8 and 12 after the start of therapy and were analysed for the HCV-RNA level by the commercial Abbott Real-Time HCV test with a lower limit of detection of 12 IU/ml (Abbott Molecular Inc., Des Plains, Illinois, USA). Genetic polymorphism in the *IL28B* gene (rs8099917), a single nucleotide polymorphism (SNP) recently identified to be associated with treatment response,[6–8] was tested by the TaqMan SNP genotyping assay (Applied Biosystems, Foster City, California, USA).

HCV infection of chimeric mice with the liver repopulated for human hepatocytes

SCID mice carrying the uPA transgene controlled by an albumin promoter were injected with 5.0–7.5×10^5 viable hepatocytes through a small left-flank incision into the inferior splenic pole, thereafter chimeric mice were generated. The chimeric mice were purchased from PhoenixBio Co, Ltd (Hiroshima, Japan).[17] Human hepatocytes with the *IL28B* homozygous favourable allele, heterozygous allele or homozygous unfavourable allele were imported from BD Biosciences (San Jose, California, USA) (table 2). Murine serum levels of human albumin and the body weight were not significantly different among four chimeric mice groups, providing a reliable comparison for anti-HCV agents.[22] Three different serum samples were obtained from three chronic HCV patients (genotype 1b).[21 22] Each mouse was intravenously infected with serum sample containing 10^5 copies of HCV genotype 1b. Administration of peg-IFN-α2a (Pegasys; Chugai Pharmaceutical Co., Ltd., Tokyo, Japan) at the dose formulation (30 μg/kg) was consecutively applied to each mouse on days 0, 3, 7 and 10 (table 3).

HCV-RNA quantification

HCV-RNA in mice sera (days 0, 1, 3, 7 and 14) was quantified by an in-house real-time detection PCR assay with a lower quantitative limit of detection of 10 copies/assay, as previously reported.[21]

Quantification of IFN-stimulated gene-expression levels

For analysis of endogenous ISG levels, total RNA was isolated from the liver using the RNeasy RNA extraction kit (Qiagen, Valencia, California, USA) and complementary DNA synthesis

Table 2 Four lines of uPA/SCID mice from four different lots of human hepatocytes (donor) containing various SNP around the *IL28B* gene

uPA/SCID mice	Donor	Race	Age	Gender	rs8103142	rs12979860	rs8099917
PXB mice	A	African American	5 Years	Male	CC	TT	TG
	B	Caucasian	10 Years	Female	CC	TT	TG
	C	Hispanic	2 Years	Female	TT	CC	TT
	D	Caucasian	2 Years	Male	TT	CC	TT

PXB mice; urokinase-type plasminogen activator/severe combined immunodeficiency (uPA/SCID) mice repopulated with approximately 80% human hepatocytes.
SCID, severe combined immunodeficient; SNP, single nucleotide polymorphism.

Table 3 Dosage and time schedule of pegIFN-α2a* treatment for HCV genotype 1b infected chimeric mice

Donor hepatocytes†	No of chimeric mice	Inoculum	Test compound	Dose			
				Level (μg/kg)	Concentrtion (μg/ml)	Volume (ml/kg)	Frequency
A	3	Serum A	Peg-IFN-α2a	30	3	10	Day 0, 3, 7, 10
B	4	Serum A	Peg-IFN-α2a	30	3	10	Day 0, 3, 7, 10
C	3	Serum A	Peg-IFN-α2a	30	3	10	Day 0, 3, 7, 10
D	3	Serum A	Peg-IFN-α2a	30	3	10	Day 0, 3, 7, 10
A	2	Serum B	Peg-IFN-α2a	30	3	10	Day 0, 3, 7, 10
C	2	Serum B	Peg-IFN-α2a	30	3	10	Day 0, 3, 7, 10
A	2	Serum C	Peg-IFN-α2a	30	3	10	Day 0, 3, 7, 10
C	2	Serum C	Peg-IFN-α2a	30	3	10	Day 0, 3, 7, 10

*Pegasys; Chugai Pharmaceutical Co., Ltd., Tokyo, Japan.
†The *IL28B* genetic variation of the donor hepatocytes was indicated in table 2.
HCV, hepatitis C virus; peg-IFN-α, pegylated interferon α.

was performed using 2.0 μg of total RNA (High Capacity RNA-to-cDNA kit; Applied Biosystems). Fluorescence real-time PCR analysis was performed using an ABI 7500 instrument (Applied Biosystems) and TaqMan Fast Advanced gene expression assay (Applied Biosystems). TaqMan Gene Expression Assay primer and probe sets (Applied Biosystems) are shown in the supplementary information (available online only). Relative amounts of messenger RNA, determined using a FAM-Labeled TaqMan probe, were normalised to the endogenous RNA levels of the housekeeping reference gene, glyceraldehyde-3-phosphate dehydrogenase. The delta Ct method ($2^{-(\text{delta Ct})}$) was used for quantitation of relative mRNA levels and fold induction.[23 24]

Statistical analyses

Statistical differences were evaluated by Fisher's exact test or the χ^2 test with the Yates correction. Mice serum HCV-RNA and intrahepatic ISG expression levels were compared using the Mann–Whitney U test. Differences were considered significant if p values were less than 0.05.

RESULTS

Characteristics of the study patients

Genotypes (rs8099917) TT, TG and GG were detected in 34, 19 and one patient infected with HCV genotype 1, respectively. SVR rates were significantly higher in HCV patients with genotype TT than in those with genotype TG/GG (50% vs 11%, p=0.012). The initial HCV serum load was comparable between genotypes TT and TG/GG (6.0±0.7 vs 5.8±0.8 log IU/ml). There were no significant differences in sex (male%, 70% vs 50%), age (55.6±10.1 vs 54.7±11.3 years), serum alanine aminotransferase level (100.3±80.8 vs 79.3±45.0 IU/L), platelet count (17.1±9.0 vs 16.5±5.8×10^4/μl) and fibrosis stages (F3/4%, 42% vs 40%) between HCV patients with the favourable (rs8099917 TT) and unfavourable (rs8099917 TG/GG) *IL28B* genotypes (table 1).

Changes in serum HCV-RNA levels in patients treated by peg-IFN-α plus ribavirin

Figure 1 shows the initial change in the serum HCV-RNA level for 14 days after peg-IFN-α plus ribavirin therapy in patients infected with HCV genotype 1 based on the genetic polymorphism near the *IL28B* gene. The immediate antiviral response (viral drop 24 h after the first IFN injection) was significantly higher in HCV patients with genotype TT than genotype TG/GG (−1.08 vs −0.39 log IU/ml, p<0.001). Figure 2 also shows the subsequent change in the serum HCV-RNA reduction after peg-IFN-α plus ribavirin therapy in patients infected with HCV genotype 1. Similarly, during peg-IFN-α plus ribavirin therapy, a statistically significant difference in the median reduction in serum HCV-RNA levels was noted according to the genotype (TT vs TG/GG). The median reduction in the serum HCV-RNA levels (log IU/ml) at 1, 2, 4, 8 and 12 weeks between genotypes TT and TG/GG was as follows: −1.58 vs −0.62, p<0.001; −2.35 vs −0.91, p<0.001;

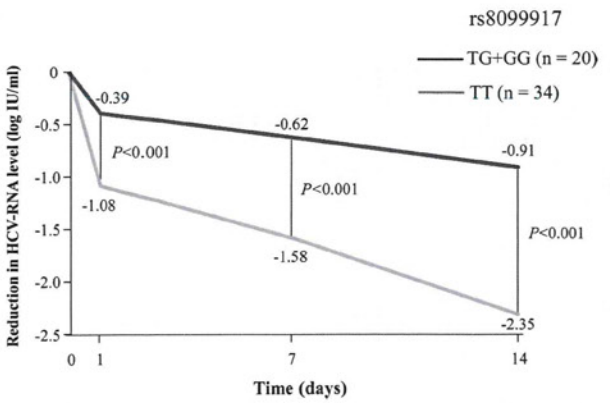

Figure 1 Rapid reduction of median hepatitis C virus (HCV)-RNA levels (log IU/ml) at 1, 7 and 14 days between *IL28B* single nucleotide polymorphisms rs8099917 genotype TT (n=34) and TG/GG (n=20) in HCV genotype 1-infected patients treated with peg-IFN-α plus ribavirin.

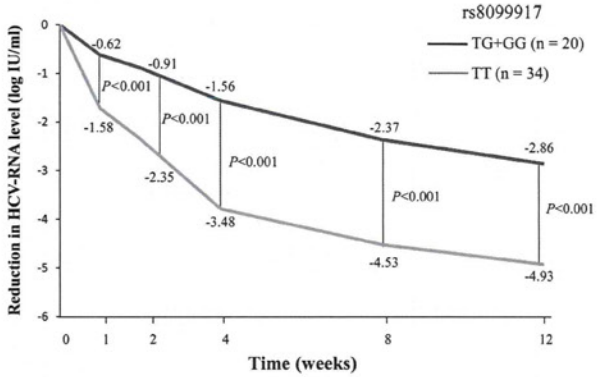

Figure 2 Weekly reduction of median hepatitis C virus (HCV)-RNA levels (log IU/ml) at 1, 2, 4, 8 and 12 weeks between *IL28B* single nucleotide polymorphisms rs8099917 genotype TT (n=34) and TG/GG (n=20) in HCV genotype 1-infected patients treated with pegylated interferon α plus ribavirin.

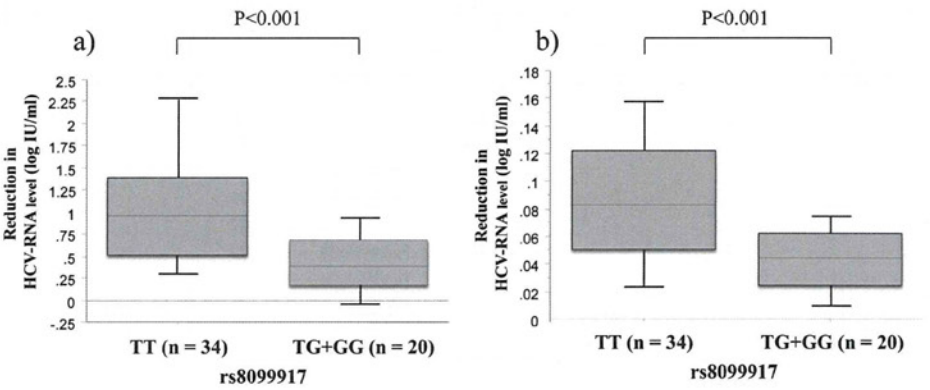

Figure 3 (A) The first-phase viral decline slope per day (Ph1/day) and (B) second-phase viral decline slope per week (Ph2/week) in hepatitis C virus (HCV) genotype 1-infected patients treated with pegylated interferon α plus ribavirin. The lines across the boxes indicate the median values. The hash marks above and below the boxes indicate the 90th and 10th percentiles for each group, respectively.

−3.48 vs −1.56, p<0.001; −4.53 vs −2.37, p<0.01; −4.93 vs −2.86, p<0.001. Furthermore, the initial first-phase viral decline slope per day (Ph1/day) and subsequent second-phase viral decline slope per week (Ph2/week) in TT genotype were significantly higher than in genotype TG/GG (Ph1/day 0.94 ±0.83 vs 0.38±0.40 log IU/ml, p<0.001; Ph2/week 0.08±0.06 vs 0.04±0.03 log IU/ml, p<0.001) (figure 3).

Changes in serum HCV-RNA levels in chimeric mice treated by peg-IFN-α

In order to clarify the association between *IL28B* alleles of human hepatocytes and the response to peg-IFN-α, we prepared four lines of uPA/SCID mice and four different lots of human hepatocytes containing various rs8099917, rs8103142 and rs12979860 SNPs around the *IL28B* gene (table 2). The chimeric mice were inoculated with serum samples from each HCV-1b patient, and then HCV-RNA levels had increased and reached more than 10^6 copies/ml in all chimeric mice sera at 2 weeks after inoculation. After confirming the peak of HCV-RNA in all chimeric mice, they were subcutaneously administered with four times injections of the bolus dose of peg-IFN-α2a for 2 weeks (table 3). Figure 4 shows the change in the serum HCV-RNA levels for 14 days during IFN injection into chimeric mice transplanted with *IL28B* favourable or unfavourable human hepatocyte genotypes. On peg-IFN-α administration, no significant difference in the median reduction in HCV-RNA levels in the serum A-infected[22] chimeric mice sera was observed between favourable (n=7) and unfavourable

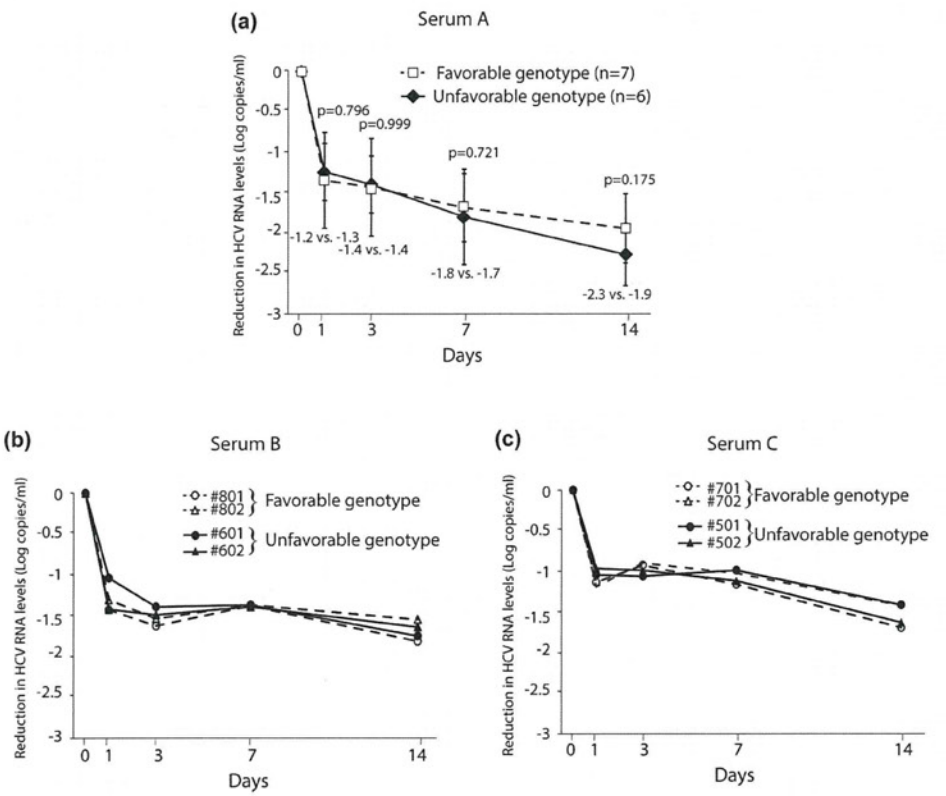

Figure 4 Median reduction of hepatitis C virus (HCV)-RNA levels (log copies/ml) after administering pegylated interferon α to chimeric mice having human hepatocytes containing various single nucleotide polymorphisms around the *IL28B* gene as favourable (rs8099917 TT) and unfavourable (rs8099917 TG) genotypes. Data are represented as mean+SD. Chimeric mice infected with a) serum A (n=7; favourable genotype, n=6; unfavourable genotype), (B) serum B (n=2, each genotype), and (C) serum C (n=2, each genotype). All serum samples were obtained from HCV-1b patients.