positive status after withdrawal of IFN were defined as virological relapsers. Patients who did not become HCV negative with IFN therapy were defined as non-virological responders.

This study was approved by the ethics committee of each institution involved. Informed consent was obtained from each patient, and the study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki.

Virological tests

Hepatitis C virus was genotyped by direct sequencing of the 5′-untranslated region and/or E1 regions, as described previously.[30,31] Genotypes were classified according to the nomenclature proposed by Simmonds *et al.*[32]

Nested PCR analysis and direct sequencing of the NS5A-ISDR were performed as previously reported for each genotype.[15,16,27,28] In brief, RNA was extracted from 140 μL serum using a QIAamp Viral RNA Kit (Qiagen, Valencia, CA, USA) and dissolved in 50 μL diethylpyrocarbonate-treated water. RNA (10 ng) was used for reverse transcription with oligo and random hexamer primers with an iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA). NS5A-ISDR was sequenced after amplification by nested PCR as previously described.[15,16,27,28]

The primers used were as follows: NS5A-ISDR of genotype 1b, sense 5′-TGGATGGAGTGCGGTTGCACA GGTA-3′ and antisense 5′-TCTTTCTCCGTGGAGGTGGT ATTG-3′; NS5A-ISDR of genotype 2a, sense 5′-ACGTCC ATGCTAACAGACCC-3′ and antisense 5′-GGGAATCT CTTCTTGGGGAG-3′; and NS5A-ISDR of genotype 2b, sense 5′-TCTCAGCTCCCTTGCGATCCTGA-3′ and antisense 5′-GATGGTATCGAAGGCTC-3′. Amplification conditions consisted of 10 min at 94°C, followed by 40 cycles of 94°C for 10 s, 55°C for 30 s and 72°C for 30 s in a thermal cycler (GeneAmp PCR System 9700; Applied Biosystems, Foster City, CA, USA). The second PCR was done using the following sets of primers: NS5A-ISDR of genotype 1b, sense 5′-CAGGTACGC TCCGGCGTGCA-3′ and antisense 5′-GGGGCCTTGGT AGGTGGCAA-3′; NS5A-ISDR of genotype 2a, sense from the first-round PCR and a new antisense primer 5′-CGAGAGAGTCCAGAACGACC-3′; and NS5A-ISDR of genotype 2b, sense 5′-AGCTCCTCAGCGAGCCA GCT-3′ and antisense 5′-GATGGTATCGAAGGCTC-3′. PCR products were separated by electrophoresis on 2% agarose gels, stained with ethidium bromide and visualized under ultraviolet light. PCR products were then purified and sequenced with the second-round PCR primers with a dye terminator sequencing kit (BigDye Terminator v1.1 Cycle Sequencing Kit; Applied Biosystems) and an ABI 310 DNA Sequencer (Applied Biosystems).

Genomic analysis

Detection of the SNP of IL-28B (rs8099917) was done by a real-time PCR system, as previously reported.[16] In brief, genomic DNA was extracted from 15 μL of whole blood using a commercial kit (QIAamp DNA Blood mini Kit; Qiagen) and dissolved in 50 μL diethylpyrocarbonate-treated water. DNA (1 ng) was used for PCR with primers and probes of commercial kit (Taqman SNP Genotyping Assays; Applied Biosystems). The SNP of IL-28B (rs8099917) was amplified, and the results were analyzed by real-time PCR in a thermal cycler (7300 Real time PCR System; Applied Biosystems).

Statistical analysis

Data are expressed as mean ± standard deviation. A paired Student's *t*-test or Fisher's exact test were used to analyze differences in variables. $P < 0.05$ was considered significant. Multiple logistic regression models were used to identify factors predictive of SVR. Statview ver. 5.0 software (SAS Institute, Cary, NC, USA) was used for all analyses.

RESULTS

Background

PATIENTS' CLINICAL CHARACTERISTICS are summarized in Table 1. HCV genotypes 1b ($n = 34$), 2a ($n = 58$), 2b ($n = 9$) and unknown ($n = 3$) were detected.

Table 1 Clinical characteristics at pretreatment

Clinical characteristics	$n = 104$
Age (years)	55.1 ± 12.5
Sex: male/female	62/42
AST (IU/L)	50.0 ± 28.2
ALT (IU/L)	62.7 ± 47.3
Platelet count (10^4/uL)	18.4 ± 5.7
HCV RNA level (KIU/mL)	36 (1.6–100)
HCV genotype (1b/2a/2b/unknown)	34/58/9/3
IFN length (weeks) (24/48/<17)	49/45/10
Body mass index	22.7 ± 3.2

Data are expressed as mean ± standard deviation.
HCV RNA level was shown by median (range).
ALT, alanine aminotransferase; AST, aspartate aminotransferase; HCV, hepatitis C virus; IFN, interferon.

Table 2 Virological response in each group

(a) Virological response according to durations of IFN therapy				
	Overall ($n = 102$)	24W ($n = 48$)	48W ($n = 45$)	<17W ($n = 9$)
RVR	81.4% ($n = 83$)	87.5% ($n = 42$)	73.3% ($n = 33$)	88.9% ($n = 8$)
ETR	100% ($n = 102$)	100% ($n = 48$)	100% ($n = 45$)	100% ($n = 9$)
SVR	92.2% ($n = 94$)	93.8% ($n = 45$)	91.1% ($n = 41$)	88.9% ($n = 8$)
(b) Virological response according to HCV genotypes				
	Overall ($n = 102$)	1b ($n = 32$)	2a ($n = 58$)	2b ($n = 9$)
RVR	81.4% ($n = 83$)	81.3% ($n = 26$)	81.0% ($n = 47$)	88.9% ($n = 8$)
SVR	92.2% ($n = 94$)	87.5% ($n = 28$)	93.1% ($n = 54$)	100% ($n = 9$)

ETR, end of treatment response; HCV, hepatitis C virus; IFN, interferon; RVR, rapid virological response; SVR, sustained virological response; W, weeks.

All patients had serum HCV RNA levels of 100 KIU/mL or less, and the median HCV RNA level was 36 KIU/mL.

One hundred and four patients were initially included in this study; 49 patients were treated with PEG IFN-α-2a for 24 weeks, and 45 patients were treated for 48 weeks. Ten patients withdrew from IFN therapy within 17 weeks, and two of these 10 patients could not be followed. The reasons for discontinuing therapy were fatigue ($n = 3$), depression ($n = 1$), rash ($n = 1$), appetite loss ($n = 1$), liver failure ($n = 1$) and unknown ($n = 3$). The two patients who withdrew from follow up were excluded from the analysis, and the remaining 102 patients were followed for 6 months after the ETR.

Virological response

Virological response is shown in Table 2. Rapid virological response (RVR), which was defined as negativity for HCV after 4 weeks of treatment, for the overall group, the 48 weeks' group, the 24 weeks' group and the under 17 weeks' group was 81.4% (83/102), 73.3% (33/45), 87.5% (42/48) and 88.9% (8/9), respectively. Virological response at the ETR was 100% among all patients. Finally, 94 (92.2%) of 102 patients achieved SVR.

There was no significant difference in virological response between patients treated for 24 weeks and those treated for 48 weeks. The virological response according to HCV genotype is shown in Table 2(b). Patients with genotype 1b had a lower SVR rate than genotypes 2a and 2b, but no significant differences in genotype were noted.

Genetic heterogeneity in NS5A-ISDR and response to IFN therapy

The prevalences of the number of amino acid substitutions in ISDR according to HCV genotypes are summarized in Figure 1. The ISDR were examined by direct sequencing, and classification involved counting the number of amino acid substitutions compared to consensus strains of each genotype, as previously reported.[15,24,27,28]

Interferon sensitivity-determining region sequences were obtained in 81 patients. Five patients did not have serum at pretreatment, and 16 patients could not be amplified by PCR. Sixty-one patients (84.7%) had one mutation or more. SVR according to the ISDR is shown in Figure 2. All patients with three or more mutations in the ISDR achieved SVR, but 18 (69.2%) of 26 patients with two or less mutations in the ISDR achieved SVR. Patients with two or less mutations in the ISDR were poor responders to IFN therapy.

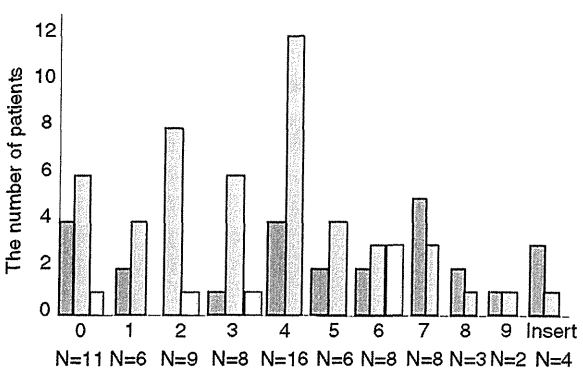

Figure 1 Number of amino acid substitutions in interferon sensitivity-determining region (ISDR) according to hepatitis C virus (HCV) genotypes. ▩, HCV genotypes 1b; □, HCV genotypes 2a; □, HCV genotypes 2b.

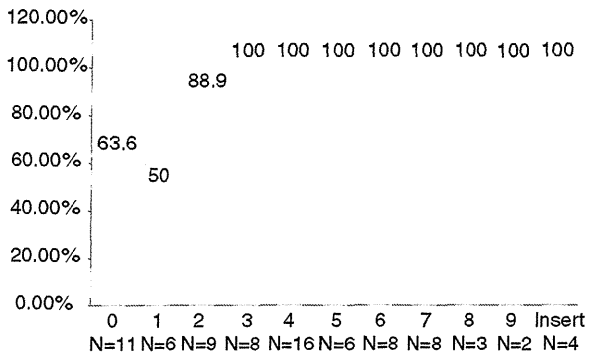

Figure 2 Sustained virological response (SVR) according to the number of amino acid substitutions in interferon sensitivity-determining region (ISDR).

Prevalence of the SNP of IL-28B (rs8099917) T (major allele) and G (minor allele) and response to IFN therapy

The frequencies of the IL-28B genotypes were: major homozygotes (TT), 73; heterozygotes (TG), 18; and minor homozygotes (GG), two. The rates of SVR in the patients with TT, TG and GG were 94.5% (69/73), 77.8% (14/18) and 100% (2/2), respectively. The SVR rate of patients with G allele of the IL-28B genotype was 80.0% (16/20), and that with T allele was 94.5% (69/73). Patients with T allele of the IL-28B genotype had a slightly higher SVR rate than did those with G allele, but there were no significant differences ($P = 0.0623$).

Analysis for factors predictive of SVR

The results of univariate analysis for factors predictive of SVR are shown in Table 3. HCV RNA levels were lower in patients with SVR than in those without SVR ($P = 0.0154$). SVR was achieved in 41.2% of patients with less than two mutations in the ISDR and 98.4% of patients with two or more mutations in the ISDR ($P = 0.0001$). HCV RNA levels and ISDR were associated with SVR on univariate analyses.

Results of multivariate analyses of factors predictive of SVR are shown in Table 4. Variables were recorded categorically as ordinal data. Background factors were age (<60 vs ≥60 years), sex (male vs female), platelet count ($<15 \times 10^4/mm^3$ vs $\geq 15 \times 10^4/mm^3$), HCV RNA level (<50 vs ≥50 KIU/mL), ALT levels (<70 vs ≥70 IU/L), aspartate aminotransferase (AST) levels (<60 vs ≥60 IU/L), HCV genotype (1 vs 2), ISDR (<2 vs ≥2 mutations), IL-28B (TT vs TG and GG) and RVR (yes vs no). As can be seen in Table 4, factors such as age, sex, platelet count, HCV RNA level, ALT levels, AST levels, HCV genotype, IL-28B and RVR did not have any effect on SVR. In contrast, the ISDR was the most influential factor.

DISCUSSION

THE HCV RNA level is one of the most important factors affecting response to IFN therapy. Patients with high HCV RNA levels respond poorly to IFN therapy, whereas patients with low HCV RNA levels have a high SVR rate to IFN therapy. Thus, most patients with low HCV RNA levels have achieved SVR, but other therapeutic options for patients who fail IFN therapy are needed. Several studies have attempted to reduce the duration of treatment, reduce the dose of IFN and/or ribavirin, or use standard IFN without risk of relapse.[8–10] The present study confirmed the high SVR rate (92.2%) in patients with low HCV RNA levels (≤100 KIU/mL)

Table 3 Univariate analysis: factors predictive of SVR

Factors	SVR ($n = 94$)	Non-SVR ($n = 8$)	*P*-value
Age (years)	54.6 ± 12.6	57.4 ± 8.8	0.5528
Sex: male/female	58/36	2/6	0.0619
ALT (IU/L)	63.2 ± 48.3	56.3 ± 32.5	0.7126
AST (IU/L)	50.7 ± 28.6	41.4 ± 21.6	0.4043
PLT ($\times 10^4/mm^3$)	18.5 ± 5.8	18.0 ± 5.0	0.8292
HCV RNA level (KIU/mL)	42.5 ± 34.8	75.0 ± 45.7	0.0154
HCV genotype: 1/2	29/63	4/3	0.4337
ISDR: <2/≥2	10/63	7/1	0.0001
IL-28B: TT/TG, GG	69/16	4/4	0.0623
RVR: yes/no	78/16	5/3	0.1661

ALT, alanine aminotransferase; AST, aspartate aminotransferase; HCV, hepatitis C virus; IL-28B, interleukin-28B; ISDR, interferon sensitivity-determining region; PLT, platelets; RVR, rapid virological response; SVR, sustained virological response.

Table 4 Multivariate analysis: factors predictive of SVR

Factors	*P*-value	Risk ratio	95% CI	
Age: <60 years	0.4556	2.837	0.183	43.891
Sex: male	0.8712	0.756	0.026	22.166
AST: <60 IU/L	0.7806	2.131	0.010	438.334
ALT: <70 IU/L	0.6063	0.239	0.001	55.563
Platelet count: $<15 \times 10^4$/uL	0.6873	0.463	0.011	19.680
HCV RNA: <50 KIU/mL	0.1046	13.170	0.585	296.318
Genotype: 2	0.1693	14.110	0.324	614.872
ISDR: <2	0.0074	0.004	0.001	0.235
IL-28B: TT	0.2684	5.978	0.252	141.852
RVR: yes	0.7495	1.756	0.055	55.696

95% CI, 95% confidence interval; ALT, alanine aminotransferase; AST, aspartate aminotransferase; HCV, hepatitis C virus; IL-28B, interleukin 28B; ISDR, interferon sensitivity-determining region; RVR, rapid virological response; SVR, sustained virological response.

treated by PEG IFN-α-2a monotherapy. Although the effects of shortened treatment duration of PEG IFN-α with ribavirin for patients with low HCV RNA levels are unclear, PEG IFN-α-2a monotherapy could reduce the cost and adverse events of ribavirin while maintaining a high SVR rate. This treatment would be a good therapeutic option for patients with low HCV RNA levels. However, selection by HCV RNA level alone was insufficient to predict IFN responsiveness completely, and other factors would be necessary to improve the positive predictive values for SVR in patients infected with low HCV RNA levels.

Hepatitis C virus genotype is another major factor, in addition to HCV RNA levels, that is associated with response to IFN therapy. In the present study, the SVR rates of genotypes 1 and 2 were 87.5% and 94.0%, respectively. Patients infected with genotypes 2 had a slightly higher SVR rate than did those with genotype 1, but there were no significant differences in our small study. The difference in SVR according to genotype may exist, but HCV genotype did not have enough power to be a determinant of IFN response completely among patients with low HCV RNA levels because of the bias for HCV RNA levels. However, patients infected with low HCV RNA levels respond differently to IFN therapy, suggesting that an additional factor associated with resistance to IFN exists.

The heterogeneity of the HCV NS5A region is an important factor that may affect response to IFN in patients with HCV genotype 1b and was named the ISDR.[17] Mutations in the ISDR affect the interaction with PKR and may inhibit viral replication. Therefore, ISDR of other HCV genotypes, in addition to 1b, could be used as predictors of IFN responsiveness.[23–28] In the present study, it was hypothesized that the amino acid substitutions in the ISDR would explain differences in IFN resistance in patients infected with low HCV RNA levels. Therefore, the utility of substitutions of amino acids in the ISDR for predicting IFN responsiveness was investigated. The ISDR was the most influential factor for SVR on multivariate analyses. All patients with three or more mutations in the ISDR achieved SVR, and 18 of 26 patients with less than three mutations in the ISDR achieved SVR. Thus, patients with less than three mutations in the ISDR would be resistant to PEG IFN-α-2a monotherapy and may need to receive much more powerful treatment, even if they have low HCV RNA levels. The ISDR system could be used as a diagnostic tool to predict SVR in patients infected with low HCV RNA levels. An individualized treatment strategy based on HCV RNA levels and the ISDR in patients with chronic hepatitis C would be an important consideration to achieve optimal therapy and avoid unnecessary treatment.

Some studies of SVR to PEG IFN-α-2b and ribavirin and/or telaprevir combination therapy for chronic hepatitis C patients with genotype 1 and high viral load identified genetic variation near the IL-28B gene associated with IFN responsiveness.[13,14,16] However, the effects of genetic variation near the IL-28B gene on SVR in patients with low HCV RNA levels treated with PEG IFN monotherapy are unknown. Therefore, the utility of the SNP of IL-28B for predicting IFN responsiveness was investigated. Patients with IL-28B (rs8099917) genotypes TG and GG had a lower SVR rate than genotype TT, but no significant differences in genotype were found in this study. The SNP of IL-28B would be associated with the response to IFN, especially for poor responders, and

was partially associated with SVR in a study of patients with HCV genotype 2 who were treated with PEG IFN-α-2b and ribavirin.[13,14,16,33,34] The clear suggestion of a correlation between the SNP of IL-28B with IFN responsiveness would not be supported in patients with low HCV RNA levels because of the high SVR rate and predominant genotype 2.

Viral factors associated with SVR have been studied, and several regions, including 5'-untranslated region, core, E2, NS5A and NS5B, have been suggested to play important roles in IFN responsiveness.[14,16,35–38] Further studies need to investigate whether these other viral factors, especially interferon and ribavirin resistance-determining region of NS5A and core amino acid substitutions, among patients with low HCV RNA levels affect the response to PEG IFN monotherapy.

Hepatitis C virus RNA levels could be easy to measure using commercial kits and would be useful for clinical practice, but sequencing analysis, which involves much effort and cost, would be needed to characterize the ISDR. SVR was achieved in 95.1% of patients with lower HCV RNA levels (<50 KIU/mL) and 98.4% of patients with mutant type. ISDR was a better factor, but HCV RNA level might be used as a predictive factor instead of measurement of ISDR.

The definition of the low HCV RNA level that was related to a good response to IFN therapy has varied widely, from 100–600 KIU/mL.[7,9–11] Zeuzem *et al.* reported that 24 weeks of therapy with PEG IFN-α-2b plus ribavirin is insufficient for the treatment of patients with HCV genotype 1 and a HCV RNA level of 600 KIU/mL or less.[10] They suggested that patients with HCV RNA of 250 KIU/mL or less would have a good response to PEG IFN-α-2b and ribavirin combination therapy for 24 weeks. Most reports from Japan defined 100 KIU/mL as the cut-off level for low HCV levels and used standard IFN monotherapy.[4,7,9,11] The outcome that would maximize the efficacy of IFN therapy would depend on the relationships between the cut-off HCV RNA level and therapeutic regimens. The optimal cut-off level for low HCV levels and the matching therapeutic regimens are not well understood, and further studies are needed to clarify these issues.

Based on the SVR in patients receiving therapy for 24 weeks compared to those treated for 48 weeks, there was no difference in IFN responsiveness by duration in this small study. However, this study was not a randomized study. Further studies are needed to investigate the optimal duration of PEG IFN-α-2a monotherapy for patients with low HCV RNA levels.

Pascu *et al.* performed a meta-analysis for the correlation between SVR and ISDR in patients with HCV genotype 1b infection who received standard IFN therapy.[19] They found that 11 of 21 European patients with mutant type ISDR and HCV RNA levels of less than 6.6 log copies/mL achieved SVR, but 67 of 69 Japanese patients with mutant type ISDR and HCV RNA levels of less than 6.6 log copies/mL achieved SVR. The mode of HCV infection and geographical and racial differences would have effects on the prediction of SVR by ISDR.[39,40] As a result, the ISDR system is more suitable for predicting SVR in Asian than in European patients. Although validation of these observations in larger cohorts is required, mutations in the ISDR were useful for predicting the response to PEG IFN-α-2a monotherapy in patients with low HCV levels.

In conclusion, in patients with HCV infection, low HCV levels and more than two mutations in the ISDR are significantly associated with a good response to PEG IFN-α-2a monotherapy. An individualized treatment strategy based on HCV RNA levels and the ISDR in patients with chronic hepatitis C would be useful in clinical practice.

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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

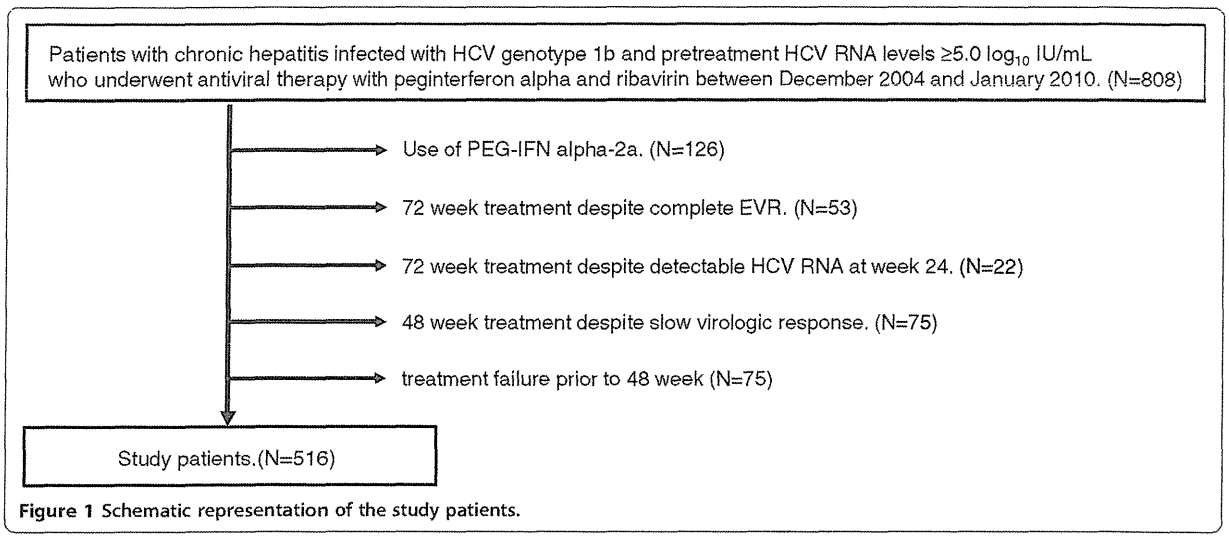

Figure 1 Schematic representation of the study patients.

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

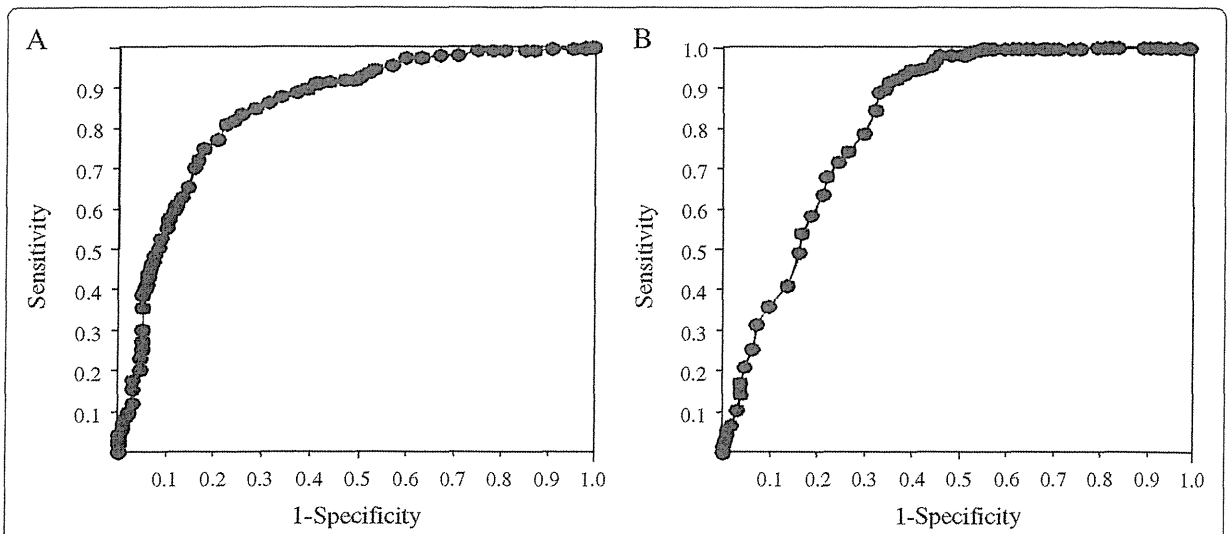

Figure 2 The receiver operating characteristics (ROC) analysis for the prediction of the sustained virologic response to combination therapy with peginterferon alpha-2b and ribavirin according to the reduction in serum HCV RNA levels in all patients who underwent response-guided therapy based on the 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).

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, $p = 0.9634$).

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 ($\times 10^3/\mu L$)	< 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).

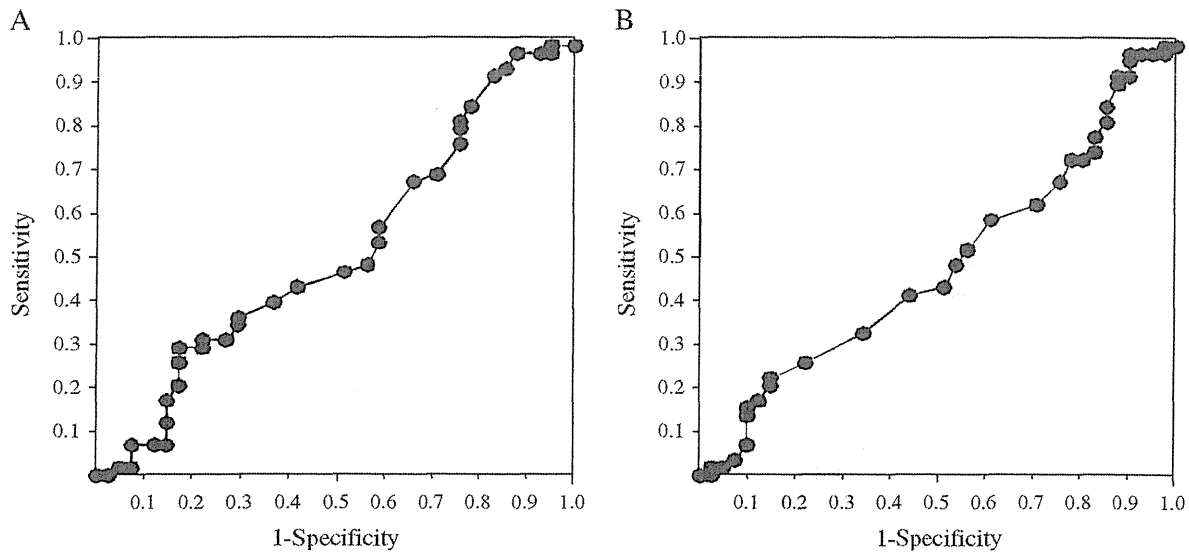

Figure 3 The receiver operating characteristics (ROC) analysis for the prediction of the sustained virologic response to combination therapy with peginterferon alpha-2b and ribavirin according to the reduction in serum HCV RNA levels in patients with delayed virologic response who underwent an extended 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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CLINICAL STUDY

Placement of a Sodium Hyaluronate Solution onto the Liver Surface as a Supportive Procedure for Radiofrequency Ablation of Hepatocellular Carcinomas Located on the Liver Surface: A Preliminary Report

Hidenori Toyoda, MD, PhD, Takashi Kumada, MD, PhD, Toshifumi Tada, MD, Yuji Kaneoka, MD, PhD, and Atsuyuki Maeda, MD, PhD

ABSTRACT

Purpose: To evaluate safety and efficacy of the placement of sodium hyaluronate solution onto the liver surface as a supportive procedure for radiofrequency (RF) ablation of hepatocellular carcinomas (HCCs) located on the liver surface as a possible alternative to RF ablation via laparoscopic approach or with the creation of artificial ascites.

Materials and Methods: Changes in temperature of a sodium hyaluronate layer placed onto an egg white were measured during coagulation of the egg white by an RF ablation needle. A phase I study was performed to evaluate the safety of intraperitoneal injection of a maximum of 20 mL of sodium hyaluronate solution into humans by observing for the occurrence of intraperitoneal inflammation and adhesion. After these studies, RF ablation with ultrasound-guided injection of sodium hyaluronate onto the liver surface was performed, targeting 28 HCC nodules located on the liver surface. Treatment outcomes and complications of this procedure were investigated.

Results: In the in vitro experiment, the maximum temperature of sodium hyaluronate solution was 41°C during RF ablation. No intraperitoneal inflammation or adhesions were observed after intraperitoneal injection of sodium hyaluronate in the phase I study. HCC was completely ablated with sufficient margins after one session of RF ablation, without any burn injuries to the abdominal wall or adjacent organs. Local recurrence was observed in one of 28 patients (3.6%) during 30.1 months of follow-up.

Conclusions: RF ablation can be safely and effectively performed on HCCs located close to the liver surface with placement of sodium hyaluronate onto the liver surface, thereby preventing burn injuries to abdominal wall or adjacent organs.

ABBREVIATIONS

CRP = C-reactive protein, HCC = hepatocellular carcinoma, RF = radiofrequency, WBC = white blood cell

Radiofrequency (RF) ablation is currently one of the standard treatment modalities for hepatocellular carcinoma (HCC) and, along with hepatic resection, is potentially curative (1–3). However, problems have been reported with the use of RF ablation for HCCs that are located close to the liver surface; these adverse effects include damage to the abdominal wall or to adjacent organs such as the gallbladder and gastrointestinal tract (4–6). RF ablation via laparoscopic approach (7–9) and RF ablation with the creation of artificial ascites (10,11) have been employed in the ablation of tumors located near the liver surface. These supportive procedures for RF ablation are used to prevent damage to the abdominal wall or adjacent organs by separating the ablation area from adjacent structures.

From the Departments of Gastroenterology (H.T., T.K., T.T.) and Surgery (Y.K., A.M.), Ogaki Municipal Hospital, 4-86 Minaminokawa, Ogaki, Gifu 503-8502, Japan. Received May 26, 2012; final revision received August 16, 2012; accepted August 24, 2012. Address correspondence to H.T.; E-mail: tkumada@he.mirai.ne.jp

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This article includes Figures E1 and E2, which are available online at *www.jvir.org*.

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However, RF ablation via laparoscopic approach usually requires general anesthesia. In addition, RF ablation via laparoscopic approach or creation of artificial ascites may cause vascular and respiratory complications as a result of increased intraperitoneal pressures (12,13).

Sodium hyaluronate solutions have been used in various clinical fields. In orthopedics, a sodium hyaluronate solution is used in intraarticular injections for osteoarthritis (14,15). In ophthalmology, the solution is used in intralenticular injections during cataract surgery (16). In gastroenterology, the solution is used as a submucosal fluid cushion during the endoscopic resection of mucosal neoplasms in the stomach or colon (17–20). Because sodium hyaluronate has a high viscosity (limiting viscosity of 11.8–19.5 dL/g) and remains at the injection site for a considerable length of time, the solution can effectively continue to separate the two objects between which it is injected. The compound creates a durable separation between the submucosal and muscle layers during endoscopic mucosal resections. Therefore, it may be possible to create a continuous separation between the liver surface and the abdominal wall or adjacent organs by injecting sodium hyaluronate onto the liver surface. Moreover, it may be possible to perform RF ablation of HCCs that are located close to the liver surface without using the laparoscopic approach or creating artificial ascites while still preventing damage to the abdominal wall or adjacent organs.

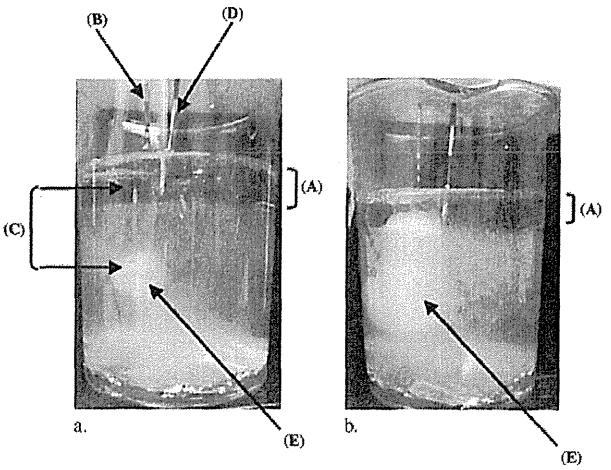

Figure 1. In vitro determination of temperature increases in the sodium hyaluronate solution associated with thermal ablation by an RF ablation needle. A 0.4% sodium hyaluronate solution layer was placed onto a layer of egg whites in a beaker (*A*). Subsequently, an RF ablation needle electrode for thermal ablation (*B*) was inserted with the tip in the egg white layer and the proximal end of the exposed tip (*C*) in the sodium hyaluronate layer. Another electrode to monitor the temperature of the sodium hyaluronate solution (*D*) was inserted into the sodium hyaluronate solution layer. The images display the results after 3 minutes **(a)** and 8 minutes **(b)** of thermal ablation. The volume of the coagulated egg whites increased with thermal ablation (*E*). (Available in color online at *www.jvir.org.*)

MATERIALS AND METHODS

In Vitro Experiment: Sodium Hyaluronate Temperature Determination during RF Ablation

The change in temperature of sodium hyaluronate solution during the RF ablation procedure was initially investigated in an in vitro experiment. In a beaker, 20 mL of a 0.4% sodium hyaluronate solution (MucoUp; Johnson and Johnson, Tokyo, Japan) was placed on the surface of raw egg whites, creating an approximately 1.8-cm layer of the sodium hyaluronate solution. An RF electrode that was equipped with a 3-cm exposed metallic tip (Cool-tip; Covidien, Mansfield, Massachusetts) was inserted into this beaker. The electrode that was used for thermal ablation was positioned with its tip in the egg white layer and part of its exposed portion in the sodium hyaluronate layer. Temperature changes in the sodium hyaluronate layer were continuously monitored by using another RF electrode, the tip of which was placed in the middle of the sodium hyaluronate solution when the egg white was heated (**Fig 1**). This procedure was continued until the heated egg white coagulated. The experiment was repeated three times.

Phase I Study: Safety of Intraperitoneal Sodium Hyaluronate Injection

After obtaining approval from the institutional ethics committee and written informed consent from each individual, a phase I study was performed to evaluate the safety of intraperitoneal injection of the sodium hyaluronate solution into humans. A 0.4% sodium hyaluronate solution (MucoUp; Johnson and Johnson) was injected percutaneously onto the liver surface under ultrasound (US) guidance with a 21-gauge needle (PEIT needle; Hakko, Nagano, Japan) that is usually used for ethanol injection therapy. The sodium hyaluronate solution was injected into six patients in a dose-escalating manner, ie, 5 mL, 10 mL, and 20 mL in two patients each. Laboratory tests were performed on day 3 after the injection to determine whether white blood cell (WBC) counts and C-reactive protein (CRP) values would increase as a result of inflammation caused by intraperitoneal injection of the sodium hyaluronate solution. The US examination was performed 5–7 days after the injection to determine whether ileus would occur as a result of peritoneal adhesions caused by the intraperitoneal injection.

RF Ablation of HCC with Sodium Hyaluronate Solution on the Liver Surface

After obtaining approval from the institutional ethics committee (based on the in vitro experiment and the phase I study) and written informed consent from each subject, RF ablation of the HCCs was performed with the placement of a sodium hyaluronate solution onto the liver surface. Between July 2009 and December 2011, 294 patients underwent RF ablation as a treatment of primary or recurrent HCCs. Patients with no more than three ($\leq$ 3) HCC tumors at

largest 3 cm in maximum diameter were considered to be candidates for RF ablation. Patients with Child–Pugh Class C cirrhosis and patients with platelet counts of less than 50×10^3 were not considered for RF ablation. HCC tumors were located on the liver surface or close to the liver surface in 28 patients who were treated with RF ablation with placement of sodium hyaluronate solution onto the liver surface. For all patients, the diagnosis of HCC was made by observing the appropriate imaging characteristics based on criteria in the guidelines issued by the American Association for the Study of Liver Diseases (21).

All patients were placed in the supine position for treatment. A sodium hyaluronate solution was placed onto the liver surface where the HCC was located by injecting the solution percutaneously through a 21-gauge needle (PEIT needle; Hakko) under real-time US guidance. The sodium hyaluronate solution was injected until the thickness of the sodium hyaluronate layer reached approximately 1–2 cm. The needle was withdrawn after the sodium hyaluronate solution was injected and before RF ablation was performed.

After placement of the sodium hyaluronate solution onto the liver surface, RF ablation was performed with use of a 20-cm-long, 17-gauge RF electrode equipped with a 2-cm or 3-cm exposed metallic tip and connected to a 500-kHz RF generator (Cool-tip; Covidien). The electrode was inserted percutaneously under real-time US guidance and positioned accurately within the tumor. The length of the exposed metallic tip (2 or 3 cm) was determined based on the HCC tumor size. RF ablation durations were 8 minutes with a 2-cm exposed tip and 12 minutes with a 3-cm exposed tip, according to the manufacturer's recommendations. If there were fewer than four occurrences of power roll-off during these ablation periods, the duration of ablation was increased until four power roll-offs occurred. The distance between the tumor and adjacent structures was monitored by real-time US throughout the RF ablation procedure.

The occurrence of abdominal pain or fever was prospectively surveyed by clinical examination during the 3 days after RF ablation, and WBC counts and CRP levels were measured on the first day after RF ablation to determine whether there were signs or symptoms of burn injuries to the abdominal wall or other organs adjacent to the tumor treated by RF ablation. Evaluation of treatment response was performed 1–3 days after the RF ablation procedure by imaging examinations, including contrast-enhanced US and either contrast-enhanced computed tomography (CT) or magnetic resonance (MR) imaging (22–28). All patients were followed for the recurrence of HCCs after RF ablation for a median of 9.7 months (range, 2.0–30.1 mo) at our institution with US every 3 months and CT or MR imaging every 6 months until March 2012. Regular monitoring of serum tumor markers (α-fetoprotein, *Lens culinaris* agglutinin–reactive fraction of α-fetoprotein, and des-γ-carboxy prothrombin) was performed every 3 months. If an increase in tumor marker levels was detected, an additional imaging examination (usually CT or MR imaging) was performed to check for HCC recurrence.

The entire protocol was approved by the institutional ethics committee and was performed in compliance with the Declaration of Helsinki. Informed consent to treatment and participation in the study was obtained in writing from all subjects.

Statistical Analyses

Continuous variables are expressed as mean ± standard deviation or as median and range. Categoric variables are expressed by using absolute numbers and percentages. The JMP statistical software package (version 4.0; SAS, Cary, North Carolina) was used for all statistical analyses.

RESULTS

In Vitro Experiment: Sodium Hyaluronate Temperature Determination

The temperature of the sodium hyaluronate solution layer was monitored during the heating of the egg white by an RF electrode with a 3-cm exposed tip. The egg white was heated and coagulated (**Fig 1**). The temperature of the egg white, which was measured at the tip of the RF electrode that was placed in the egg white layer, increased to a maximum of 86°C at 5 minutes after heating was initiated. The procedure was completed in 8 minutes with the coagulation of the egg white. The temperature of the sodium hyaluronate solution increased 2 minutes after the heating was initiated, reached the maximum temperature of 41.3°C at 6 minutes, and remained constant until the end of the procedure.

Phase I Study: Safety of Intraperitoneal Sodium Hyaluronate Injection

No difficulties were encountered while injecting the sodium hyaluronate solution via the 21-gauge needle. Based on the real-time US observation, the sodium hyaluronate solution created a layer (emitting hyperechoic and hypoechoic signals) that maintained a space between the abdominal wall and liver surface until 2 hours after the injection. The layer was not detectable by US on the following morning, which was approximately 15–18 hours after the injection.

None of the patients reported any symptoms, such as fever or abdominal pain, during or after the injection of the sodium hyaluronate solution. **Table 1** presents the WBC and CRP results obtained before and 3 days after the injection. No increase in WBC or CRP values that would indicate marked inflammation was observed in any patient regardless of the dose of sodium hyaluronate solution injected. In addition, no findings suggestive of ileus were observed based on abdominal US.

RF Ablation of HCC with Sodium Hyaluronate Solution on the Liver Surface

Table 2 displays the characteristics of the patients and HCC tumors. The treated HCCs were recurrent in 75% of

Table 1. Demographic Details of Peritoneal Injection of Sodium Hyaluronate Solution (N = 6)

Pt. No./Age (y)/Sex	Dose Injected (mL)	WBC Count (/μL)		CRP (mg/dL)	
		Baseline	After Injection*	Baseline	After Injection*
1/64/F	5	3,710	3,970	0.06	0.58
2/74/M	5	4,340	3,540	0.69	2.26
3/78/M	10	4,260	4,320	0.14	0.77
4/71/M	10	7,180	6,780	0.12	0.63
5/64/M	20	6,240	6,280	0.25	1.96
6/64/F	20	2,550	3,150	0.06	0.03

CRP = C-reactive protein, WBC = white blood cell.
* Tested 3 d after injection of sodium hyaluronate solution.

patients. Tumors were smaller than 3 cm in all cases. All tumors were located on the liver surface in various sections of the liver except for segments I and VIII. The HCCs were located adjacent to the gallbladder in three patients, the gastrointestinal tract in one patient, and the right kidney in one patient based on US imaging. HCC thermal ablation was performed for 8 minutes with an electrode with a 2-cm exposed tip in 19 patients and for 12 minutes with an electrode with a 3-cm exposed tip in nine patients. None of the patients required a prolonged duration of thermal ablation. In 23 of 28 patients, the proximal end of the exposed metallic tip was located within the sodium hyaluronate solution layer above the liver surface rather than in the tumor or liver tissue.

During the RF ablation procedure, the sodium hyaluronate solution layer on the liver surface did not flow outside of the injection area (**Fig 2**). No changes in the thickness or in the real-time US appearance of the sodium hyaluronate layer were observed throughout the RF ablation procedure. Based on the clinical evaluations and laboratory tests, no patients experienced clinically significant burn injuries of the abdominal wall or other organs, including those patients who underwent RF ablation for HCCs that were adjacent to the gallbladder (**Fig 3**) or gastrointestinal tract (**Fig E1**, available online at *www.jvir.org*). Based on the imaging examinations, the HCCs were ablated and necrotized with sufficient margins with only one session of RF ablation in all cases (**Figs E1 and E2**, available online at *www.jvir.org*). Local recurrence was observed in one of 28 patients (3.6%) during a follow-up of 30.1 months after treatment. The time interval between RF ablation and recurrence was 14.8 months. No patients experienced tumor seeding after RF ablation.

Table 2. Characteristics of Patients and HCC Tumors

Detail	Value
Age (y)	
Mean ± SD	70.8 ± 6.5
Range	58–83
Sex	
Male	23 (82.1)
Female	5 (17.9)
Etiology	
HBV	4 (14.3)
HCV	21 (75.0)
Non-HBV/non-HCV	3 (10.7)
ALT (IU/L)	52.6 ± 38.7
Platelet count ($\times 10^3$/mL)	120 ± 52
Prothrombin (%)	82.5 ± 14.2
AFP (ng/mL)	
Median	9.8
Range	0.6–521.7
DCP (mAU/mL)	
Median	101.0
Range	6.0–763.0
History of HCC	
Primary	7 (25.0)
Recurrence	21 (75.0)
Tumor location	
Segment II	2 (7.2)
Segment III	6 (21.4)
Segment IV	2 (7.2)
Segment V	7 (25.0)
Segment VI	9 (32.0)
Segment VII	2 (7.2)
Tumor size (mm)	
Mean ± SD	14.8 ± 4.8
Range	8.0–26.0
Exposed electrode tip length	
2 cm	19 (67.9)
3 cm	9 (32.1)

AFP = α-fetoprotein, ALT = alanine aminotransferase, DCP = des-γ-carboxy prothrombin, HBV = hepatitis B virus, HCC = hepatocellular carcinoma, HCV = hepatitis C virus, SD = standard deviation.
Values presented as means ± SD where applicable. Values in parentheses are percentages.

DISCUSSION

The injection of sodium hyaluronate solution into the human body is performed in clinical practice (including intraarticular injections, intralenticular injections, and submucosal injections in the gastrointestinal tract), and the safety of the use of this material in the human body has been established (14–20). In the United States, the use of sodium hyaluronate solution has been approved by the