was reduced. On the other hand, immunodepletion with a control anti-C4 antibody or without antibody did not reduce the 8.1 k m/z peak (Fig. 2).

Profiling the C3a of sera from patients with HCC and without HCC

The 8.1 k m/z peak was confirmed as the complement C3a fragment using an immunodepletion assay. However, C3a was stabilized as C3adesArg with a molecular weight of approximately 8.9 k m/z. Figure 3a, b compares the expression of the 8.1 k m/z peak in the sera of HCV-HCC or HCV-CLD patients and healthy controls. The intensities

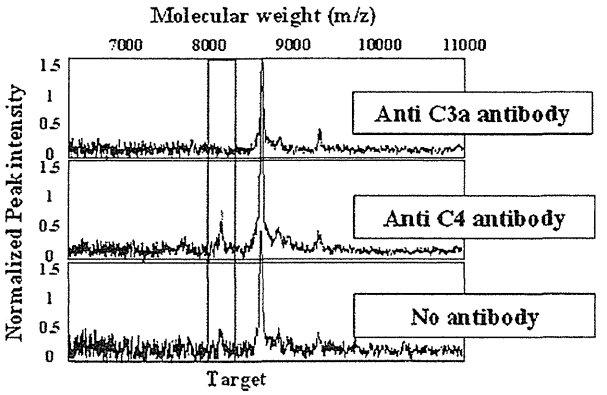

Fig. 2 Immunodepletion assay of the C3a fragment. Analysis of supernatant that had been immunodepleted with an anti-C3a antibody showed that only the 8.1 k m/z peak corresponding to complement C3a was reduced. Supernatants that had been immunodepleted with either a control anti-C4 antibody or without antibody did not have reduced 8.1 k m/z peaks by the ProteinChip system

in HCC patient sera were significantly higher than those in the HCV-CLD patients or healthy controls. The expression of the 8.9 k m/z peak in HCV-HCC patients was also higher than that in HCV-CLD patients or healthy controls (Fig. 3c, d). Although the 8.9 k m/z peak was not identified as C3adesArg, it is possible that both the 8.1 and 8.9 k m/z peaks were specific tumor markers for HCC. Furthermore, we analyzed sera from 10 HCV-HCC patients who were diagnosed with HCC within 1 or 2 years and sera from five patients who had received curative treatments using RFA, PEIT, and TACE for HCC. The 8.1 k m/z C3a fragment in the HCV-HCC patients was significantly increased in the year of disease onset compared to the pre-onset year. After treatment, expression of the C3a fragment significantly decreased in all five of the patients who had measurable samples after treatment (Fig. 4a). In contrast, the 8.9 k m/z peak did not change regardless of the occurrence of HCC over time (Fig. 4b). Thus, the 8.1 k m/z C3a fragment appears to be the most discriminatory tumor marker for HCV-HCC.

Relationship between the C3a fragment and other tumor markers

AFP and DCP levels were measured in sera from 83 of 87 patients with HCV-associated liver disease. The recommended cutoff levels for these tumor markers, AFP and DCP, are 20 ng/ml and 40 mAU/ml, respectively. AFP-L3 in 26 patients with HCV-associated liver disease was also investigated among measurable samples in which AFP in a total 35 patients was higher than 20 ng/ml. The cutoff level of AFP-L3 was set at 10%. When samples from patients

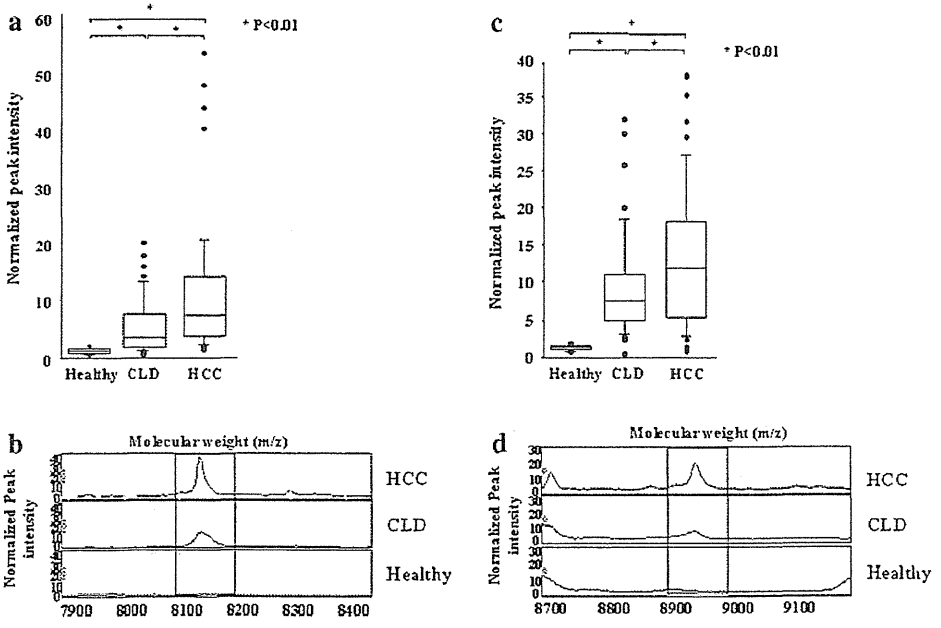

Fig. 3 **a** and **c** Comparisons of the expression profiles of the 8.1 and 8.9 k m/z peaks in HCV-HCC, HCV-CLD, and healthy sera. *Boxes* indicate the median ± 25th percentile. The *lower* and *upper bars* represent the 10th and 90th percentiles, respectively. **b** and **d** Representative spectra of the 8.1 and 8.9 k m/z peaks from patients in each group. The *horizontal axis* indicates the protein molecular weight, while the *vertical axis* designates the relative intensity

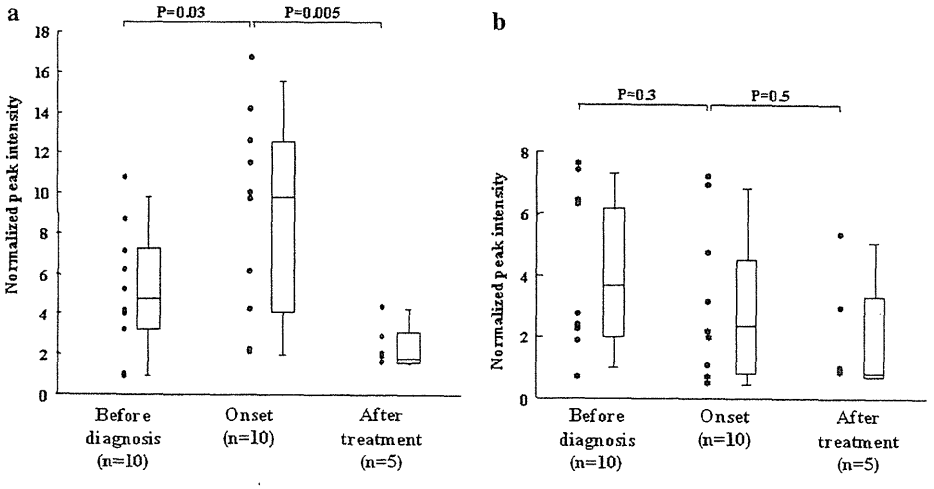

Fig. 4 Comparisons of the expression profiles of the 8.1 k m/z (**a**) and 8.9 k m/z (**b**) peaks in sera from HCV-HCC patients before diagnosis, during disease onset, and after treatment. The samples in the before diagnosis group included sera collected 1 or 2 years before the onset of HCC. *Boxes* indicate the median ± 25th percentile, the *lower bar* indicates the 10th percentile and the *upper bar* indicates the 90th percentile

Table 2 Diagnostic rates for hepatocellular carcinoma in the HCV infected patients

Markers	Sensitivity (%)	Specificity (%)	ROC AUC
AFP[a] (>20 ng/ml)	38 (17/45)	47 (18/38)	0.53
DCP[b] (>40 mAU/ml)	45 (20/44)	74 (29/39)	0.68
AFP-L3[c] (>10%)	58 (8/14)	50 (6/12)	0.58
C3a fragment (>3.5)	78 (37/45)	52 (22/42)	0.70
C3a fragment + AFP	91 (41/45)	26 (10/38)	0.72
C3a fragment + DCP	93 (41/44)	33 (13/39)	0.77
AFP + DCP	64 (28/44)	34 (12/35)	0.70
C3a fragment + AFP + DCP	98 (43/44)	20 (7/35)	0.80

[a] Alpha fetoprotein

[b] Des-γ-carboxy prothrombin

[c] Alpha fetoprotein, lectin lens culinaris agglutin-bound fraction

with HCV-HCC and HCV-CLD without HCC were compared, the sensitivity and specificity of AFP were 38 and 47%, whereas those of DCP were 45 and 74% and those of AFP-L3 were 58 and 50%, respectively. When the cutoff level for the relative intensity of the C3a fragment was set at 3.5, the sensitivity and specificity were 78 and 52%, respectively; the C3a fragment had the most sensitivity for the diagnosis of HCC. Furthermore, the ROC AUC of the C3a fragment, AFP, DCP, and AFP-L3 was 0.70, 0.53, 0.68, and 0.58, respectively (Table 2). There was no relationship between the C3a fragment and several other tumor and inflammation markers [AFP, DCP, AFP-L3, alanine aminotransferase (ALT), and high-sensitivity C-reactive protein (hs-CRP)], and each of these markers was independent of the diameter and number of tumors. The ROC AUC using AFP and DCP was highly similar to the ROC AUC with the C3a fragment alone. In addition, we investigated a combination assay that included the C3a fragment, AFP and DCP. This combination test, in which at least AFP, DCP, or the C3a fragment was positive, had a positive identification rate of 98%, although the specificity of this assay was too low at 20%. The ROC AUC of the combination test using AFP, DCP, and the C3a fragment was higher than those of any other markers. This result indicates that this combination assay using three markers is more useful than the combination assay using AFP ± DCP, which are measured worldwide to detect HCC (Table 2).

Profiling C3a expression in culture medium

C3a reacted with HCC cell lines, and the C3a peak in the culture medium was monitored by the ProteinChip system. The C3a fragment (approximately 8.1 k m/z) was not detected in the supernatants of HuH-7 and HepG2 cell cultures. However, the 8.9 k m/z peak was detected in the culture medium. This 8.9 k m/z peak was considered to be a stabilized form of C3a. This result indicated that the stabilized form of C3a (8.9 k m/z) was not undergoing proteasome-mediated degradation to yield the C3a fragment (8.1 k m/z) in these HCC cell lines.

Discussion

Because the HCC disease-associated mortality rate remains high, it is highly important to develop early diagnostic tools and treatments for HCC. Our study indicates that an 8.1 k m/z peak, which was identified as the C3a fragment by both peptide sequencing and an immunoassay, is up-regulated in the serum of HCC patients, 93% (42/45) of whom were TNM stage I or II. The C3a fragment in some HCC cases was also significantly higher in the year of HCC onset compared to the pre-onset year and decreased after curative treatment. Therefore, the C3a fragment appears to

be a promising simple tumor marker for the diagnosis of early HCC. In addition, a combination serum HCC diagnostic test that included AFP, DCP, and the C3a fragment had higher sensitivity than each individual marker. These results suggest that this combination test may be a useful HCC screening method, although the low specificity may pose challenges. Further examinations are needed to determine whether the C3a fragment or a combination test can be used to detect early HCC.

The results of our study demonstrated that the C3a fragment (8.1 k m/z) is a highly expressed novel tumor marker that is abundant in the sera of early HCC patients but not in the sera of healthy volunteers or HCV-CLD patients. A similar study by Lee et al. [17] used the ProteinChip SELDI system to show that C3a is a potential candidate biomarker for HCV-HCC. However, Lee et al. found that the molecular weight of C3a was represented by an approximately 8.9 k m/z peak. C3a has a very short half-life and is immediately cleaved into the more stable C3adesArg (8.9 k m/z), which is the anaphylatoxin C3a that lacks the C-terminal arginine and is stable state in the serum [23]. In our study, the 8.9 k m/z peak was also significantly different among HCV-HCC patients, HCV-CLD patients, and healthy volunteers (Fig. 3c, d). However, the discriminatory power of the 8.9 k m/z peak (ROC AUC was 0.60) was lower than the 8.1 k m/z peak (ROC AUC was 0.70) to distinguish between HCV-HCC and HCV-CLD. In addition, unlike the 8.1 k m/z peak, the levels of the 8.9 k m/z peak did not significantly increase with time as HCC progressed in 10 HCV-HCC cases (Fig. 4b). In contrast, Li et al. identified two proteins (8926 m/z and 8116 m/z) as complement component C3adesArg and a C-terminal truncated form of C3adesArg; the latter was a C-terminal truncation of C3adesArg that lacked the C-terminal sequence RASHLGLA (referred to as C3adesArgΔ8) in breast cancer patients [24]. However, these two biomarkers cannot be used to discriminate between breast cancers and benign tumors, and there were minimal differences in the peak intensities between breast cancer patients and healthy controls. Therefore, the C3a fragment with a molecular weight of 8.1 k m/z appears to be a potential diagnostic marker for HCC, although we cannot explain why the 8.1 k m/z fragment of C3a is overexpressed in HCC patients and did not confirm whether our C3a fragment (8.1 k m/z) is C3adesArgΔ8.

C3a, including C3adesArg, was also previously identified as a tumor marker for lymphoid malignancies, breast and colorectal cancers using the ProteinChip SELDI system [24–26]. Complement activation and subsequent deposition of complement components on tumor tissues has been demonstrated in cancer patients [27]. Malignant ovarian cells isolated from ascitic fluid samples had C3 activation products deposited on their cell surface [28]. Complement components are important mediators of inflammation and help regulate the immune response. C3a is biologically active and binds to mast cells and basophils, triggering the release of their vasoactive contents [29]. We investigated C3a expression by immunochemical examination of HCC tissues and Western blot analysis of proteins extracted from human HCC cell lines, including HepG2 and HuH-7. However, specific C3a expression, including the C3a fragment (8.1 k m/z), was not detected.

The complement system can be activated after exposure to tumor antigens [30]. It is speculated that small tumors can trigger a systematic reaction. Therefore, elevated C3a (8.9 k m/z) levels in the serum of HCV-HCC patients may reflect both a systematic immune response to HCV infection and non-specific tumor antigens rather than a specific immune response to HCC [24–26, 31]. In contrast, it is possible that overexpression of the C3a fragment (8.1 k m/z) is specific for HCC in addition to non-specific C3 activation.

In contrast to our results, Steel et al. [32] searched for HCC biomarkers using HCC-associated HBV-infected patient sera and found that the C-terminal fragment of complement C3 was down-regulated. Kawakami et al. [33] searched for characteristic alterations in the sera of HBV- and HCV-HCC-infected patients who had undergone curative radiofrequency ablation treatment and showed that C3 was up-regulated after treatment. In these studies, C3 was separated and identified using 2-DE of a mixture of proteins from a small number of patient sera samples, and this process identified various molecular weights for C3. In addition, we analyzed the sera of 25 patients with HCC-associated HBV infections, and the profile of several proteins was different between HCV- and HBV-infected patients. Although 35 protein peaks, including the C3a fragment, were overexpressed in the sera of both HCV-HCC and HBV-HCC patients compared to sera from healthy volunteers, the C3a fragment (8.1 k m/z) was particularly overexpressed in the sera of HCV-HCC patients and was not significantly different between HBV-HCC patients and HCV-CLD patients without HCC (data not shown). The biologic and pathogenic activities of HCV and HBV are different, and the molecular mechanisms underlying the development of hepatitis and hepatocarcinogenesis may differ between HBV and HCV infections [34–36]. Although the number of samples, cause of liver disease, and method of protein identification may affect these results, we speculate that the C3a fragment with a molecular weight of 8.1 k m/z is a candidate tumor marker for HCV-HCC but not HBV-HCC.

AFP, which is a commonly used HCC tumor marker, is elevated not only during HCC, but also during hepatocyte regeneration following liver damage. Previous reports revealed that AFP was abnormally elevated in the sera of patients with acute hepatitis, chronic hepatitis, and liver

cirrhosis. This lack of specificity for HCC means that AFP has a comparatively high false-positive rate [37]. The C3a fragment may also be elevated during hepatocyte regeneration following liver damage [38], and early diagnosis of small HCC tumors may be difficult with one marker alone. Therefore, the false-positive rates for HCC must be carefully considered [39–41]. Also, a combination of markers, including AFP, DCP, and the C3a fragment, in the serum should be verified to improve the diagnostic rate.

The ProteinChip SELDI system can separate and partially characterize multiple proteins in tissue and serum samples. Our previous report used a panel of proteins to diagnose early HCC with the ProteinChip SELDI system [15]. This panel diagnosis of seven protein peaks included a discriminant peak of 4060 m/z. This 4060 m/z peak may be a double-charged 8130 m/z peak, although the C3a fragment (8130 m/z) was not used to develop this diagnostic method. These results suggest that the C3a fragment is a useful HCC biomarker, regardless of whether this fragment carries a single or double charge. In addition, the panel diagnosis method is more useful than measuring the C3a fragment alone to diagnose and predict the occurrence of HCC. However, this method must be performed using the ProteinChip SELDI system, which is expensive and does not detect putative interactions between various proteins. Identifying a specific HCC protein such as the C3a fragment will also further our understanding of the molecular mechanisms of hepatocarcinogenesis. Therefore, the C3a fragment should not only be considered a simple HCC tumor marker, but should also be evaluated for its contribution to HCC carcinogenesis.

In conclusions, serum profiling with the ProteinChip SELDI system may be used to distinguish HCC from chronic liver disease without HCC and to detect early HCC in HCV-infected patients. Because we identified the C3a fragment (8.1 k m/z) in serum samples from HCC patients, the C3a fragment is a promising marker that can be used to screen for HCV-HCC and to develop new therapeutic targets.

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Research Article

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Efficacy of pegylated interferon alpha-2b and ribavirin treatment on the risk of hepatocellular carcinoma in patients with chronic hepatitis C: A prospective, multicenter study☆

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Background & Aims: The effects of pegylated interferon (PegIFN) α and ribavirin (RBV) treatment of chronic hepatitis C on the incidence of hepatocellular carcinoma (HCC) have not been well established. This study investigated the impact of treatment outcome on the development of HCC by chronic hepatitis C patients treated with PegIFNα2b and RBV.

Methods: This large-scale, prospective, multicenter study consisted of 1013 Japanese chronic hepatitis C patients with no history of HCC (non-cirrhosis, n = 863 and cirrhosis, n = 150). All patients were treated with PegIFNα2b and RBV and the follow-up period started at the end of the antiviral treatment (median observation period of 3.6 years). The cumulative incidence rate of HCC was estimated using the Kaplan–Meier method, according to treatment outcome.

Results: Forty-seven patients (4.6%) developed HCC during the observation period. In the non-cirrhosis group, the 5-year cumulative incidence rates of HCC for the sustained virological response (SVR) (1.7%) and transient virological response (3.2%) (TVR: defined as relapse or breakthrough) groups were significantly lower than those of the non-virological response (NVR) group (7.6%) ($p = 0.003$ and $p = 0.03$, respectively). A significantly low rate of incidence of HCC by TVR patients in comparison with NVR patients was found for patients aged 60 years and over, but not for those under 60 years of age. In the cirrhosis group, the 5-year cumulative incidence rates of HCC for the SVR (18.9%) and TVR groups (20.8%) were also significantly lower than those of the NVR group (39.4%) ($p = 0.03$ and $p = 0.04$, respectively).

Conclusions: SVR and complete viral suppression during treatment with relapse (TVR) were associated with a lower risk of HCC development when compared with NVR.

Keywords: Hepatitis C; Pegylated interferon; Ribavirin; Hepatocellular carcinoma.

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Abbreviations: HCV, hepatitis C virus; HCC, hepatocellular carcinoma; SVR, sustained virological response; IFN, interferon; PegIFN, pegylated interferon; RBV, ribavirin; NVR, non-virological response; TVR, transient virological response; KULDS, Kyushu University Liver Disease Study; AFP, α-fetoprotein; HIV, human immunodeficiency virus; EASL, European Association for the Study of the Liver; ALT, alanine aminotransferase; HbA1c, hemoglobin A1c; EPV, events per predictor variable; HR, hazard ratio; CI, confidence interval; DAAs, direct acting antivirals.

Introduction

Hepatitis C virus (HCV) is a major human pathogen responsible for chronic hepatitis, which often progresses to cirrhosis and hepatocellular carcinoma (HCC) [1–3]. While recent advances in HCV have led to a markedly improved treatment, HCC is at present the sixth most common cancer and the third cause of cancer death worldwide [4]; moreover, its incidence is increasing due to HCV infection [5].

Previous studies have reported that patients who achieved a sustained virological response (SVR) after interferon (IFN) monotherapy demonstrated improvement in liver fibrosis and a

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reduction in the incidence of decompensated liver disease and HCC compared with non-SVR patients [6–9]. In the past 10 years, a combination of pegylated IFN (PegIFN) α and ribavirin (RBV) has become the standard treatment and has resulted in an increased SVR rate [10–12]. Therefore, whether or not PegIFNα and RBV treatment is effective in preventing HCC is important, but its effect on the incidence of HCC has not been adequately studied, particularly in a large prospective study.

A recent prospective study from the United States reported that the cumulative incidence rate of HCC in an SVR group was significantly lower than in a non-virological response (NVR) group. It was also lower in a transient virological response (TVR) group than in an NVR group, although the difference did not reach statistical significance [13]. The number of aging chronic hepatitis C patients has been increasing in Japan, earlier than in other countries [14], thus investigation into the development of HCC by Japanese chronic hepatitis C patients treated with PegIFNα and RBV is highly important. Furthermore, the risk factors for the development of HCC by patients who achieve an SVR after treatment with PegIFNα and RBV have not been adequately clarified in a prospective study, although a recent report suggested that SVR reduced the risk of all-cause mortality in patients treated with PegIFNα and RBV [15]. Clarification of the demographic and clinical factors associated with HCC development, such as advanced age, lower albumin, lower platelet count and higher α-fetoprotein (AFP) level, is important.

The aim of this large-scale, multicenter, prospective study was to evaluate the relationships among pretreatment clinical factors, virological response, and development of HCC by chronic hepatitis C patients with no history of HCC, who were treated with PegIFNα2b and RBV.

Cancer

Patients and methods

Patients

The Kyushu University Liver Disease Study (KULDS) Group consists of the Kyushu University Hospital and affiliated hospitals in the Northern Kyushu area of Japan. We conducted a prospective study to investigate the efficacy and safety of PegIFNα2b and RBV for chronic hepatitis C patients. The design of the KULDS project has been described previously [12,16,17]. This prospective study consisted of 1013 Japanese patients with chronic HCV infection aged 18 years or older, treated with PegIFNα2b and RBV between December 2004 and November 2009.

The exclusion criteria were: (1) history of HCC; (2) HCC development during antiviral treatment; (3) previous PegIFNα and RBV treatment; (4) positivity for antibody to human immunodeficiency virus (HIV) or positivity for hepatitis B surface antigen; (5) clinical or biochemical evidence of hepatic decompensation at entry; (6) excessive active alcohol consumption (a daily intake of more than 40 g of ethanol) or drug abuse; (7) other forms of liver disease (e.g., autoimmune hepatitis, alcoholic liver disease, hemochromatosis); or (8) treatment with antiviral or immunosuppressive agents prior to enrollment.

The study was conducted in accordance with the ethical principles of the Declaration of Helsinki and was approved by the Ethics Committee of each participating hospital. Informed consent was obtained from all patients before enrollment.

Antiviral treatment and patient follow-up

All HCV genotype 1 patients received a combination treatment of PegIFNα2b (PEG-Intron; MSD, Tokyo, Japan) and RBV (Rebetol; MSD) for 48 weeks: the same regimen was prescribed for 24 weeks for genotype 2 patients. In order to investigate the incidence of HCC after treatment, the length of the follow-up period was calculated from the end of antiviral treatment to the diagnosis of HCC or last follow-up visit. Serum AFP and abdominal imaging (ultrasonographic examination, or computed tomography) were performed every 3–6 months, for each patient. The HCC diagnosis was based on histology or non-invasive criteria according to the guidelines of the European Association for the Study of the Liver (EASL) [18].

Clinical and laboratory assessment

Clinical parameters included serum albumin, alanine aminotransferase (ALT), serum AFP, hemoglobin, platelet count, hemoglobin A1c (HbA1c), HCV genotype, and HCV RNA. All were measured by standard laboratory techniques in a commercial laboratory (SRL Laboratory, Tokyo, Japan). The HbA1c levels that we report are expressed as National Glycohemoglobin Standardization Program units (%). Body mass index was calculated as weight in kilograms/height in square meters.

Assessment of liver fibrosis

Liver biopsy for 613 (60.5%) of the 1013 patients was performed by experienced hepatologists. The antiviral treatment was initiated within 1 month after liver biopsy. The minimum length of liver biopsy was 15 mm and at least 10 complete portal tracts were necessary for inclusion. For each specimen, the stage of fibrosis was established according to the METAVIR score [19]. Liver cirrhosis in patients with no liver biopsy was diagnosed by ultrasonographic findings (nodules in the hepatic parenchyma, portal vein >16 mm) (mandatory inspection) at the time of antiviral treatment initiation. Moreover, the diagnosis of liver cirrhosis was made based on at least one of the following: (1) endoscopic findings (varices, portal gastropathy); (2) serological markers (aspartate aminotransferase to platelet ratio index >2.0; the cut-off value that indicates a negative predictive value for cirrhosis is 93%) [20]; or (3) transient elastography (FibroScan value ⩾14.9 kiloPascal; the cut-off value that indicates that the negative predictive value for cirrhosis is 100%) [21]. The EASL HCV guidelines of 2011 describe the accuracy of these non-invasive tests of liver fibrosis as sufficient for identifying patients with cirrhosis [22].

Efficacy of treatment

Successful treatment was an SVR, defined as undetectable HCV RNA at 24 weeks after the end of treatment. A TVR was defined as relapse of serum HCV RNA after treatment of patients whose HCV RNA level was undetectable at the end of treatment and the reappearance of HCV RNA at any time during treatment after virological response (breakthrough). An NVR was defined as a decrease in the HCV RNA level of less than 2 $\log_{10}$ IU/ml at week 12 (null response) and a more than 2 $\log_{10}$ IU/ml decrease in the HCV RNA level from baseline at week 12, but detectable HCV RNA at weeks 12 and 24 (partial response).

HCV RNA level and HCV genotype

Clinical follow-up of HCV viremia was done by real-time reverse transcriptase PCR assay (COBAS TaqMan HCV assay) (Roche Diagnostics, Tokyo, Japan), with a lower limit of quantitation of 15 IU/ml and an outer limit of quantitation of 6.9×10^7 IU/ml (1.2 to 7.8 log IU/ml referred to $\log_{10}$ IU/ml). HCV genotype determination was by sequence determination in the 5′ non-structural region of the HCV genome, followed by phylogenetic analysis [23].

Statistical analysis

Statistical analyses were conducted using SPSS Statistics 19.0 (IBM SPSS Inc., Chicago, IL, USA). Baseline continuous data are expressed as median (first-third quartiles) and categorical variables are reported as frequencies and percentages. Univariate analyses were performed using the Chi-square, Fisher's Exact, Mann–Whitney U tests or analysis of variance (ANOVA) as appropriate. Variables with $p < 0.05$ in univariate analysis were evaluated using multivariate logistic regression to identify those significantly associated with the incidence of HCC. As a rule of thumb, 10 events per predictor variable (EPV) are needed when performing a logistic regression analysis. However, 5 to 9 EPV with a large sample size (over 1000) showed robust results of as much as 10 to 16 EPV [24]. Thus, our sample size and 5 to 9 EPV might be sufficient to insure the robustness of our model. Results are expressed as hazard ratios (HR) and their 95% confidence interval (CI).

The main outcome of this study was HCC incidence. Cumulative incidence curves of HCC according to response to antiviral treatment were plotted using the Kaplan–Meier method. Differences between groups were assessed using

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log-rank tests. The time frame for HCC incidence was defined as the time from the end of antiviral treatment to the diagnosis of HCC. A p value less than 0.05 was regarded as statistically significant in all analyses.

Results

Patient characteristics

The baseline characteristics of the 1013 studied patients at the start of antiviral treatment, as classified by the existence of cirrhosis and treatment outcome, are shown in Table 1. HCV genotype 1 was detected in 710 patients and genotype 2 in 303. Of all patients, 151 (14.9%) discontinued antiviral treatment because of adverse effects or other reasons (e.g., poor virological response, economic reasons, or dropout). The discontinuation rate of patients with HCV genotype 1 (129 of 710, 18.2%) was significantly higher than that of those with HCV genotype 2 (22 of 303, 7.3%) (p <0.001). Of the studied patients, 557 achieved SVR (55.0%), 304, including 20 with breakthrough, were TVR (30.0%), and 152 (15.0%) were NVR. The SVR rate of patients infected with HCV genotype 1 was 43.9% (312 of 710), significantly lower than the 80.9% (245 of 303) found for patients with genotype 2 (p <0.001).

In the non-cirrhosis group (n = 863), the three treatment outcome groups differed significantly for age, sex, HCV genotype, and laboratory values associated with liver and metabolic disease (e.g., ALT, platelet count, AFP and HbA1c). The SVR group was more likely to be infected with HCV genotype 2 and to have mild liver fibrosis, but less likely to have laboratory values associated with advanced liver and metabolic disease (e.g., low platelet count, or high AFP and HbA1c level) than the TVR and NVR groups. Independent comparisons of SVR and TVR patients extracted age (p <0.001), sex distribution (p = 0.01), ALT level (p = 0.01), platelet count (p <0.001) and HCV genotype (p <0.001). Likewise, independent comparisons of TVR and NVR patients extracted only AFP level (p = 0.01).

Liver cirrhosis was diagnosed according to clinical (n = 77) and histological (n = 73) findings. In the cirrhosis group (n = 150), however, no significant differences, except for ALT and HCV genotype, were found among the clinical and biochemical parameters of the three treatment outcome groups.

SVR and TVR patients had fewer deaths from any cause (four [0.7%] and four [1.3%], respectively) in comparison to NVR patients (six [3.9%]). Similarly, the frequency of SVR and TVR patients who developed ascites and encephalopathy, symptoms of hepatic decompensation, was lower than that of NVR patients (ascites: two [0.4%], six [2.0%] and eight [5.3%], and encephalopathy: two [0.4%], two [0.7%] and five [3.3%] patients with SVR, TVR and NVR, respectively). None of the patients underwent liver transplantation during the observation period.

Risk of HCC classified by treatment outcome

Of 1013 patients who were followed for a median of 3.6 (range 0.3–7.0) years, 47 (4.6%) developed HCC during the observation period. The baseline characteristics of these patients classified by the development of HCC are shown in Table 2. By univariate analysis, the development of HCC was associated with older age, male sex, higher ALT level, lower serum albumin, lower platelet count, higher AFP level, cirrhosis, and NVR. No significant difference in the duration of HCV RNA negativity was found between the HCC (median [first-third quartiles]: 30.0 [24.0–48.5] weeks) and non-HCC group (41.0 [27.0–48.0] weeks) (p = 0.36) in patients with TVR.

Multivariable logistic regression analysis of possible predictors of HCC development is shown in Table 3. We examined eight factors (age [<60 *vs.* ⩾60 years], sex [men *vs.* women], ALT [<40 *vs.* ⩾40 IU/L], platelet count [<150 *vs.* ⩾150 × 10^9/L], AFP [<10 *vs.* ⩾10 ng/ml], serum albumin [<40 *vs.* ⩾40 g/L], liver pathophysiology [non-cirrhosis *vs.* cirrhosis] and treatment outcome [SVR *vs.* TVR *vs.* NVR]). Significant independent pretreatment predictors of HCC were age 60 years and over (HR 2.81; 95%CI 1.39–5.69; p = 0.004), male sex (HR 2.98; 95%CI 1.46–6.05; p = 0.003), low platelet count (<150 × 10^9/L) (HR 4.04; 95%CI 1.57–10.44; p = 0.004), higher AFP level (⩾10 ng/ml) (HR 2.50; 95%CI 1.09–5.78; p = 0.03), cirrhosis (HR 3.22; 95%CI 1.28–8.13; p = 0.01), and NVR (HR 3.72; 95%CI 1.69–8.18; p = 0.001). Baseline ALT level, serum albumin level, and TVR were not associated with the development of HCC.

Table 1. Pretreatment characteristics of 1013 patients with chronic hepatitis C classified by the existence of cirrhosis and treatment outcome.

Characteristic	Non-cirrhosis n = 863				Cirrhosis n = 150			
	SVR n = 504	TVR n = 255	NVR n = 104	p value*	SVR n = 53	TVR n = 49	NVR n = 48	p value*
Age (yr)	54 (46-63)	61 (55-67)	61 (53-67)	<0.001	61 (57-67)	63 (53-68)	60 (54-68)	0.94
Male, n (%)	263 (52.2)	109 (42.7)	52 (50.0)	0.05	30 (56.6)	19 (38.8)	25 (52.1)	0.18
Body mass index (kg/m²)	22.9 (20.8-25.2)	23.3 (21.3-25.7)	23.1 (21.2-25.1)	0.12	23.0 (20.4-25.6)	23.7 (21.9-26.7)	24.6 (22.8-26.9)	0.07
ALT (IU/L)	52 (34-91)	47 (33-78)	51 (31-80)	0.02	88 (69-127)	65 (53-107)	66 (48-102)	0.01
Albumin (g/L)	42 (40-44)	42 (39-44)	42 (39-44)	0.26	37 (35-39)	37 (35-40)	37 (33-39)	0.87
Platelet count (x10⁹/L)	177 (144-212)	158 (129-194)	159 (130-197)	<0.001	103 (89-116)	97 (84-111)	99 (84-118)	0.26
Hemoglobin (g/L)	137 (129-148)	136 (128-147)	138 (127-149)	0.49	130 (122-140)	133 (123-142)	137 (126-147)	0.37
Ferritin (ng/ml)	156 (75-280)	174 (92-316)	213 (116-361)	0.16	200 (127-317)	202 (134-327)	250 (170-452)	0.05
α-fetoprotein (ng/ml)	4.1 (2.9-6.0)	4.8 (2.9-7.8)	5.9 (3.4-8.9)	<0.001	14.0 (9.2-36.0)	14.1 (9.3-31.3)	30.2 (15.4-42.9)	0.24
Hemoglobin A1c (%)	5.8 (5.7-6.3)	5.9 (5.7-6.4)	6.0 (5.7-6.7)	0.005	5.8 (5.4-6.4)	5.6 (5.3-6.4)	6.0 (5.4-6.6)	0.73
HCV genotype (1/2), n (%)	288/216 (57.1/42.9)	220/35 (86.3/13.7)	92/12 (88.5/11.5)	<0.001	24/29 (45.3/54.7)	43/6 (87.8/12.2)	43/5 (89.6/10.4)	<0.001

Data are expressed as number (%) or median (first-third quartiles).
SVR, sustained virological response; TVR, transient virological response; NVR, non-virological response; HCV, hepatitis C virus; ALT, alanine aminotransferase.
*Comparison among the three groups.

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Table 2. Risk factors for the development of HCC by chronic hepatitis C patients treated with PegIFNα2b and RBV.

Characteristic	All patients n = 1013	HCC n = 47	non-HCC n = 966	p value*
Age (yr)	58 (50-65)	67 (58-71)	58 (49-65)	<0.001
Male, n (%)	498 (49.2)	32 (68.1)	466 (48.2)	0.007
Body mass index (kg/m²)	23.0 (21.1-25.2)	23.6 (21.6-25.7)	23.0 (21.1-25.2)	0.15
ALT (IU/L)	54 (35-89)	74 (46-100)	54 (34-89)	0.008
Albumin (g/L)	41 (39-44)	40 (37-42)	44 (41-46)	0.002
Platelet count (x10⁹/L)	159 (120-199)	110 (88-132)	161 (123-201)	<0.001
Hemoglobin (g/L)	136 (127-147)	136 (128-149)	136 (127-147)	0.89
Ferritin (ng/ml)	165 (84-376)	187 (80-462)	167 (80-306)	0.68
α-fetoprotein (ng/ml)	4.9 (3.0-9.3)	11.7 (6.8-32.7)	4.8 (3.0-8.7)	<0.001
Hemoglobin A1c (%)	5.5 (5.3-5.9)	5.8 (5.4-6.3)	5.5 (5.3-5.9)	0.96
HCV genotype (1/2), n (%)	710/303 (70.1/29.9)	38/9 (80.9/19.1)	672/294 (69.6/30.4)	0.09
Non-cirrhosis/cirrhosis, n	863/150 (85.2/14.8)	19/28 (40.4/59.6)	844/122 (87.4/12.6)	<0.001
Treatment duration (wk)	47 (24-48)	43 (23-48)	47 (24-48)	0.58
Virological response (SVR/TVR/NVR), n (%)	557/304/152 (55.0/30.0/15.0)	13/13/21 (27.7/27.7/44.7)	544/291/131 (56.3/30.1/13.6)	<0.001

Data are expressed as number (%) or median (first-third quartiles).
All demographic and clinical data are those at the start of antiviral treatment.
HCV, hepatitis C virus; HCC, hepatocellular carcinoma; SVR, sustained virological response; TVR, transient virological response; NVR, non-virological response; ALT, alanine aminotransferase.
*Comparison between HCC and non-HCC.

Overall cumulative incidence of HCC classified by treatment outcome

The 5-year cumulative incidence rates of HCC of the SVR (3.1%) and TVR groups (5.8%) were significantly lower than those of the NVR group (18.8%) (both $p < 0.001$), and the rate of the SVR group was lower, but not significantly, than that of the TVR group ($p = 0.21$).

Cumulative incidence of HCC classified by treatment outcome in the non-cirrhosis group

The Kaplan–Meier curves for the incidence of HCC classified by treatment outcome in the non-cirrhosis group are shown in Fig. 1A ($p = 0.009$ by log-rank test). The 5-year cumulative incidence rates of HCC in the SVR (1.7%) and TVR groups (3.2%) were significantly lower than those of the NVR group (7.6%) ($p = 0.003$ and $p = 0.03$, respectively), and the rate of the SVR group was lower, but not significantly, than that of the TVR group ($p = 0.47$).

Cumulative incidence of HCC classified by treatment outcome in the cirrhosis group

The Kaplan–Meier curves for the incidence of HCC classified by treatment outcome in the cirrhosis group are shown in Fig. 1B ($p = 0.03$ by log-rank test). The 5-year cumulative incidence rates of HCC in the SVR (18.9%) and TVR groups (20.8%) were significantly lower than those of the NVR group (39.4%) ($p = 0.03$ and $p = 0.04$, respectively), and the rate of the SVR group was lower, but not significantly, than that of the TVR group ($p = 0.94$).

Adjusted rates of HCC incidence classified by treatment outcome of non-cirrhotic patients under 60 years of age

The Kaplan–Meyer curves of the estimation of the incidence of HCC by non-cirrhosis patients under 60 years of age, classified by treatment outcome, are shown in Fig. 2A ($p = 0.51$ by log-rank test). The 5-year cumulative incidence rates of HCC in the SVR (0.9%) and TVR groups (1.7%) were lower, but not significantly, than those of the NVR group (2.6%) ($p = 0.25$ and $p = 0.45$, respectively).

Table 3. Multivariate logistic regression analysis of possible predictors of HCC development.

Parameter	Hazard ratio	95% CI	p value
Age			
<60 yr	1		
≥60 yr	2.81	1.39-5.69	0.004
Sex			
Female	1		
Male	2.98	1.46-6.05	0.003
Platelet count			
≥150 x 10⁹/L	1		
<150 x 10⁹/L	4.04	1.57-10.44	0.004
α-fetoprotein			
<10 ng/ml	1		
≥10 ng/ml	2.50	1.09-5.78	0.03
Liver pathophysiology			
Non-cirrhosis	1		
Cirrhosis	3.22	1.28-8.13	0.01
Treatment outcome			
SVR	1		
TVR	1.50	0.65-3.44	0.34
NVR	3.72	1.69-8.18	0.001

HCC, hepatocellular carcinoma; SVR, sustained virological response; TVR, transient virological response; NVR, non-virological response.

Adjusted rates of HCC incidence classified by treatment outcome of non-cirrhotic patients aged 60 years and over

The Kaplan–Meyer curves of the estimation of the incidence of HCC in non-cirrhosis patients, aged 60 years and over classified by treatment outcome, are shown in Fig. 2B ($p = 0.05$ by log-rank test). The 5-year cumulative incidence rates of HCC in the SVR (3.5%) and TVR groups (4.2%) were significantly lower than those

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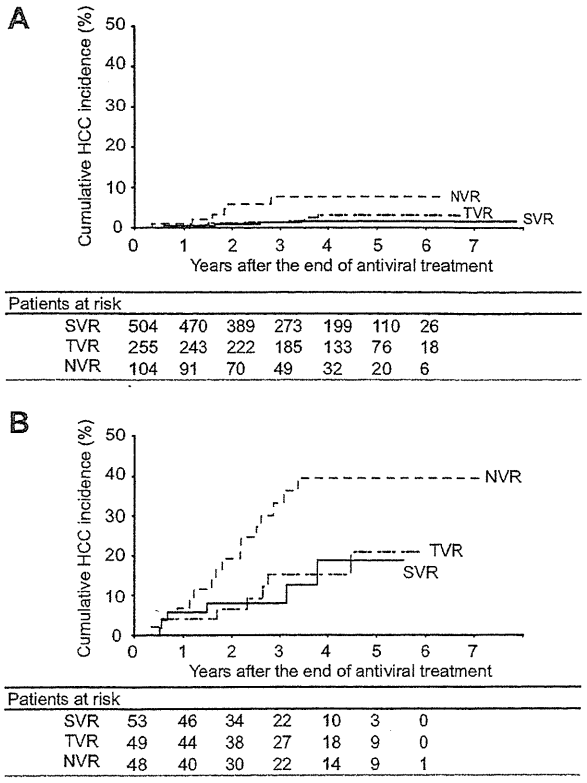

Fig. 1. Cumulative incidence of HCC after PegIFNα2b and RBV treatment stratified by treatment outcome (SVR: continuous line, TVR: long dashed-dotted line, NVR: dashed line). (A) Non-cirrhosis group (overall: $p = 0.009$; SVR *vs.* TVR: $p = 0.47$; SVR *vs.* NVR: $p = 0.003$; and TVR *vs.* NVR: $p = 0.03$ by log-rank test). (B) Cirrhosis group (overall: $p = 0.03$; SVR *vs.* TVR: $p = 0.94$; SVR *vs.* NVR: $p = 0.03$; and TVR *vs.* NVR: $p = 0.04$ by log-rank test).

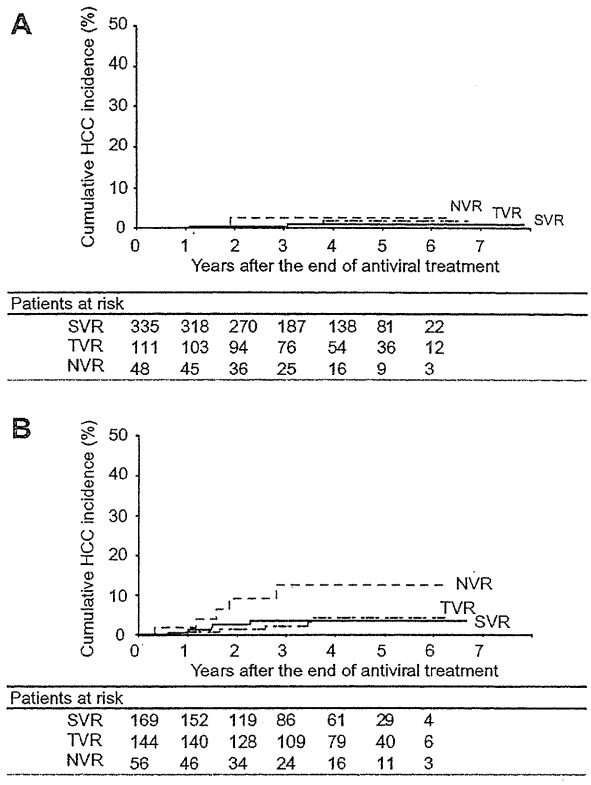

Fig. 2. Cumulative incidence of HCC after PegIFNα2b and RBV treatment stratified by treatment outcome of the non-cirrhosis group (SVR: continuous line, TVR: long dashed-dotted line, NVR: dashed line). (A) Under 60 years of age (overall: $p = 0.51$; SVR *vs.* TVR: $p = 0.94$; SVR *vs.* NVR: $p = 0.25$; and TVR *vs.* NVR: $p = 0.45$ by log-rank test). (B) Aged 60 years and over (overall: $p = 0.05$; SVR *vs.* TVR: $p = 0.96$; SVR *vs.* NVR: $p = 0.04$; and TVR *vs.* NVR: $p = 0.03$ by log-rank test).

of the NVR group (12.4%) ($p = 0.04$ and $p = 0.03$, respectively), and the rate of the SVR group was slightly lower, but not significantly, than that of the TVR group ($p = 0.96$).

The development of HCC by SVR patients

Thirteen patients who achieved SVR (2.3%) (6 non-cirrhotic and 7 cirrhotic patients) developed HCC during the follow-up period. Their individual pretreatment characteristics are shown in Table 4. Of these patients, 3 (patients 1–3) under 55 years of age had liver cirrhosis and the period from the end of antiviral treatment to the diagnosis of HCC was over 3 years. Of the remaining 10 patients (patients 4–13) aged 55 years and over, 6 did not have cirrhosis and the period from the end of antiviral treatment to the diagnosis of HCC was under 2.5 years.

Discussion

We here report the results of a prospective, long-term follow-up study done to evaluate the effect of treatment outcome on the development of HCC in a large cohort of Japanese patients with chronic hepatitis C, who were treated with PegIFNα2b and RBV. We found that those patients who achieved SVR or TVR had a lower risk of developing HCC within 5 years after the end of PegIFNα2b and RBV treatment when compared with NVR, in both cirrhosis and non-cirrhosis groups. Although SVR patients have been reported to have little risk of HCC incidence, a small number of our patients who achieved SVR did develop HCC, showing the necessity of a continued screening of patients with SVR.

Previously, the likelihood of HCC development by PegIFNα- and RBV-treated patients was difficult to determine because of the paucity of adequate long-term prospective studies. Based on the results of this prospective study, sex, age, platelet count, AFP level, and treatment outcome are significant, independent factors for the development of HCC. In addition to our present data, the incidence rate of HCC has been shown to be significantly lower for patients with TT genotype at rs8099917 and CC genotype at rs12979860 near the *IL28B* gene, which are associated with good response to antiviral treatment (data not shown). Of particular interest, the adjusted cumulative incidence of HCC was not significantly different between SVR and TVR for the 5 years after the end of treatment. Two randomized studies of maintenance therapy with low-dose PegIFNα to prevent hepatic decompensation and HCC have been recently reported [25,26]. However, maintenance therapy did not prevent HCC in presence of HCV viremia for at least 5 years, regardless of the degree of viral suppression. Our results showed that complete HCV sup-

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Table 4. Individual characteristics of SVR patients who developed HCC.

Patient number	Age (yr)	Sex	Liver pathophysiology	Time to HCC* (yr)	HCV genotype	ALT (IU/L)	Albumin (g/L)	Platelet count (x10⁹/L)	AFP (ng/ml)	HbA1c (%)
1	47	F	Cirrhosis	3.1	1	44	40	134	3.3	7.1
2	53	M	Cirrhosis	3.1	2	105	42	68	31.0	6.1
3	54	M	Cirrhosis	3.8	1	86	36	88	13.9	5.9
4	59	M	Non-cirrhosis	1.1	2	227	44	131	4.4	6.6
5	63	F	Cirrhosis	1.5	2	81	33	130	16.3	5.3
6	64	F	Non-cirrhosis	1.5	2	72	38	120	6.6	6.8
7	64	M	Non-cirrhosis	1.5	1	29	46	124	20.7	5.1
8	66	F	Cirrhosis	0.7	2	169	42	105	106.0	6.4
9	66	M	Non-cirrhosis	0.6	1	36	35	147	6.2	5.5
10	71	M	Cirrhosis	0.6	2	80	32	106	10.6	5.5
11	71	M	Non-cirrhosis	1.0	1	47	42	108	4.3	5.7
12	74	M	Non-cirrhosis	2.3	1	47	43	143	12.9	6.9
13	77	M	Cirrhosis	0.5	1	73	30	124	11.6	5.4

All data are those at the start of antiviral treatment.
SVR, sustained virological response; HCC, hepatocellular carcinoma; F, female; M, male; HCV, hepatitis C virus; ALT, alanine aminotransferase; AFP, α-fetoprotein; HbA1c, hemoglobin A1c.
*The time frame for HCC incidence starts from the end of antiviral treatment.

pression during antiviral treatment played an important role in preventing the development of HCC.

A recent prospective study that included Caucasian, Hispanic, and Black patients treated with PegIFNα2a and RBV reported that the adjusted mortality from any cause or liver transplantation, or of any liver-related outcome, was significantly lower in TVR patients than in NVR patients [13]. Similarly, the risk of decompensated liver disease, HCC and liver-related death was also lower in TVR patients than in NVR patients, although these differences did not reach statistical significance [13]. Therefore, the significantly low incidence rate of HCC, for the patients of this study with TVR in comparison with NVR, is an original finding, but the trend was true for cirrhotic patients of all ages and for non-cirrhotic patients aged 60 years and over. One possible explanation for this difference may be related to the rising incidence of HCC for NVR patients aged 60 years and over. Our results indicate that the duration of clinical benefit may outlast the period of actual viral suppression in the 5 years after treatment, however, it remains unclear how older age would explain why TVR resulted in a lower incidence of HCC that matched the incidence in SVR. Therefore, it will be necessary to investigate the development of HCC in SVR and TVR patients beyond five years.

Recently, a number of direct-acting antivirals (DAAs) have been designed and developed. Among them, telaprevir and boceprevir, non-structural 3/4A protease inhibitors, have shown promising results in various clinical trials and have led to an increased SVR rate when given in combination with PegIFNα and RBV, as compared with PegIFNα and RBV alone [27,28]. Furthermore, several IFN-free clinical trials, using regimens that combine several potent DAAs, are ongoing. As a result of advances in antiviral treatment, almost all patients can experience complete HCV suppression during treatment. We showed that TVR patients had a lower incidence rate of HCC than did NVR patients. It will be necessary to study the impact of virological response on the development of HCC by patients who undergo DAAs with and without IFN antiviral treatment.

Findings on the effect of SVR on liver-related preferable clinical outcomes have been reported in many previous reports [13,29–31], however, the analysis of the effect of SVR on the development of HCC is statistically difficult, because the number of events is too small to draw meaningful conclusions. In fact, there were only 13 patients with SVR who developed HCC during the observation period, reducing the validity of the analysis. Additional prospective studies that include a larger number of patients with SVR will be necessary to evaluate the relationship between SVR and the development of HCC.

Risk factors for HCV-related HCC have been reported previously, such as older age, male sex, obesity, diabetes mellitus, alcohol consumption, HCV genotype 1b, insulin resistance, complicated hepatic steatosis, and co-infection with hepatitis B virus or HIV [32,33]. Unfortunately, this study lacks data on insulin resistance and hepatic steatosis. Homeostasis Model Assessment of Insulin Resistance value is also related to a profound effect on PegIFNα2b and RBV treatment outcome [34], thus, there may be a significant difference in HbA1c level between the SVR, TVR and NVR non-cirrhotic groups, indicating differences in glucose metabolism. Moreover, it is known that hepatic steatosis occurs in about 40% of the chronic hepatitis C patients, when all common factors of fatty liver, such as alcohol abuse, obesity, and diabetes, have been excluded [35]. Therefore, it remains unclear whether or not there is a significant bias due to different rates of patients with insulin resistance or hepatic steatosis. Another limitation is the generalizability of the extremely high cumulative incidence rate of HCC, especially for cirrhotic NVR patients. The reasons for this exceedingly high rate are not well understood, although it may be explained by the increasing number of aging chronic hepatitis C patients in Japan, earlier than other countries [14]. Our results, therefore, may not be generalized to other ethnic groups that do not have such high rates of HCC.

In summary, this prospective study demonstrated that SVR and TVR patients had a significantly lower rate than NVR patients of HCC incidence within five years after the end of treatment, both for patients with and without cirrhosis. Because the risk of developing HCC remains present even after HCV eradication, long-term screening of patients with SVR is important.

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Conflict of interest

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

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ORIGINAL ARTICLE

Estimation of two real-time RT-PCR assays for quantitation of hepatitis C virus RNA during PEG-IFN plus ribavirin therapy by HCV genotypes and IL28B genotype

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Abstract Hepatitis C virus (HCV) RNA values measured with two real-time PCR methods (Cobas Ampliprep/Cobas TaqMan, CAP/CTM, and the Abbott real-time PCR test, ART) vary among patients with genotype 1. We investigated HCV RNA values measured by two real-time PCR assays during pegylated interferon plus ribavirin (PEG-IFN/RBV) therapy. We evaluated 185 cases of chronic hepatitis C patients, among which 97 patients received the PEG-IFN/RBV therapy. HCV RNA values of CAP/CTM for genotype 1 were significantly higher than those of ART ($p < 0.05$) The difference in HCV RNA values (CAP/CTM minus ART) of genotype 1 was significantly higher than those in genotype 2 ($p < 0.0001$). The positive rate (>0) of the difference of HCV RNA values in genotype 1 was 100 % (55/55), which was significantly higher than the 78.6 % (33/42) of genotype 2 ($p < 0.001$). There was no difference between TT and TG/GG genotype groups in terms of difference of HCV RNA values (CAP/CTM minus ART). After PEG-IFN/RBV therapy was administered, reduction of HCV measurements was observed from day 1 for both assays regardless of genotype. The HCV value of CAP/CTM during PEG-IFN/RBV therapy was consistently higher than the value of ART, although the difference in these two values gradually became smaller during the course of therapy, and eventually no significant difference was observed near the detection level. No correlation was observed between the sustained virological response (SVR) rate and the difference between the CAP/CTM HCV values and the ART HCV value before treatment.

Keywords Abbott real-time PCR test · Cobas Ampliprep/Cobas TaqMan · Hepatitis C virus · Genotype · PEG-IFN plus ribavirin therapy · Real-time RT-PCR assay

Introduction

Approximately 80 % of patients infected by hepatitis C virus (HCV) develop chronic hepatitis [1, 2]. Currently, there are more than 100 million HCV carriers worldwide. Chronic hepatitis C could gradually progress to cirrhosis and liver cancer. The first treatment option for chronic hepatitis C is the pegylated interferon plus ribavirin (PEG-IFN/RBV) combination therapy [3, 4]. Several virological predictive factors for sustained virological response (SVR) of PEG-IFN/RBV combination therapy are HCV genotype, baseline viral loads, and early virological response [5–7]. The SVR rate of PEG-IFN/RBV therapy is approximately 50 % for genotype 1 and 80 % for genotype 2. HCV RNA monitoring early in PEG-IFN/RBV therapy is an important predictive factor for SVR for either genotype 1 or genotype 2 [8, 9]. Detection of HCV RNA during PEG-IFN/RBV therapy is important in determining the length of IFN treatment [10]. Currently, Cobas Ampliprep/Cobas TaqMan (CAP/CTM) and Abbott real-time PCR test (ART) are used for HCV RNA measurement. The HCV RNA value in genotype 1 measured by CAP/CTM assay was significantly higher than values by ART assay [11]. The HCV RNA

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value in genotype 1 was measured by two real-time polymerase chain reaction (PCR) methods in this study to investigate whether there is a significant difference in HCV RNA values during PEG-IFN/RBV therapy.

Materials and methods

Of patients with chronic hepatitis C who visited Shin-Kokura hospital from April 2009 to December 2010, 185 were enrolled in this study. Of these 185 patients, 92 subjects were male and 93 were female, 96 subjects were 60 years old or older, and 89 were younger than 60 years old. The study protocol was in compliance with the Good Clinical Practice Guidelines and the 1975 Declaration of Helsinki and was approved by the Institutional Review Board. Each patient gave informed consent before participating in this trial. Of the 185 subjects in the study, 97 patients received the PEG-IFNa-2b plus ribavirin combination therapy: 55 patients had genotype 1, and 42 patients had genotype 2. PEG-IFNa-2b (PEG-Intron; MSD, Tokyo, Japan) was injected subcutaneously at a median dose 1.5 μg/kg (range, 1.3–1.5 μg/kg) once a week. Ribavirin (Rebetol; MSD, Tokyo, Japan) was administered at 200–600 mg twice a day after breakfast and dinner (daily dose, 600–1,000 mg). Patients were considered to have an SVR if HCV RNA remained undetectable at 24 weeks after the completion of treatment. The SVR rate was evaluated separately in patients with genotype 1 and genotype 2. Fifty-two of 55 cases of genotype 1 that received PEG-IFN/RBV were evaluated because the treatment was discontinued in 3 patients. Forty-one of 42 cases of genotype 2 were evaluated; treatment was discontinued in 1 patient.

Two real-time reverse transcriptase-polymerase chain reaction (RT-PCR) assays, CAP/CTM (Roche Molecular Systems, Pleasanton, CA, USA) and Abbott real-time HCV test (ART; Abbott Molecular, Abbott Park, IL, USA) were used for the quantitative measurement of HCV RNA concentrations before PEG-IFN/RBV treatment and at day 1, week 1, and week 2 during PEG-IFN/RBV treatment.

Abbott real-time HCV test

The Abbott real-time HCV test is based upon RT-PCR followed by real-time fluorescent detection of HCV RNA (RT-PCR assay). The assay has adopted the second international WHO standard for HCV RNA (code 96/798) for calibration. HCV RNA concentration is expressed in IU/ml. The ART assay has a lower limit of detection (LOD) of 12 IU/ml with a linear quantitation range of 12×10^7 IU/ml.

Cobas Ampliprep/Cobas TaqMan assay

The Cobas Ampliprep/Cobas TaqMan assay is based upon RT-PCR followed by real-time fluorescent detection of HCV RNA from 850 ml serum. CAP/CTM is standardized against the first WHO international standard for HCV RNA (code 96/798). HCV RNA concentration is reported in IU/ml. CAP/CTM assay has an LOD of 15 IU/ml with a linear quantitation range of $43–6.9 \times 10^7$ IU/ml.

We genotyped 115 patients for a single nucleotide polymorphism (SNP): rs8099917, an IL28B SNP previously reported to be associated with PEG-IFN/RBV therapy outcome. Samples were genotyped using the Illumina Human Hap 610-Quad Genotyping Bead Chip, with the Invader, or TaqMan assay, as described elsewhere [11–13].

Data analysis

Statistical analysis was performed using PASW Statistics, version 18 (SPSS) and R, version 2.11. Categorical data were analyzed using the chi-squared test and Fisher's exact tests, and continuous data were analyzed using the nonparametric Mann–Whitney U test. p values (two-tailed) <0.05 were considered statistically significant. Correlation coefficient (R) was assessed by the Spearman's correlation coefficient implemented in STATA software version 8.0 (Stata-Corp. LP, College Station, TX, USA).

Results

Figure 1 shows the correlation between the HCV RNA measurements obtained by the two real-time PCR assays: CAP/CTM versus ART in the study variables. A strong correlation was noted between the two real-time RT-PCR assays with an overall coefficient of correlation (R^2) of 0.8975 ($p < 0.0001$).

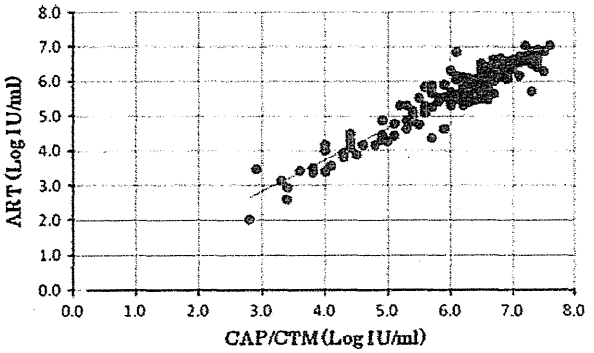

Fig. 1 Correlation between hepatitis C virus (HCV) RNA measurements obtained by two real-time RT-PCR assays: Cobas Ampliprep/Cobas TaqMan (*CAP/CTM*) versus Abbott real-time HCV test (*ART*)

Table 1 Correlation between hepatitis C (HCV) RNA measurements obtained by two real-time RT-PCR assays: CAP/CTM versus ART for study variables ($y = 0.9064x + 0.1176$, $R^2 = 0.8975$, $p < 0.0001$)

Study variables	n	CAP/CTM (log IU/ml)		ART (log IU/ml)		Correlation coefficient	
		Mean	SD	Mean	SD	R^2	p value
Gender							
Male	92	6.18	1.06	5.71	0.97	0.9009	<0.0001
Female	93	6.18	0.92	5.72	0.92	0.8972	<0.0001
Age (years)							
≥60	96	6.04	1.03	5.56	1.00	0.9142	<0.0001
<60	89	6.33	0.92	5.89	0.89	0.8709	<0.0001
Platelet counts (10^9/l)							
≥150	126	6.24	0.96	5.78	0.94	0.9106	<0.0001
<150	59	6.04	1.03	5.58	0.96	0.8705	<0.0001
IL28B							
TT	84	6.06	1.06	5.67	1.06	0.8826	<0.0001
TG/GG	31	5.91	1.18	5.45	1.07	0.9461	<0.0001
Genotype							
1	55	6.06	1.09	5.52	1.09	0.9647	<0.0001
2	42	5.94	1.21	5.65	1.15	0.9233	<0.0001

CAP/CTM Cobas Ampliprep/Cobas TaqMan, *ART* Abbott real-time HCV test, *IL28B* interleukin 28B

Table 1 shows the correlation between the HCV RNA measurements obtained by the two real-time RT-PCR assays, CAP/CTM versus ART, in the study variables. All the coefficient of correlation (R^2) values based on the variables, such as gender, age (≥60 or >60 years), and number of platelets (≤150 × 10^9/l or >150 × 10^9/l), were more than 0.8700 ($p < 0.0001$) and were strongly correlated with the HCV RNA values obtained by the two real-time RT-PCR assays. The coefficients of correlation (R^2) for IL28B genotype (TT, TG/GG) were 0.8826 ($p < 0.000$) and 0.9461 ($p < 0.0001$), respectively, and a strong correlation was observed also for the HCV RNA values obtained by the two real-time RT-PCR assays. Similarly, the coefficients of correlation (R^2) for the HCV genotypes (genotypes 1, 2) were 0.9647 ($p < 0.0001$) and 0.9233 ($p < 0.0001$), respectively, and a strong correlation was observed also for the HCV RNA values obtained by the two real-time PCR assays.

Table 2 shows HCV RNA concentrations of study variables as measured by the two real-time RT-PCR assays. HCV RNA values measured by CAP/CTM were significantly higher than those by ART for all variables, such as gender, age (≥60 or >60 years), and the number of platelets (≥150 × 10^9/l or >150 × 10^9/l) ($p < 0.05$). The HCV RNA values of the IL28B group with TT genotype measured by CAP/CTM were significantly higher than those by ART ($p < 0.05$); however, no difference was observed for the TG/GG genotypes. The difference of HCV RNA values (CAP/CTM minus ART) between the TT genotype and the TG/GG genotypes was not statistically significant (Fig. 2). The positive rates of the difference of HCV RNA values in the TT genotype and the TG/GG genotypes (CAP/CTM minus ART) were 90.5 and 90.3 %, respectively, and were not statistically significant. The difference of HCV RNA values in the TG/GG genotypes were not statistically significant, which can be explained by the small number of subjects enrolled in this study. The HCV RNA values in genotype 1 measured by CAP/CTM were significantly higher than those by ART ($p < 0.05$); however, the difference was not statistically significant in genotype 2. The difference of HCV RNA values (CAP/CTM minus ART) was significantly higher in genotype 1 than in genotype 2 ($p < 0.0001$) (Fig. 3). The positive rate (>0) of the difference of HCV RNA values (CAP/CTM minus ART) in genotype 1 was 100 % (55/55), significantly higher ($p < 0.001$) compared to the positive rate of 78.6 % (33/42) in genotype 2.

Table 3 shows HCV RNA concentrations of HCV genotypes 1 and 2 during PEG-IFN/RBV treatment as measured by the two real-time PCR assays. The HCV RNA values decreased during PEG-IFN/RBV therapy for both genotype 1 and genotype 2. The difference of HCV RNA values (CAP/CTM minus ART) in the genotype 1 group decreased gradually. The difference was not statistically significant at day 1 of treatment. The HCV RNA values of CAT/CTM in the genotype 1 group were higher than those of ART at day 1, week 1, and week 2; however, the difference was not statistically significant.

Table 4 shows the SVR rate in patients who received PEG-IFN/RBV therapy by differences of HCV RNA values between CAT/CTM and ART before PEG-IFN/RBV

Table 2 HCV RNA concentrations for study variables as measured by two real-time RT-PCR assays: CAP/CTM and ART

Study variables	*n*	CAP/CTM (log IU/ml)		ART (log IU/ml)		Average HCV RNA level (CAP/CTM−ART)		Quantitation difference		*p* value*
		Mean	SD	Mean	SD	Mean	SD	Mean	SD	
Gender										
Male	92	6.18	1.06	5.71	0.97	5.95	1.00	0.47	0.33	0.0019
Female	93	6.18	0.92	5.72	0.92	5.95	0.91	0.45	0.30	0.0010
Age (years)										
≥60	96	6.04	1.03	5.56	1.00	5.80	1.00	0.48	0.30	0.0010
<60	89	6.33	0.92	5.89	0.89	6.11	0.87	0.44	0.33	0.0016
Platelet counts (10^9/l)										
≥150	126	6.24	0.96	5.78	0.94	6.01	0.94	0.46	0.29	0.0002
<150	59	6.04	1.03	5.58	0.96	5.81	0.98	0.47	0.37	0.0123
IL28B										
TT	84	6.06	1.06	5.67	1.06	5.86	1.04	0.40	0.34	0.0131
TG/GG	31	5.91	1.18	5.45	1.07	5.68	1.12	0.45	0.29	0.1199
Genotype										
1	55	6.06	1.09	5.52	1.09	5.00	1.09	0.54	0.21†	0.0161
2	42	5.94	1.21	5.65	1.15	5.79	1.17	0.28	0.34†	0.2734

CAP/CTM Cobas Ampliprep/Cobas TaqMan, *ART* Abbott real time HCV test, *IL28B* interleukin 28B

* CAP/CTM versus ART

† $p < 0.0001$, genotype 1 versus genotype 2

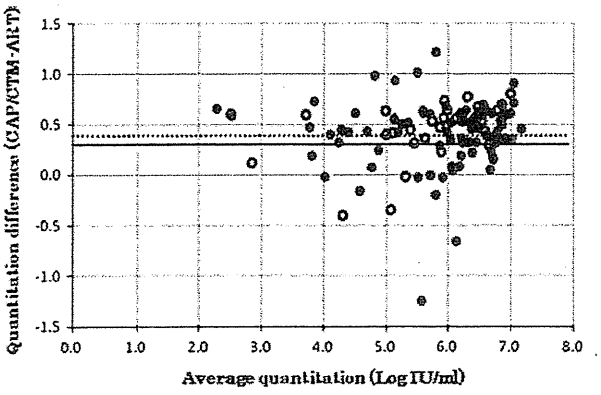

Fig. 2 Genotype-specific HCV RNA level difference in HCV RNA measurements by CAP/CTM versus those by ART test in samples with interleukin 28B (IL28B) genotypes; TT and TG/GG, before pegylated interferon plus ribavirin (PEG-IFN/RBV) treatment [*closed circles* genotype TT, *open circles* genotype TG/GG, *solid line* mean HCV RNA values of the difference (CAP/CTM minus ART) in TT, *dotted line* mean HCV RNA values of the difference (CAP/CTM minus ART) in TG/GG]

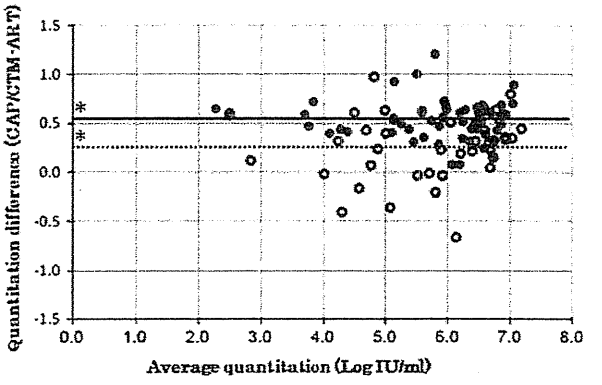

Fig. 3 Genotype-specific HCV RNA level difference in HCV RNA measurements by CAP/CTM versus those by ART test in samples with HCV subtypes 1 and 2 before PEG-IFN/RBV treatment [*closed circles* genotype 1, *open circles* genotype 2, *solid line* mean HCV RNA values of the difference (CAP/CTM minus ART) in HCV genotype 1, *dotted line* mean HCV RNA values of the difference (CAP/CTM minus ART) in HCV genotype 2]. *$p < 0.0001$, genotype 1 versus genotype 2

therapy. Group L comprises patients with a difference of HCV RNA values of 0.5 IU/ml or more (CAP/CTM minus ART), and group S comprises patients with a difference of HCV RNA values of less than 0.5 IU/ml (CAP/CTM minus ART). The SVR rate of genotype 1 (55.8 %) was significantly higher than that of genotype 2 (78.0 %, $p = 0.015$). For genotype 1, the SVR rate of IL28B genotype TT was significantly higher ($p = 0.016$) than that of genotype TG or GG. The SVR rates in group L and group S were not significantly different for IL28B genotypes TT, TG, or GG. The SVR rate of genotype TT was significantly higher than that of genotype TG or GG. The SVR rates in groups L and S were evaluated for the CAP/CTM HCV RNA values and ART before therapy, but no significant difference was

Table 3 HCV RNA concentrations for HCV genotypes 1 and 2 during PEG-IFN/RBV treatment as measured by two real-time RT-PCR assays: CAP/CTM and ART

Genotype	*n*	CAP/CTM (log IU/ml)		ART (log IU/ml)		Average HCV RNA level (log IU/ml)		Quantitation difference (CAP/CTM−ART)		*p* value*
		Mean	SD	Mean	SD	Mean	SD	Mean	SD	
Genotype 1										
Before treatment	55	6.06	1.09	5.52	1.09	5.00	1.09	0.54	0.21	0.0161
Day 1	53	4.64	1.11	4.23	1.12	4.44	1.12	0.41	0.15	0.0662
Week 1	55	3.79	1.93	3.41	1.74	3.60	1.83	0.38	0.38	0.2739
Week 2	55	3.06	2.05	2.80	1.74	2.93	1.88	0.26	0.49	0.4966
Genotype 2										
Before treatment	42	5.94	1.21	5.65	1.15	5.79	1.17	0.28	0.34	0.2734
Day 1	39	4.55	1.19	4.49	1.08	4.52	1.13	0.06	0.27	0.8618
Week 1	41	3.39	1.51	3.21	1.48	3.30	1.47	0.18	0.45	0.6946
Week 2	42	1.98	1.30	1.89	1.06	1.96	1.14	0.09	0.29	0.8014

CAP/CTM Cobas Ampliprep/Cobas TaqMan, *ART* Abbott real time HCV test

* CAP/CTM versus ART

observed. The SVR rate of patients with less than 6.0 log IU/ml of the HCV RNA values measured by both CAP/CTM and assay was higher than that with 6.0 log IU/ml or more, but this difference was not significant. For genotype 2, the SVR rate of IL28B genotype TT was higher than that of genotype TG or GG, but this difference also was not significant. There was no difference in the SVR rates between less than 6.0 and 6.0 log IU/ml or more. The SVR rates in group L and group S were not significantly different. The SVR rate of patients with genotype 2 was high regardless of HCV RNA values and IL28B genotype.

Discussion

The study revealed that the HCV RNA values measured by CAP/CTM were higher than those by ART among the subjects with genotype 1; however, no difference was observed among the patients with genotype 2. The difference of HCV RNA values (CAP/CTM minus ART) in genotype 1 was significantly higher than those in genotype 2 ($p < 0.0001$). The positive rate (>0) of the difference of HCV RNA values (CAP/CTM minus ART) in genotype 1 was 100 %, which was significantly higher than the positive rate of 78.6 % in the genotype 2 group ($p < 0.001$). The differences of HCV RNA values (CAP/CTM minus ART) in the genotype 1 group were all positive (>0), and all the viral load measurements obtained from CAP/CTM were higher than those from ART (Fig. 3). Ohnishi et al. [11] reported that the difference of the WHO standard versions used for each assay calibration might be the reason for the findings. CAP/CTM adopted the First International HCV RNA WHO standard for the assay calibration, while ART adopted the Second International HCV RNA WHO standard. Direct comparison of the two assays for measuring the WHO standard revealed a consistently higher quantitation of the WHO standard by CAP/CTM than by ART. The HCV RNA values of CAT/CTM in genotype 2 were reported to be lower those of ART [14]. Some cases in this evaluation also had lower CAP/CTM HCV RNA values (Fig. 3). No consistency was observed in genotype 2; some CAT/CTM HCV RNA values were higher than ART and some were lower. Base substitution is thought to contribute to this inconsistency [14]; this could have resulted from the differences between the two PCR methods. Also, this is consistent with a previous study in which CAP/CTM values were relatively higher for genotype 1 and lower for genotype 2 [2].

The difference of the HCV RNA values between CAP/CTM and ART was investigated in this study based on viral kinetics from the early stage of PEG-IFN/RBV treatment. After administration of PEG-IFN/RBV treatment, reduction of HCV RNA measurements obtained from both the CAP/CTM assay and the ART assay was observed from day 1 regardless of genotype (1 or 2). The HCV RNA values of CAP/CTM were consistently higher than those of ART during PEG-IFN/RBV therapy. The difference between these two values eventually became smaller because of the effect of PEG-IFN/RBV therapy, and a significant difference was no longer observed.

The IL28B genotype is one of the predictors of PEG-IFN/RBV therapy outcome before administration of treatment [15, 16]. In this study, for the genotype 1 patients, the SVR rate of IL28B genotype TT was significantly higher than the SVR rates of genotype TG or GG.

Table 4 Sustained virological response (SVR) rate in patients who received PEG-IFN/RBV therapy by difference between CAT/CTM HCV value and ART value before PEG-IFN/RBV therapy

	Group S SVR/*n* (%)	Group L SVR/*n* (%)	*p* value*	Total SVR/*n* (%)
Genotype 1				
IL28B				
TT	15/19 (79)	10/19 (53)	0.087	25/38 (66)
TG or GG	1/5 (20)	2/9 (22)	0.481	3/14 (21)
p value	0.012	0.128		0.004
CAP/CTM				
<6.0 log IU/ml	6/8 (75)	5/7 (71)	0.875	11/15 (73)
≥6.0 log IU/ml	10/16 (63)	7/21 (33)	0.077	17/37 (46)
p value	0.540	0.077		0.072
ART				
<6.0 log IU/ml	8/10 (80)	8/14 (57)	0.241	16/24 (67)
≥6.0 log IU/ml	8/14 (57)	4/14 (29)	0.126	12/28 (43)
p value	0.241	0.126		0.085
Total	16/24 (67)	12/28 (43)	0.086	28/52 (54)†
Genotype 2				
IL28B				
TT	19/22 (86)	4/6 (67)	0.264	23/28 (82)
TG or GG	5/7 (71)	4/6 (67)	0.852	9/13 (69)
p value	0.362	1.000		0.112
CAP/CTM				
<6.0 log IU/ml	11/15 (73)	3/4 (75)	0.946	14/19 (74)
≥6.0 log IU/ml	12/14 (86)	6/8 (75)	0.531	18/22 (82)
p value	0.411	1.000		0.530
ART				
<6.0 log IU/ml	14/15 (93)	4/5 (80)	0.717	18/20 (90)
≥6.0 log IU/ml	10/14 (71)	4/7 (57)	0.305	14/21 (67)
p value	0.564	0.407		0.293
Total	24/29 (83)	8/12 (67)	0.257	32/41 (78)†

Group L, ≥0.5 log IU/ml (CAP/CTM−ART); group S, <0.5 log IU/ml (CAP/CTM−ART)

* Group S versus group L

† $p = 0.015$, genotype 1 versus genotype 2

The HCV RNA values obtained from the two real-time PCR assays were analyzed based on the IL28B genotypes in this study. The HCV RNA values in the TT genotype group measured by CAP/CTM were significantly higher than those by ART; however, there was no significant difference in the TG or GG genotype groups. IL28B genotypes TT, TG, or GG were evaluated by differences of HCV RNA values between the CAP/CTM and ART: only the genotype TT group had a higher SVR rate. No SVR rate difference depending on the difference of HCV RNA values between CAP/CTM and ART was observed for genotypes 1 and 2. Clinically, a higher SVR rate was observed in the genotype TT group. It is assumed that the HCV RNA values of CAP/CTM were significantly higher than those of ART in genotype 1 patients because 73 % were in the genotype TT group. Therefore, there is assumed to be no correlation between IL28B and the difference of HCV RNA values between CAP/CTM and ART.

The data were also analyzed based on gender, age, and the number of platelets. For all variables, HCV RNA values as measured by CAP/CTM were significantly higher than those by ART; however, there was no difference in the HCV RNA values measured by CAP/CTM and ART when the measurements were compared against each variable.

The difference in HCV RNA measurements is suggested to be the result of HCV genotype. The prevalence of genotype 1 is higher in Japanese; therefore, the difference was observed in the measurements obtained from both assays.

The details of primer design and the PCR protocol for the products of both manufacturers used for this evaluation

are not disclosed. The PCR protocol of CAP/CTM method has two steps whereas the ART method has a three-step protocol. For the CAP/CTM method, elongation and probe hybridization are conducted simultaneously in the low-temperature step, and the temperature is generally 50–60 °C . In the ART method, a single-stranded linear probe is used instead of a TaqMan Probe and it has three steps, although it is also a real-time PCR method. Also, probe hybridization takes place at a lower temperature than for the CAP/CTM method, which is thought to optimize the tolerance level for HCV detection.

The newly developed ART features nucleic acid extraction using m2000 system, automated real-time PCR analysis, and high processing capacity. The assay results correlate well with the CAP/CTM assay, which suggests the wide application of the platform in clinical settings in the future. Additionally, the sample volume is 0.5 or 0.2 ml, which is highly practical for pediatric patients or when only a limited amount of patient sample is available. Also, some research has suggested that the genotype reactivity of ART is superior [17, 18].

In this study, the SVR rate was higher in genotype 2 than in genotype 1. For genotype 1, the SVR rate in IL28B genotype TT was higher than that in genotype TG or GG. For genotype 2, there was no difference of SVR rate between genotype TT and genotype TG or GG. These results were similar to the results of a previous study.

In summary, the HCV RNA values in genotype 1 obtained from the CAP/CTM assay were significantly higher compared to the values obtained from ART; however, no difference was observed in genotype 2. The HCV RNA values decreased during PEG-IFN/RBV therapy regardless of genotype. The HCV RNA value for CAP/CTM during PEG-IFN/RBV therapy was consistently higher than that for ART. However, the difference in these two values gradually became less during the course of therapy, and eventually no significant difference was observed near the detection level. No correlation was observed between the SVR rate and the difference between the CAP/CTM HCV values and the ART HCV value before treatment. Both CAP/CTM assay and ART assay were useful for PEG-IFN/RBV therapy. In this study, it was not clear which of the two HCV RNA assays was useful regarding the effects of IFN therapy. More detailed study is necessary.

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ORIGINAL ARTICLE

An inadequate dose of ribavirin is related to virological relapse by chronic hepatitis C patients treated with pegylated interferon alpha-2b and ribavirin

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Abstract The aim of this large-scale analysis was to assess the effect of 48-week pegylated interferon (PEG-IFN) α-2b and ribavirin (RBV) therapy on virological relapse by patients infected with hepatitis C virus (HCV) genotype 1. The relationship between virological relapse and the dose of PEG-IFNα-2b and RBV was investigated in 619 patients who had once cleared HCV RNA during PEG-IFNα-2b and RBV treatment for 48 weeks. The overall virological relapse rate was 34.1% (211 of 619). The relapse rate was 59.5% (22 of 37) for patients who received <6 mg/kg/day of RBV, even if a sufficient dose of PEG-IFNα-2b (≥1.5 μg/kg/day) was received. In contrast, the relapse rate was 28.1% (16 of 57) for patients who received ≥12 mg/kg/day of RBV, irrespective of the PEG-IFNα-2b dose. The relapse rates were significantly increased with the reduction of the RBV dose for both PEG-IFNα-2b doses of ≥1.2 and <1.2 μg/kg/week ($P < 0.0001$ and $P = 0.0006$, respectively). Moreover, the relapse rate was 41.2% (35 of 85) for patients with an early virological response (EVR) who received <6 mg/kg/day of RBV. The relapse rates were significantly increased with the reduction of the RBV dose in both those patients with an EVR and those with a late virological response ($P = 0.0006$ and $P = 0.0088$, respectively). To summarize, for HCV genotype 1 patients treated with PEG-IFNα-2b and RBV, the virological relapse of HCV was RBV dose-dependent, irrespective of the dose of PEG-IFNα or the effect of early viral kinetics.

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