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ORIGINAL ARTICLE

Abbott RealTime PCR assay is useful for evaluating virological response to antiviral treatment for chronic hepatitis C

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Abstract This study was done to evaluate the utility of the Abbott RealTime PCR assay (ART) for the monitoring of chronic hepatitis C patients. The serum samples of 183 patients infected with hepatitis C virus (HCV) genotype 1b who had completed a 48-week period of pegylated interferon (PEG-IFN) alpha-2b plus ribavirin treatment were prospectively analyzed. Serum HCV RNA levels were measured both by ART and by the Roche COBAS Amplicor Monitor test, version2.0 (CAM) at baseline and at weeks 4, 12, 24, 36, and 48 of treatment, and at 24 weeks after the end of treatment (EOT). A significant positive correlation of pretreatment HCV RNA levels was found between ART and CAM ($r = 0.595$, $P < 0.0001$). Of the 183 patients, 66 (36.0%) achieved a sustained virological response (SVR). The logarithmic decline of the HCV RNA level from the pretreatment level determined by ART in SVR patients was significantly higher than that in non-SVR patients at all time points tested. The logarithmic decline determined by CAM in SVR patients was significantly higher than that in non-SVR patients only at week 4, but there was no significant difference at other weeks. Of 124 patients who were HCV RNA-negative at EOT by ART, 58 (46.8%) had a relapse of viremia at 24 weeks after EOT, whereas 77 of 143 patients (53.8%) who were HCV RNA-negative at EOT by CAM had a relapse. The relapse rate was lower when determined by ART than by CAM, but not significantly so. ART is more useful than CAM for evaluating the virological response to antiviral treatment for chronic hepatitis C.

Keywords Hepatitis C · Interferon · Ribavirin · RealTime PCR

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Introduction

The number of patients infected with hepatitis C virus (HCV) is estimated to be approximately 170 million worldwide and 2.0 million in Japan. In Japan, chronic hepatitis C is the main cause of liver cirrhosis and hepatocellular carcinoma [1].

Interferon (IFN) is a cytokine produced to defend the host against infection. Type-I IFNs, including IFN α and IFN β, are induced by the activation of two pathways, Toll-like receptor 3 and cytosolic retinoic acid-inducible gene-I (RIG-I) like helicases, by which HCV is recognized [2]. All type-I IFNs bind to the same cell surface receptor, the IFN α receptor (IFNAR), consisting of two chains, and induce intracellular signaling through the Jak-signal transducer and activator of transcription (STAT) pathway. The important role of IFN α in antiviral responses is based on direct antiviral actions through the transcriptional activation of hundreds of IFN-stimulated genes (ISGs). Induction of these ISG-encoded proteins and their related pathways can lead to a block in viral transcription, degradation of viral RNA, inhibition of translation, or interference with various steps of viral replication [3, 4].

Table 1 Demographic characteristics of the183 studied patients

Characteristics	Total no. = 183	SVR no. = 66	Non-SVR no. = 117	*P* value
Male no. (%)	83 (45.4)	37 (56.1)	46 (43.9)	0.0289
Age (years)	58.7 ± 10.4	55.0 ± 10.7	60.7 ± 9.7	0.0003
Body mass index (kg/m^2)	23.2 ± 3.0	22.8 ± 3.0	23.4 ± 3.0	0.2017
Creatinine (mg/dl)	0.67 ± 0.17	0.70 ± 0.16	0.65 ± 0.18	0.1054
Creatinine clearance (ml/min)	95.1 ± 27.7	96.8 ± 27.7	94.1 ± 27.6	0.5224
Albumin (g/dl)	4.2 ± 0.4	4.2 ± 0.3	4.2 ± 0.4	0.2451
Alanine aminotranseferase (IU/l)	68.1 ± 48.8	71.7 ± 54.7	66.0 ± 45.2	0.4441
γ-Glutamyl-transpeptidase (IU/l)	46.5 ± 31.9	31.5 ± 16.7	55.0 ± 35.2	<0.0001
White blood cell count (/mm^3)	4900 ± 1367	5018 ± 1232	4833.4 ± 1438	0.3811
Hemoglobin (g/dl)	13.5 ± 1.5	13.7 ± 1.5	13.3 ± 1.4	0.0813
Platelet count (10^9/l)	16.4 ± 4.6	17.3 ± 4.7	15.9 ± 4.5	0.0671
Serum HCV RNA levels (log IU/ml)[a]	5.7 ± 0.5	5.6 ± 0.6	5.7 ± 0.5	0.1062
Histological hepatic fibrosis				
Stage 0 no. (%)	16 (8.7)	9 (13.6)	7 (6.0)	
Stage 1 no. (%)	56 (30.6)	22 (33.3)	34 (29.1)	0.0526
Stage 2 no. (%)	46 (25.1)	16 (24.2)	30 (25.6)	
Stage 3 no. (%)	23 (12.6)	3 (4.6)	20 (17.1)	
Stage 4 no. (%)	19 (10.4)	5 (7.6)	14 (12.0)	
Untested no. (%)	23 (12.6)	11 (16.7)	12 (10.2)	

Data are shown as numbers (%) or means ± SD

HCV hepatitis C virus, *SVR* sustained virological response

[a] Measured by Abbott RealTime PCR assay

The most common current antiviral treatment for chronic hepatitis C is the combination of pegylated interferon α (PEG-IFN α) and ribavirin (RBV) [5], which has brought about a higher rate of sustained virological response (SVR) than standard IFN monotherapy [6, 7]. Before initiating antiviral therapy, factors such as age, sex, HCV genotype, HCV viral load, and the stage of liver fibrosis, which influence the rate of success, must be considered. During therapy, the rate of success is influenced by adherence to the treatment regimen and viral load kinetics [8–12].

In large multicenter studies, positive and negative predictors of SVR, using viral load kinetics, have been established that are now used for recommendations on the management of antiviral treatment by the American and European international consensus conferences. Therefore, accuracy of the quantification of HCV RNA is essential for the clinical management of patients under treatment [13, 14]. Real-time polymerase chain reaction (PCR) assays have recently become available for sensitive HCV RNA quantification. In this study, we evaluated the utility of the Abbott RealTime PCR assay (ART) for the management of patients undergoing antiviral treatment by comparing it to the Roche COBAS Amplicor Monitor test, version2.0 (CAM).

Patients and methods

Patients

A total of 183 Japanese patients [83 male (45.3%), mean age 58.7 years] infected with HCV genotype-1 were enrolled for the study. Their clinical characteristics are shown in Table 1. All received therapy with PEG-IFN α2b plus RBV. Serum HCV RNA levels were prospectively analyzed by both ART and CAM at baseline, and at weeks 4, 12, 24, 36, and 48 of treatment, and at 24 weeks after the end of treatment (EOT). Written informed consent was obtained from each patient, and the study was approved by the local ethics committee in accordance with the Declaration of Helsinki.

Therapeutic protocol

All patients were treated with PEG-IFN α2b (1.5 μg/kg of body weight by subcutaneous injection once a week) plus RBV (600–1000 mg/day orally according to body weight) for 48 weeks. The duration and dosages are those approved by the Japanese Ministry of Health, Labor and Welfare. The dose of PEG-IFN α2b was reduced if patients had

adverse psychological effects or a decrease in the white blood cell and platelet counts. Likewise, the dose of RBV was reduced if the hemoglobin level decreased to under 100 g/l. Both PEG-IFN α2b and RBV were discontinued if the hemoglobin level and white blood cell and platelet counts fell below 85 g/l, 1×10^9/l, and 2.5×10^9/l, respectively.

The present study did not set a treatment discontinuation rule as was used in other studies [15, 16]. This rule recommends that antiviral therapy should be discontinued for patients whose HCV RNA levels have decreased by less than 2 log IU/ml at week 12 or those in whom HCV RNA has remained detectable after week 24.

Definition of SVR, relapse, non-responder, and virological breakthrough

SVR was defined as undetectable serum HCV RNA by ART or CAM at 6 months after EOT. Relapse was defined as serum HCV RNA becoming undetectable during the treatment and sustained until EOT, but reappearing positive after EOT. Non-responder was defined as serum HCV RNA never becoming undetectable during the treatment or after EOT. Virological breakthrough was defined as undetectable serum HCV RNA during the treatment and the reappearance of serum HCV RNA positivity during the treatment.

Determination of HCV RNA level

We prospectively analyzed the serum HCV RNA levels of all patients by both ART and CAM.

ART was carried out following the manufacturer's protocols (Abbott Molecular, Des Plaines, IL, USA). Briefly, nucleic acid was extracted from a 500-μl serum sample and amplified with a control. Quantification was performed automatically and was based on a stored calibration curve derived from replicate testing of high and low calibrators, provided by Abbott, which are standardized to the WHO reference material [17]. ART provides a lower detection limit of 1.08 log IU/ml, a specificity of more than 99.5%, and a linear amplification range from 1.08 to 8.0 log IU/ml independent of the HCV genotype [18–20].

CAM was done following the manufacturer's instructions (Roche Molecular Systems, Branchburg, NJ, USA). In brief, 100 μl of serum was subjected to chaotropic lysis in the presence of known amounts of an internal quantitation standard (QS). The target viral RNA and the QS were resuspended in Specimen Diluent (Roche), and the mixture was mixed with an equal volume of amplification ready solution (Master Mix; Roche) containing the primers KY78 (biotinylated) and KY80 (nonbiotinylated), deoxynucleoside triphosphates, AmpErase, and r*Tth* DNA polymerase. Amplification, amplicon dilution, detection, and quantitation were automatically performed by the COBAS Amplicor analyzer [21]. The dynamic range of CAM is 500 to approximately 5,100,000 IU/ml with a specificity of almost 100%, independent of the HCV genotype [22–24]. To compare serum HCV RNA levels determined by ART with those determined by CAM, we transformed the levels determined by CAM (IU/ml) into logarithmic levels (log IU/ml). Therefore, the range of CAM was 2.7 to 6.7 log IU/ml.

Dynamic changes of serum HCV RNA levels during treatment

We analyzed the serum HCV RNA levels of the patients by both ART and CAM at the same time and compared the logarithmic declines of HCV RNA levels with the pretreatment levels. Serum HCV RNA levels that were undetectable by both ART and CAM were treated as 0 log IU/ml.

Determination of HCV genotype

HCV genotype was determined using type-specific primers from the core region of the HCV genome. The protocol for genotyping was carried out as described earlier [25].

Statistical analysis

Statistical analysis was done with BMDP statistical software for the IBM 3090 computer system (BMBD Statistical Software, Los Angeles, CA, USA). Continuous data were expressed as mean values or means ± SD. A paired *t*-test, unpaired *t*-test, Mann–Whitney *U*-test, or Kruskal–Wallis non-parametric analysis of variance was used to compare HCV dynamics. A "*P*" value of less than 0.05 was regarded as statistically significant.

Results

Correlation of ART and CAM pretreatment HCV RNA levels

Figure 1 shows the ART and CAM pretreatment HCV RNA levels for 66 SVR and 117 non-SVR patients infected with HCV genotype 1. The levels determined by ART ranged from 4.20 to 6.90 log IU/ml (median 5.66 log IU/ml) and those by CAM ranged from 4.40 to 6.75 log IU/ml (median 6.08 log IU/ml). We found a significant positive

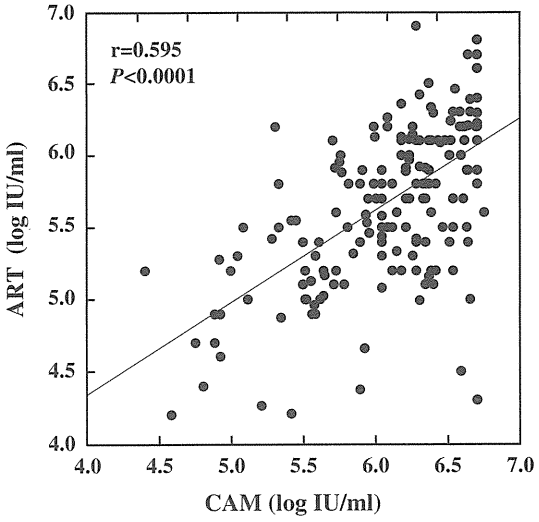

Fig. 1 Correlation of pretreatment hepatitis C virus (HCV) RNA levels determined by Abbott RealTime PCR assay (*ART*) and Roche COBAS Amplicor Monitor (*CAM*)

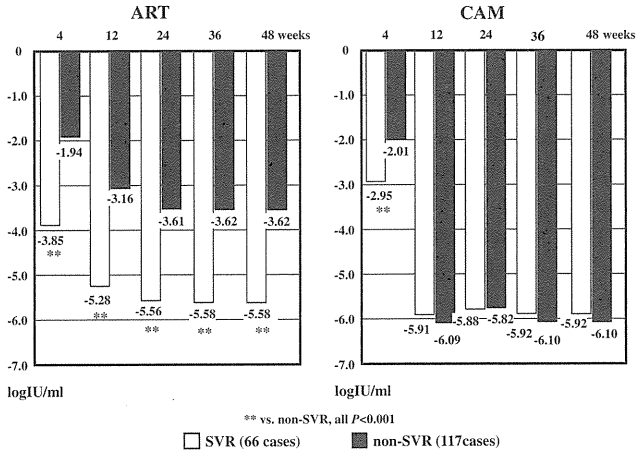

Fig. 2 The logarithmic declines from pretreatment hepatitis C virus (HCV) RNA levels determined by Abbott RealTime PCR assay (*ART*) and Roche COBAS Amplicor Monitor (*CAM*) during pegylated interferon alpha 2b plus ribavirin treatment. *White* and *black bars* indicate sustained virological response (*SVR*) and non-SVR patients, respectively

correlation between the ART and CAM pretreatment HCV RNA levels of the 183 patients ($r = 0.595$, $P < 0.0001$).

Comparison of ART with CAM in the logarithmic decline of HCV RNA levels

The logarithmic decline during treatment from the pretreatment HCV RNA level was compared between SVR and non-SVR patients (Fig. 2). The logarithmic declines of the HCV RNA levels of SVR patients determined by ART (−3.85, −5.28, −5.56, −5.58, and −5.58 at weeks 4, 12, 24, 36, and 48, respectively) were significantly higher than those of the non-SVR patients (−1.94, −3.16, −3.61, −3.62, and −3.62 at weeks 4, 12, 24, 36, and 48, respectively) (all $P < 0.001$). The logarithmic declines of the HCV RNA levels of SVR patients determined by CAM (−2.95) were significantly higher than those of the non-SVR patients (−2.01) ($P < 0.001$) at week 4, but there was no significant difference at weeks 12, 24, 36, and 48 (SVR, −5.91, −5.88, −5.92, and −5.92, vs. non-SVR, −6.09, −5.82, −6.10, and −6.10, respectively).

Virological response: comparison of ART and CAM

Of the 183 patients, 66 (36.0%) were shown to have achieved an SVR determined by both ART and CAM. By ART, 58 patients relapsed during the treatment (31.7%) and by CAM 77 (42.1%) relapsed ($P = 0.051$); 59 were non-responders (32.2%) by ART and 40 (21.8%) were non-responders by CAM ($P = 0.034$). All 19 patients whose HCV RNA was undetectable at EOT by CAM but detectable by ART had relapsed at the end of follow-up (EOF).

Comparison of ART and CAM for determining the time HCV RNA became undetectable

We analyzed the distribution of the time at which HCV RNA became undetectable by ART and by CAM for 66 SVR and relapsed patients ($n = 58$ by ART and $n = 77$ by CAM). For the 66 SVR patients, the percentages of patients for whom HCV RNA became undetectable at weeks 4, 12, and 24 were 27.2% ($n = 18$), 50.0% ($n = 33$), and 22.7% ($n = 15$) by ART and 62.1% ($n = 41$), 36.4% ($n = 24$), and 1.5% ($n = 1$) by CAM. For the 58 relapsed patients determined by ART, HCV RNA was undetectable in the serum of 5.2% ($n = 3$), 32.7% ($n = 19$), 43.1% ($n = 25$), 15.5% ($n = 9$), and 3.4% ($n = 2$) at weeks 4, 12, 24, 36, and 48, respectively. For the 77 relapsed patients determined by CAM, the percentages were 18.1% ($n = 14$), 55.8% ($n = 43$), 19.5% ($n = 15$), 1.3% ($n = 1$), and 5.2% ($n = 4$) at weeks 4, 12, 24, 36, and 48, respectively.

Comparison of the positive predictive value (PPV) and negative predictive value (NPV) rates for SVR determined by ART and CAM

The PPV and NPV rates were calculated for each treatment period (at weeks 4, 12, 24, 36, and 48) based on undetectable HCV RNA determined by ART and CAM. The PPV rates for SVR determined by ART were higher than those by CAM at all weeks (ART, 85.7, 70.8, 58.6, 57.4, and 55.9% vs. CAM, 74.5, 53.3, 47.8, 47.8, and 46.8%, respectively). There was a significant difference at week 12 ($P = 0.032$), but there were no significant differences at the other testing time points. The NPV rates for SVR

Table 2 Comparison of ART and CAM for the detection of HCV RNA in the serum of 13 patients with virological breakthrough by ART

Patients	ART (week)						CAM (week)					
A, H[a], HCV RNA	4	12	24	36	48	EOF	4	12	24	36	48	EOF
1. 55M, F1, 5.1 log	+	−	−	+	−	+	+	+	−	−	−	+
2. 60M, NT, 5.9 log	+	−	−	−	+	+	+	+	−	−	−	+
3. 55F, F1, 5.5 log	+	−	−	+	−	+	+	+	−	−	−	+
4. 71F, F2, 5.6 log	+	−	−	+	−	+	+	+	−	−	−	+
5. 64F, F1, 5.0 log	+	−	−	+	−	+	+	−	−	−	−	+
6. 54F, F1, 5.8 log	+	−	+	−	−	+	−	−	−	−	−	+
7. 56F, F2, 5.1 log	+	+	−	−	+	+	−	−	−	−	−	+
8. 66F, F0, 5.0 log	+	+	−	+	−	+	+	−	−	−	−	+
9. 65M, F3, 6.1 log	+	+	−	−	+	+	+	+	−	−	−	+
10. 35F, NT, 6.4 log	+	+	−	+	−	+	+	+	−	−	−	+
11. 58F, F3, 5.1 log	+	+	−	−	+	+	−	−	−	−	−	+
12. 67F, F2, 6.2 log	+	+	−	+	+	+	+	+	−	−	−	+
13. 68F, F2, 6.4 log	+	+	+	−	+	+	+	+	−	−	−	+

HCV hepatitis C virus, *ART* Abbot RealTime PCR assay, *CAM* Roche COBAS Amplicor Monitor, *A* age (years) and sex (M/F), *H* histology, *NT* not tested, *EOF* end of follow-up (week 24 after end of treatment)

[a] Fibrosis only

Table 3 Comparison of ART and CAM for the detection of HCV RNA in the serum of 3 patients with virological breakthrough by CAM

Patients	CAM (week)						ART (week)					
A, H[a], HCV RNA	4	12	24	36	48	EOF	4	12	24	36	48	EOF
1. 55M, F1, 5.1 log	+	−	+	−	−	+	+	+	+	−	−	+
2. 64F, F1, 5.9 log	+	−	+	−	−	+	+	+	+	+	+	+
3. 58M, F1, 6.0 log	+	+	−	+	−	+	+	+	+	+	+	+

HCV hepatitis C virus, *ART* Abbot RealTime PCR assay, *CAM* Roche COBAS Amplicor Monitor, *A* age (years) and sex (M/F), *H* histology, *EOF* end of follow-up (week 24 after end of treatment)

[a] Fibrosis only

determined by ART were lower than those by CAM at all testing time points (ART, 70.4, 86.5, 98.6, 100, and 100%, vs. CAM, 80.5, 98.4, 100, 100, and 100%, respectively), but there were no significant differences.

Comparison of HCV RNA detection by ART and CAM among patients with virological breakthrough

We defined virological breakthrough as undetectable HCV RNA during the treatment and the reappearance of serum HCV RNA positivity. Both ART and CAM analyses were done of the HCV viremia of patients who had a virological breakthrough. Virological breakthrough was detected by ART in 13 patients and by CAM in 3 patients. Table 2 shows the transition of HCV RNA detection for the 13 patients with virological breakthrough detected by ART, and Table 3 shows the transition of HCV RNA detection for the 3 patients with virological breakthrough detected by CAM. Of the 13 patients with virological breakthrough detected by ART, none was positive for serum HCV RNA by CAM after week 24. This suggests that ART is more useful than CAM for monitoring virological breakthrough. To the contrary, of the 3 patients with virological breakthrough detected by CAM, 2 (66.7%) were positive for serum HCV RNA by ART during the treatment. For the other patient, HCV viremia was not detected only at weeks 36 and 48 by ART, which suggests that patients who had been thought to have relapsed might actually have continually had HCV viremia.

Discussion

As part of the treatment strategy for chronic hepatitis C, measurement of the serum HCV RNA level is absolutely necessary because the earlier HCV RNA becomes

undetectable, the higher the probability of SVR [11, 15, 16, 26]. Therefore, the accuracy, sensitivity, lower limit of detection, and a broad dynamic range to monitor viral load change during antiviral therapy are important for the measurement of viremia. In the present study, we compared ART, a RealTime PCR assay, to CAM, the conventional method, and the results showed that ART was superior to CAM for monitoring viremia during antiviral therapy and more useful for the early prediction of virological response.

This study showed that the logarithmic declines of HCV RNA levels in SVR patients at all time points were significantly higher than those in non-SVR patients when measured with ART, but that the only significant difference was at week 4 when measured with CAM. These results indicate that ART is more useful than CAM for the prediction of SVR when patients have rapid declines of HCV RNA levels during the early phase of treatment. The reasons for this are that ART is an advanced quantitative assay based on the real-time PCR method, it is more sensitive, and has a broader range of determination than conventional methods [20, 27–29]. The lower limit of detection of ART is 12 IU/ml and the range of measurement is from 1.08 to 8.0 log IU/ml, but the detection limit of CAM is 500 IU/ml and the range of CAM is 2.7 to 6.7 log IU/ml.

PEG-IFN plus RBV treatment is currently the standard care for chronic hepatitis C patients, because it remarkably improves the SVR rate. However, there are still some patients who relapse during treatment and others who are negative at the end of the treatment but have flare-ups after the treatment. A suitable marker for the prediction of HCV viremia flare-up has not yet been identified. Minimal residual viremia detected by transcription-mediated amplification assay at the end of therapy in CAM-negative cases has been reported, which would be useful for predicting post-therapy relapse [30, 31]. Likewise, in the present study, all 13 patients with virological breakthrough detected by ART but not by CAM had relapsed after the treatment, and all 19 patients whose HCV RNA was undetectable at EOT by CAM but detectable by ART had relapsed. ART was superior to CAM for detecting virological breakthrough.

The differences in the limits of detection between the two assays would be responsible for this difference between ART and CAM in the virological dynamic change determined after the start of treatment. The principle of real-time PCR assays is to detect amplicon synthesis and deduce the number of viral genomes in the starting clinical sample during the PCR rather than at the end, and it is the reason that real-time PCR assays are more adequate for monitoring viral kinetics during antiviral treatment than end-point PCR assays [32].

Although there is a synergistic effect between IFN and RBV [33, 34], the logarithmic decline of serum HCV RNA induced by IFN in the early stages is significantly higher than that induced by RBV: IFN can induce several orders of logarithmic decline of serum HCV RNA, while RBV induces lower than 0.5 logarithmic decline [35, 36], so a more highly sensitive assay such as ART can detect virological breakthrough.

The results of the IDEAL (individualized dosing efficacy versus flat dosing to assess optional pegylated interferon therapy) trial showed that a logarithmic decline of HCV viremia at treatment week 4 of more than 3 log IU/ml or HCV viremia that becomes undetectable at week 12 are good indications of SVR [37, 38]. Our findings were similar to those of the IDEAL trial. Previously, it was recommended that antiviral therapy should be discontinued for patients whose HCV RNA levels had decreased to less than 2 log IU/ml at week 12 or those whose levels remained detectable after week 24 [15, 16]. The present study did not have such rules for stopping treatment. Moreover, most of our patients strongly hoped to complete the scheduled treatment even if they showed non-virological response. In fact, our previous multicenter study showed that only 5.9% of 273 patients, when given the option of stopping treatment because of non-virological response, stopped treatment [10]; thus, we had the data of such patients during the course of treatment and can corroborate such an application of the stopping rule.

We concluded that because of its accuracy and sensitivity for measuring HCV viremia, the Abbott RealTime PCR Assay is useful for monitoring HCV viremia during antiviral therapy and can be used for the prediction of SVR in patients chronically infected with HCV genotype 1.

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Original Article

Longitudinal assessment of liver stiffness by transient elastography for chronic hepatitis B patients treated with nucleoside analog

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Aim: To evaluate the association between liver stiffness measured by transient elastography (FibroScan) and the efficacy of long-term nucleoside analog (NA) treatment for patients with chronic hepatitis B.

Methods: Study 1: Forty-four chronic HBV patients had liver stiffness measured by FibroScan and underwent liver biopsy. Study 2: Group A: 22 patients started NA treatment at entry and FibroScan was done annually for 3 years. Group B: 23 patients started NA treatment prior to pretreatment FibroScan measurement, and FibroScan was done for from 3 to 5 years after the start of NA treatment.

Results: Study 1: The FibroScan values were significantly correlated with fibrosis stage ($r = 0.672$, $P < 0.0001$). Optimal cutoff of FibroScan values were 6.1 kPa for ≥ F1, 6.3 kPa for ≥ F2, 8.9 kPa for ≥ F3 and 12.0 kPa for F4. Study 2: For Group A, the baseline median FibroScan value was 8.2 kPa. FibroScan values significantly decreased annually for 3 years after the start of NA treatment (6.4 kPa, 5.8 kPa and 5.3 kPa at years 1, 2 and 3, respectively). For Group B, the FibroScan values did not significantly improve over the 3 years after the start of NA treatment.

Conclusions: Liver stiffness, measured by transient elastography, of chronic hepatitis B patients treated with NA showed a rapid decline in the first 3 years followed by a more steady transition for from 3 to 5 years irrespective of long term virological effect.

Key words: breakthrough hepatitis, hepatitis B virus, liver fibrosis, nucleoside analog, transient elastography

INTRODUCTION

CHRONIC HEPATITIS B virus (HBV) infection is a main cause of viral hepatitis. It is estimated that more than 350 million people are infected with HBV worldwide.[1,2] Morbidity and mortality by chronic HBV infection are a major public health concern. Lamivudine (LMV), an oral cytosine nucleoside analog (NA), was introduced for the treatment for HBV infection in 1998.[3,4] LMV can provide suppression of viral replication and biochemical improvement, which reduces the risk of developing serious liver diseases such as cirrhosis and hepatocellular carcinoma (HCC).[5–7]

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Although LMV has been shown to be highly effective in inhibiting HBV replication, the incidence of LMV-resistant virus is high, occurring in approximately 24% of patients after one year of treatment and in as many as 70% of patients after 4 years of treatment.[8] The emergence of LMV-resistance owing to the emergence of genotypic resistance by tyrosine, methionine, aspartate, or aspartate (YMDD) mutation may lead to viral and biochemical breakthrough and sometimes hepatitis flare-ups and rapid decompensation.[9] Adefovir dipivoxil (ADV) has been introduced for the treatment of chronic HBV infection, and ADV as an add-on to LMV has been used to reduce breakthrough.

Liver biopsy was for many years the gold standard for staging fibrosis. However, liver biopsy is no longer considered a perfect methodology because of the invasive nature of the procedure, sampling error, and inter-observer variability,[10] making improved testing

strategies necessary for accurate assessment of the liver status of patients with liver diseases. Recently, transient elastography (FibroScan; Echosens, Paris, France) has been proposed as a reliable, rapid, noninvasive and reproducible method for measuring liver stiffness.[11] FibroScan is increasingly being used as a noninvasive method for assessing liver fibrosis, and there have been many reports to date on patients with various liver diseases, such as chronic hepatitis C, primary biliary disease, non-alcoholic steatohepatitis, and chronic hepatitis B.[12–14] However, there are few reports of an association between the values as measured by FibroScan (FibroScan values) and the effectiveness of treatments for chronic liver diseases. We previously showed that FibroScan values were significantly correlated with the histological stage of percutaneous liver biopsy of patients with chronic hepatitis B and C.[15] Longitudinal assessment by FibroScan among patients with chronic hepatitis C treated with pegylated interferon alpha-2b and ribavirin was also done, with FibroScan shown to be a useful tool for the diagnosis of liver fibrosis and follow-up assessment of antiviral treatment.[16]

The aims of the present study were as follows: (Study 1) to test the correlation between liver histology and liver stiffness measured by FibroScan before NA treatment and (Study 2) to evaluate the association between liver stiffness measured by FibroScan and the efficacy of NA treatment for long-term in chronic HBV patients.

METHODS

Patients

IN STUDY 1, to evaluate the relationship between histological findings and FibroScan values, the liver stiffness of 44 patients with chronic HBV infection was measured by FibroScan and the patients underwent liver biopsy around the same time. The baseline laboratory results of the study population are summarized in Table 1.

In Study 2, 45 patients with chronic HBV infection undergoing long-term NA treatment had annual measurements of liver elasticity by FibroScan. These patients were divided into two groups according to the time of initiation of NA treatment: Since 2005 and between 2001 and 2004 (Groups A and B, respectively). In Group A, 22 patients started NA treatment at entry and FibroScan was done annually for 3 years (17 patients included in Study 1). In Group B, 23 patients started NA treatment prior to our pretreatment FibroScan measurement program, so FibroScan was done from 3 to 5 years after the start of NA treatment. The baseline laboratory results of the study population are summarized in Table 2.

Table 1 Baseline of characteristics of patients with chronic hepatitis B virus (HBV) infection (Study 1)

Characteristics	Liver biopsy $n = 44$
Male (%)	29 (64.4)
Age (years)	47.0 ± 13.9
Alanine aminotransferase (IU/L)	50.4 ± 29.0
Platelet count (10^9/L)	163 ± 53
Prothrombin time (%)	90.6 ± 11.3
α-fetoprotein (ng/mL)	5.5 ± 10.9
Serum type IV collagen (ng/mL)	165 ± 81
HBeAg positive No. (%)	19 (42.2)
Serum HBV DNA level (Log copies/mL)†	5.3 ± 1.5
FibroScan values (kPa)	6.3 (3.3–25.7)
Liver histology	
Stage of fibrosis (F0/F1/F2/F3/F4)	6/18/12/4/4
Grade of activity (A0/A1/A2/A3)	0/19/23/2

†Logarithmic transformed copies/mL.
Data is shown by the mean ± standard deviation, median (range) or *n* (%).
HBeAg, hepatitis B e antigen; HBV, hepatitis B virus; NA, nucleoside analog.

All patients satisfied the following criteria: (1) positive for hepatitis B surface antigen and (2) a history of an increased alanine aminotransferase (ALT) level for over 6 months. Exclusion criteria for the study were: (1) positive for antibody to human immunodeficiency virus or positive for anti-hepatitis C virus; (2) clinical or biochemical evidence of hepatic decompensation; (3) excessive active alcohol consumption (> 60 g/day converted into ethanol) or drug abuse; (4) suspected hepatocellular carcinoma at entry; or (5) treatment with immunosuppressive agents within 12 months prior to enrollment. Patients who fulfilled the above criteria were recruited for treatment at Kyushu University Hospital.

Informed consent was obtained from all patients before enrollment. The study was conducted in accordance with the ethical guidelines of the 1975 Declaration of Helsinki.

Clinical and laboratory assessment

Clinical parameters included ALT, platelet count, α-fetoprotein (AFP), type IV collagen, prothrombin time (PT), HBV genotype and HBV DNA. Body mass index (BMI) was calculated as weight in kilograms/height in square meters. Serum levels of ALT, platelet

Table 2 Baseline (proior to NA treatment) of characteristics of patients with chronic hepatitis B virus (HBV) infection (Study 2)

Characteristics	Group A $n = 22$	Group B $n = 23$	*P*-values
Male (%)	14 (63.6)	16 (69.6)	0.4567
Age (years)	49.8 ± 8.1	50.7 ± 10.3	0.7652
Body mass index (kg/m^2)	22.9 ± 2.5	22.4 ± 2.5	0.1786
Alanine aminotransferase (IU/L)	54.6 ± 30.4	56.1 ± 28.3	0.8613
Platelet count (10^9/L)	137 ± 54	156 ± 51	0.2128
Prothrombin time (%)	88.1 ± 11.2	80.2 ± 9.9	0.0239
α-fetoprotein (ng/mL)	5.0 ± 3.0	5.5 ± 4.0	0.6135
Serum type IV collagen (ng/mL)	172 ± 58	Not evaluated	–
HBeAg positive No. (%)	7 (31.8)	7 (30.4)	0.9202
Serum HBV DNA level (Log copies/mL)†	5.9 ± 1.5	6.2 ± 1.3	0.4448
FibroScan values (kPa)	8.2 (4.2–28.5)	Not evaluated	–
Liver histology			
Stage of fibrosis (F0/F1/F2/F3/F4)	2/5/7/1/2	0/3/5/7/5	0.1051
Grade of activity (A0/A1/A2/A3)	0/3/13/1	0/3/13/4	0.5166
Not determined (*n*)	5	3	

†Logarithmic transformed copies/mL.
Data is shown by the mean ± standard deviation, median (range), or *n* (%).
HBeAg, hepatitis B e antigen; HBV, hepatitis B virus; NA, nucleoside analog.

count, AFP, type IV collagen and PT were measured by standard laboratory techniques at a commercial laboratory (MBC Laboratory, Tokyo, Japan).

HBV genotyping and HBV DNA measurement

Hepatitis B virus genotyping was determined serologically by the polymerase chain reaction (PCR)-invader method with genotype specific probes.[17] Quantification of serum HBV DNA was performed by quantitative PCR assay (Amplicor HBV Monitor, Roche Diagnostics, Mannheim, Germany), over a detection range from 2.6 (corresponding to 400 copies/mL) to 7.5 log copies/mL. Virological breakthrough was defined as the reappearance of serum HBV DNA to a level more than 10-fold (1 log) the minimum during treatment.[18]

Monitoring the emergence of LMV-resistant mutants

The emergence of LMV-resistant mutants is mainly based on point mutation from methionine to valine/isoleucine at rt204 (rt204V/L) in the YMDD motif. YMDD mutation was detected by rapid PCR amplification across the YMDD-encoding gene locus and analysis of the hybridization kinetics of an integrated probe to infer its sequence using the LightCycler (Roche Diagnostics).[19,20]

Nucleoside analog treatment

Of the 45 patients treated with NA, 38 (84.4%) received LMV (Zeffix; Glaxo Smith Kline, UK) in a single oral daily dose of 100 mg and the other seven (15.6%) received entecavir (ETV) (Baraclude; Bristol-Myers Squibb, USA) in a single oral daily dose of 0.5 mg. Breakthrough hepatitis (BTH) by drug-resistant YMDD mutants developed in 13 (28.9%) of these patients. BTH patients received 10 mg ADV (Hepsera; Glaxo Smith Kline) in addition to LMV daily.

Transient elastography (FibroScan)

FibroScan was done for the right lobe of the liver through the intercostal spaces with the patient lying in the dorsal decubitus position with the right arm in maximal position. The tip of the probe transducer was covered with coupling gel and placed on the skin between the ribs at the level of the right lobe of the liver. The operator, assisted by an ultrasonic time-motion image, located a liver portion at least 6 cm thick and free of large vascular structures. Once the measurement area had been located, the operator pressed the probe button to start acquisition. The elasticity was automatically calculated by the apparatus, with the data shown as kilopascal (kPa). All examinations were performed by accomplished operators of our department. Only liver stiffness measurements

Table 3 Optimal cutoff of FibroScan values for the determination of histological fibrosis stage in 44 biopsy-received patients with chronic hepatitis B virus (HBV) infection (Study 1)

	Histological fibrosis stage by liver biopsy			
	F ≥ 1	F ≥ 2	F ≥ 3	F = 4
Cutoff value* (kPa)	6.1	6.3	8.9	12.0
AUROC	0.67	0.86	0.87	0.89
Sensitivity (%)	65.9	95.2	87.5	75.0
Specificity (%)	71.4	74.0	75.0	88.6
Positive predictive value (%)	93.1	74.1	41.2	37.5
Negative predictive value (%)	26.3	95.2	96.8	97.5
Positive likelihood ratio	2.30	3.66	3.50	6.58

*The optimal cutoff value is the one that gives the higher total sensitivity and specificity.
AUROC, area under the receiver operating characteristic curve.

obtained with at least six successful acquisitions and a success rate of at least 60% were considered reliable. The validity of FibroScan values depends on an interquartile range of all successful measurements (IQR/M) of less than 30% of median values.[21] The mean IQR/M of the present study was 22.1% and no cases of IQR/M > 30% were found.

Liver histology and quantification of liver biopsy

Liver biopsy was performed by experienced hepatologists with a 16G disposable needle (Bard Monopty; C.R.Bard, Covington, USA) under ultrasound guidance. The median liver biopsy length was 18 mm (minimum length 15 mm). Liver biopsy specimens were fixed in formalin and paraffin was embedded. All biopsy specimens were analyzed by two experienced pathologists who were blinded to the clinical data. For each specimen, the stage of fibrosis and the grade of activity were established according to the METAVIR score.[22] Fibrosis was staged on a 0–4 scale as follows: F0 = no fibrosis, F1 = portal fibrosis without septa, F2 = portal fibrosis and few septa, F3 = numerous septa without cirrhosis, F4 = cirrhosis. The grading of activity, including the intensity of the necroinflammation, was scored as follows: A0 = no histological activity, A1 = mild activity, A2 = moderate activity, A3 = severe activity.

Breakthrough hepatitis

Biochemical breakthrough usually lags behind virological breakthrough, and serum ALT level may remain normal for weeks to years after the development of antiviral resistance. BTH was defined as a serum ALT level over five times the upper limit of the normal range.

Statistical analysis

Statistical analysis was done with biomedical computer programs (BMDP) statistical software for the IBM 3090 system computer (BMBD Statistical Software, Inc., Los Angeles, CA, USA). Continuous data were expressed as median or mean ± standard deviation (SD). The paired *t*-test, unpaired *t*-test, Mann-Whitney *U*-test or Kruskal–Wallis non-parametric analysis of variance (ANOVA) was used for the analysis. Area under the receiver operating characteristic curve (AUROC) analysis was done to evaluate the relationship between histological findings and FibroScan values. The cutoff values were selected from the receiver operating characteristic curve to maximize the total sensitivity and specificity. A *P*-value less than 0.05 was regarded as statistically significant.

RESULTS

Relationship between liver fibrosis and FibroScan values at baseline

ALL PATIENTS WERE infected with HBV genotype C. The median values (interquartile range) of the patients were 5.0 kPa (3.5–6.0), 5.7 kPa (5.0–6.0), 7.0 kPa (6.2–10.1), 9.4 kPa (9.2–13.3) and 18.0 kPa (12.9–21.9) for F0, F1, F2, F3 and F4, respectively. The FibroScan values were significantly correlated with fibrosis stage ($r = 0.672$, $P < 0.0001$) and were also significantly increased in accordance with the grade of activity of the patients ($r = 0.321$, $P < 0.0001$). According to the progression of liver fibrosis stage, the mean grade of activity was higher (F0: 1.2, F1: 1.4, F2 1.8, F3 2.0 and F4 2.0), but the analysis did not reach significance. Table 3 shows the optimal liver stiffness cutoff values obtained by sensitivity, specificity and positive

likelihood ratios. Four threshold FibroScan values were identified: 6.1 kPa for ≥F1 (sensitivity 65.9%, specificity 71.4%); 6.3 kPa for ≥F2 (sensitivity 95.2%, specificity 74.0%); 8.9 kPa for ≥F3 (sensitivity 87.5%, specificity 75.0%) and 12.0 kPa for F4 (sensitivity 75.0%, specificity 88.6%). The corresponding AUROC were 0.67 for ≥F1, 0.86 for ≥F2, 0.87 for ≥F3 and 0.89 for F = 4.

Longitudinal FibroScan values 1–3 years after the start of the nucleoside analog treatment (Group A, Tables 4,6)

All patients were infected with HBV genotype C. The clinical and FibroScan data of Group A are shown in Table 4. For 19 patients (86.4%) the serum HBV DNA level became undetectable and that of the other three patients was reduced to under 4.0 log copies/mL on PCR by 6 months after the start of NA treatment. ALT level (54.6 ± 30.4 to 22.1 ± 7.7 U/L, $P < 0.0001$), PT (88.1 ± 11.2 to 96.3 ± 9.1%, $P < 0.0001$), AFP (5.0 ± 3.0 to 3.0 ± 1.6 ng/mL, $P = 0.0004$) and serum type IV collagen (172 ± 58 to 142 ± 37 ng/mL, $P = 0.0023$) were significantly improved during the first 3 years of NA treatment. The baseline median FibroScan value was 8.2 kPa and the mean value was 10.5 ± 6.5 kPa in Group A. FibroScan values significantly decreased annually for 3 years after the start of NA treatment (median value 6.4 kPa [mean value 8.7 ± 5.6 kPa], 5.8 kPa [7.4 ± 4.6 kPa] and 5.3 kPa [6.8 ± 4.0 kPa] at years 1, 2 and 3, respectively).

For non-BTH patients ($n = 18$, 81.8%), the baseline median FibroScan value was 7.7 kPa and the mean value was 10.3 ± 7.0 kPa. FibroScan values showed a significant, annual decrease for 3 years after the start of NA treatment (median value 6.3 kPa [mean value 8.4 ± 5.9 kPa], 5.4 kPa [7.2 ± 5.0 kPa] and 5.0 kPa [6.5 ± 4.3 kPa] at years 1, 2 and 3, respectively). For patients with an episode of BTH and viral breakthrough due to YMDD mutation, the serum HBV DNA level became undetectable on PCR by 6 months after the additional medication (ADV added-on LMV). For BTH patients ($n = 4$, 18.2%), the baseline median FibroScan value was 10.2 kPa and the mean value was 11.2 ± 4.3 kPa. FibroScan values improved due to NA treatment for the first 2 years (median value 10.8 kPa [mean value 10.2 ± 3.9 kPa], 7.9 kPa [7.9 ± 2.4 kPa] and 8.2 kPa [8.0 ± 2.2 kPa] at years 1, 2 and 3, respectively). However, three of four patients had BTH occur in the 3 year, therefore, FibroScan values did not improve.

Longitudinal FibroScan values 3–5 years after the start of nucleoside analog treatment (Study 2: Group B, Tables 5,6)

The clinical and FibroScan data of Group B are shown in Table 5. The serum HBV DNA level of 20 patients (87.0%) became undetectable and that of the other three patients was under 4.0 log copies/mL by PCR by 6 months after the start of treatment. For patients with an episode of BTH or viral breakthrough due to YMDD mutation, the serum HBV DNA level became undetectable by PCR by 6 months after the addition of ADV to LMV. Neither liver fibrosis nor the biochemical markers (ALT: 25.2 ± 16.8 to 24.2 ± 13.8 IU/L, PT: 91.1 ± 10.8 to 92.7 ± 8.7%, AFP: 3.1 ± 2.1 to 3.3 ± 3.1 ng/mL and serum type IV collagen: 154 ± 51 to 148 ± 48 ng/mL) of patients with or without BTH statistically improved over the 3 years after the start of NA treatment. At 3, 4 and 5 years after the start of NA treatment, the median (mean) FibroScan values were 6.1 (8.1 ± 5.2) kPa, 6.7 (8.2 ± 5.2) kPa and 5.9 (8.1 ± 5.1) kPa, respectively. For BTH patients ($n = 9$, 39.1%) at 3, 4 and 5 years after the start of NA treatment, the median (mean) FibroScan values were 10.4 (11.0 ± 6.4) kPa, 10.2 (11.1 ± 6.8) kPa and 9.6 (11.1 ± 6.6) kPa, respectively. Similarly, for non-BTH patients ($n = 14$, 60.9%), the median (mean) FibroScan values were 5.2 (6.2 ± 3.3) kPa, 5.2 (6.3 ± 2.8) kPa and 5.3 (6.3 ± 2.7) kPa, respectively. In both the BTH and non-BTH groups, FibroScan values did not significantly improve over the 3 years after the start of NA treatment.

Relationship between hepatocellular carcinoma and breakthrough hepatitis

Three of the patients (13.6%) in Group A and three (13.0%) in Group B developed HCC in the follow-up period: Four (66.7%) had FibroScan values consistently over 10 kPa. Patients no. 4 in Group A and no. 20 in Group B developed HCC after the start of NA treatment, in spite of low FibroScan values. Both of these patients had YMDD mutation detected by PCR and an elevated HBV DNA level before the occurrence of HCC.

In analysis of the relation between HCC and BTH, the incidence of HCC for BTH patients (4 of 13, 30.8%) was significantly higher than that of non-BTH patients (2 of 32, 6.3%) ($P = 0.0332$).

DISCUSSION

THIS STUDY DEMONSTRATED an association between liver stiffness measured by FibroScan and the efficacy of NA treatment of patients with chronic

Table 4 Clinical data and longitudinal FibroScan values in Group A (Study 2)

Patient No.	Age (years)	Sex	BMI (kg/m²)	Histology		FibroScan values (kPa)				ALT (IU/L)				HBeAg	HBV DNA (LogIU/mL)				BTH	HCC
				F-stage	A-grade	FS-0	FS-1	FS-2	FS-3	ALT-0	ALT-1	ALT-2	ALT-3		DNA-0	DNA-1	DNA-2	DNA-3		
1	48	M	22.5	2	2	17.3	14.0	12.9	12.6	53	30	35	29	–	5.5	–	–	4.2	+ (3)	+ (3)
2	56	F	21.1	not tested	not tested	10.4	9.6	7.9	8.6	27	18	16	13	+	6.8	–	–	5.4	+ (3)	–
3	53	M	23.0	2	2	9.9	12.0	7.9	7.7	67	84	20	19	+	7.5	5.9	–	–	+ (1)	–
4	42	M	23.4	not tested	not tested	7.1	5.1	5.0	5.2	44	23	20	21	+	7.7	3.2	–	5.4	+ (3)	+ (3)
5	42	F	21.4	0	2	4.2	4.0	3.8	2.5	25	14	15	16	–	5.5	–	–	–	–	–
6	65	M	26.3	1	1	5.2	4.3	4.0	3.4	35	17	20	34	–	3.4	–	–	3.0	–	–
7	34	M	24.2	1	2	5.4	4.8	3.8	3.9	74	36	45	36	+	7.5	–	–	–	–	–
8	56	F	23.6	0	1	5.5	4.8	4.7	4.0	87	17	15	16	–	7.0	–	–	–	–	–
9	57	M	22.5	not tested	not tested	5.6	4.8	4.0	4.0	43	37	35	32	–	7.3	–	–	–	–	–
10	51	M	28.6	1	2	5.8	6.2	5.6	5.6	55	28	27	21	–	5.4	–	–	–	–	–
11	49	F	22.9	2	3	7.0	5.3	4.6	2.9	59	18	12	12	+	6.2	–	–	–	–	–
12	32	F	29.1	1	2	7.2	5.4	5.2	4.8	65	28	27	27	–	4.8	–	–	–	–	–
13	37	M	22.3	1	1	7.5	6.4	6.1	5.2	99	17	12	18	+	7.7	3.4	–	–	–	–
14	52	M	23.8	3	2	7.9	5.2	3.9	4.8	96	17	19	11	–	4.1	–	–	–	–	–
15	55	M	22.5	not tested	not tested	8.5	6.3	6.0	5.8	47	26	25	35	–	7.3	–	–	–	–	–
16	55	M	20.8	2	2	8.6	7.4	6.2	5.3	42	20	25	27	–	5.0	–	–	–	–	–
17	51	F	21.1	2	2	9.4	7.4	5.9	5.4	36	29	23	24	–	6.1	–	–	–	–	–
18	48	M	23.9	2	2	12.8	7.2	5.2	4.8	45	22	29	22	–	5.5	–	–	–	–	–
19	53	M	21.6	4	2	13.3	11.5	9.6	8.6	13	14	12	12	–	3.7	–	–	–	–	–
20	56	F	22.7	2	2	17.3	14.2	10.9	10.4	21	16	18	20	–	4.5	–	–	–	–	–
21	55	F	21.2	4	2	25.7	22.1	17.6	15.1	141	29	21	24	–	6.0	–	2.7	3.0	–	+ (3)
22	49	M	27.5	not tested	not tested	28.5	24.0	21.2	18.0	27	19	20	17	–	3.4	–	–	–	–	–
					median	8.2	6.4	5.8	5.3											

FS-0, FibroScan values at baseline; FS-1, 2 and 3, FibroScan values at 1, 2 and 3 years after the start of nucleoside analog treatment, respectively.
M, male; F, female; BMI, body mass index; F-stage, fibrosis stage; A-grade, activity grade; ALT, alanine aminotranseferase; BTH, breakthrough hepatitis; HCC, hepatocellular carcinoma.
ALT-0, ALT level at baseline; ALT-1, 2 and 3, ALT levels at 1, 2 and 3 years after the start of nucleoside analog treatment, respectively.
DNA-0, HBV DNA level at baseline; DNA-1, 2 and 3, HBV DNA levels at 1, 2 and 3 years after the start of nucleoside analog treatment, respectively.
Figure in parenthesis was the onset of BTH or HCC after the start of nucleoside analog treatment (unit: year).

Table 5 Clinical data and longitudinal FibroScan values in Group B (Study 2)

Patient No.	Age (years)	Sex	BMI (kg/m²)	Histology		FibroScan values (kPa)			ALT (IU/L)			HBeAg	HBV DNA (LogIU/mL)						BTH	HCC
				F-stage	A-grade	FS-3	FS-4	FS-5	ALT-0	ALT-3	ALT-5		DNA-0	DNA-1	DNA-2	DNA-3	DNA-4	DNA-5		
1	64	M	22.7	4	3	20.5	23.3	21.8	34	14	16	–	7.1	–	4.9	–	–	–	+ (2)	+ (2)
2	50	M	24.4	4	2	20.4	17.7	18.8	83	29	25	+	6.5	–	3.9	3.6	3.6	2.8	+ (2)	–
3	56	M	23.7	3	2	14.3	16.7	16.6	24	17	15	–	6.5	–	–	5.3	–	2.7	+ (3)	+ (5)
4	61	F	24.2	3	3	10.6	8.5	9.6	56	38	41	+	7.8	–	6.8	3.1	–	–	+ (2)	–
5	46	F	22.0	4	3	10.4	10.2	9.6	63	27	24	–	7.1	–	–	–	4.0	–	+ (4)	–
6	43	M	22.2	4	3	9.5	10.2	9.8	56	38	41	+	4.1	6.0	–	–	–	–	+ (1)	–
7	49	M	21.5	3	2	6.5	6.7	6.4	32	24	22	–	6.0	–	–	–	4.0	–	+ (4)	–
8	47	M	21.5	1	1	3.7	3.5	4.0	31	29	29	–	6.4	–	–	–	–	3.9	+ (5)	–
9	57	M	20.7	3	2	3.2	3.5	3.0	40	14	12	+	8.6	–	7.1	4.0	–	–	+ (2)	–
10	71	F	22.6	1	1	3.9	4.3	5.1	25	13	11	–	5.5	–	–	–	–	–	–	–
11	35	M	22.6	2	2	4.4	5.0	5.0	97	15	14	+	7.6	–	–	–	–	–	–	–
12	55	M	22.4	1	1	4.6	4.5	3.8	42	17	15	–	3.3	–	–	–	–	–	–	–
13	60	F	19.1	not tested	not tested	4.8	3.8	4.8	77	13	11	–	6.0	–	–	–	–	–	–	–
14	47	M	24.2	2	2	4.8	5.2	4.3	61	41	47	–	6.3	–	–	–	–	–	–	–
15	58	F	21.2	2	2	4.8	5.2	5.2	38	10	14	–	7.0	–	–	–	3.7	3.9	–	–
16	60	M	24.0	3	2	5.1	5.8	5.4	119	26	24	–	4.6	–	3.0	2.7	3.1	3.0	–	–
17	41	F	18.9	2	2	5.3	4.8	5.9	39	7	13	+	7.6	–	–	–	–	–	–	–
18	44	M	20.9	not tested	not tested	5.5	6.8	5.8	23	11	17	–	5.6	–	–	–	–	–	–	–
19	33	M	21.3	2	2	6.1	5.0	4.8	73	41	41	+	7.2	2.7	3.3	–	–	–	–	–
20	51	M	23.6	3	2	6.1	6.8	6.1	54	29	27	–	6.1	–	3.6	3.0	3.8	3.2	–	+ (2)
21	43	F	18.2	not tested	not tested	6.6	6.8	7.5	73	32	27	–	6.5	–	–	–	–	–	–	–
22	62	M	23.6	4	2	8.3	10.5	10.1	33	28	26	–	3.8	–	–	–	–	–	–	–
23	32	M	30.3	3	2	17.1	14.0	13.9	118	87	68	–	6.1	–	–	–	–	3.3	–	–
					median	6.1	6.7	5.9												

FS-3, 4 and 5, FibroScan values at 3, 4 and 5 years after the start of nucleoside analog treatment, respectively.
M, male; F, female; BMI, body mass index; F-stage, fibrosis stage; A-grade, activity grade; ALT, alanine aminotransferase; BTH, breakthrough hepatitis; HCC, hepatocellular carcinoma.
ALT-0, ALT level at baseline; ALT-3 and 5, ALT levels at 3 and 5 years after the start of nucleoside analog treatment, respectively.
DNA-0, HBV DNA level at baseline; DNA-1, 2, 3, 4 and 5, HBV DNA levels at 1, 2, 3, 4 and 5 years after the start of nucleoside analog, respectively.
Figure in parenthesis was the onset of BTH or HCC after the start of nucleoside analog treatment (unit: year).

Table 6 Longitudinal assessment of FibroScan values and biochemical parameters after the start of nucleoside analog treatment (Study 2)

	FibroScan (kPa)	*P*-value	ALT (IU/L)	*P*-value	PT (%)	*P*-value	AFP (ng/mL)	*P*-value	IV-C (ng/mL)	*P*-value
Group A										
All patients ($n = 22$)										
Baseline	8.2 (4.2–28.5)		54.6 ± 30.4		88.1 ± 11.2		5.0 ± 3.0		172 ± 58	
FS-1	6.4 (4.0–24.0)	<0.0001*	25.4 ± 14.8	0.0002*						
FS-2	5.8 (3.8–21.2)	<0.0001**	22.3 ± 8.3	0.3211**						
FS-3	5.3 (2.5–18.0)	0.0064***	22.1 ± 7.7	0.8494***	96.3 ± 9.1	<0.0001	3.0 ± 1.6	0.0004	142 ± 37	0.0023
Group B										
All patients ($n = 23$)										
Baseline	not tested		56.1 ± 28.3		80.2 ± 9.9		5.5 ± 4.0		not tested	
FS-3	6.1 (3.2–20.5)		25.2 ± 16.8	0.0001*	91.1 ± 10.8	0.0001*	3.1 ± 2.1	0.0004*	154 ± 51	
FS-4	6.7 (3.5–23.3)	0.7439#								
FS-5	5.9 (3.0–21.8)	0.6785##	24.2 ± 13.8	0.3530#	92.7 ± 8.7	0.2459#	3.3 ± 3.1	0.5401#	148 ± 48	0.1884#

*, compared to Baseline; **, ***, compared to FS-1; #, compared to FS-2; ##, compared to FS-3; ###, compared to FS-4.
Data are shown by the mean ± standard deviation or median (range).
FS-1, 2, 3, 4 and 5, FibroScan values at 1, 2, 3, 4 and 5 years after the start of nucleoside analog treatment, respectively.
ALT, alanine aminotransferase; PT, prothrombin time; AFP, α-fetoprotein; IV-C, type IV collagen.

hepatitis B. Recent reports showed that 12 months ETV treatment for chronic hepatitis B is associated with an improvement of FibroScan values.[23,24] The results suggested that a decrease in FibroScan values during the first year of NA treatment might be attributed to not only improvement of liver fibrosis but also to necroinflammation. We were able to investigate the association between the efficacy of NA treatment and FibroScan values for a much longer period than the previous reports. Suzuki *et al.*[25] found that a 3-year LMV therapy could induce histological improvements whether YMDD mutants accompanied by virological breakthrough and BTH appeared. Our findings also showed that liver stiffness markedly improved during the first 3 years of NA treatment, but that the degree of improvement was less after 3 years of NA treatment, irrespective of the long term virological effect.

First, we investigated the association between liver fibrosis by liver biopsy and FibroScan values. Liver biopsy is not always acceptable to patients and biochemical liver examinations are sometimes unreliable for determining the extent of liver fibrosis because patients who have advanced liver fibrosis may have normal ALT levels. We showed that the FibroScan values were significantly correlated with fibrosis stage ($r = 0.672$, $P < 0.0001$). However, the cutoff for chronic hepatitis B (F3 8.9 kPa, F4 12.0 kPa) was lower than that of chronic hepatitis C (F3 10.3 kPa, F4 14.9 kPa)[16] especially for predicting severe fibrosis and cirrhosis. Macronodular cirrhosis, characterized by large nodules delimited by thin septa, is commonly found in patients with HBV infection. A different type and/or extent of liver inflammatory infiltrate within liver blocks, characterized by interface and/or lobular hepatitis infected with HBV and by the portal tract lymphoid aggregate near or surrounding bile ducts infected with HCV, might account for the difference of cutoff values between chronic hepatitis B and C patients.

Usually, complete long-term suppression of HBV DNA is an essential goal of treatment for chronic hepatitis B. Liver fibrosis is potentially reversible after viral replication has subsided,[26] therefore, the longitudinal assessment of liver fibrosis treated with NA is very important. We previously showed a dramatic reduction of FibroScan values for both virological and biochemical response by chronic hepatitis C patients treated with pegylated interferon alpha-2b and ribavirin treatment.[16] In this study of chronic hepatitis B patients who underwent NA treatment, the FibroScan values showed a rapid decline without BTH in the first 3 years. In the treatment of chronic hepatitis C, sustained virological clearance is

an ultimate aim. However, even successful response to NA by chronic hepatitis B patients does not bring about clearance. In fact, our data showed that in both the BTH and non-BTH groups, FibroScan values did not significantly improve over the 3 years after the start of NA treatment.

Previous reports have shown a well-marked association between elevated HBV DNA and progression to cirrhosis and HCC.[27] Even when ALT levels were normal, HBeAg negative patients who had HBV DNA over 4.0 log copies/mL had an increased risk of developing liver cirrhosis or HCC. Although HBV genotypes also influence the pathological features of patients, the virologic and molecular mechanisms involved remain largely not understood. Epidemiologic studies have shown that each genotype has a distinct geographic and ethnic distribution.[28,29] HBV genotypes A and D occur frequently in Africa, Europe and India, while genotypes B and C are prevalent in Asia. In general, genotype C has been shown to be associated with more progressive hepatitis than genotype B.[30] Recently, the intracellular expression of HBV DNA and hepatitis B core protein were shown to be higher for genotypes B and C than for genotypes A and D in an experimental study.[31] The intracellular accumulation of HBV DNA and antigens may play a role in liver cell damage, and moreover, the higher replication capacity of genotype C may explain the association with more severe histological liver damage than is seen for other genotypes and continued higher FibroScan values than in the case of long term HCV infection, irrespective of well-controlled antiviral treatment.

The introduction of LMV has resulted in improved suppression of HBV replication, improving histological necroinflammation and fibrosis. However, LMV-resistant HBV (the emergence of YMDD mutation) has appeared with prolonged LMV treatment, which can lead to viral or biochemical breakthrough. Some patients with hepatic cirrhosis suffer severe deterioration after BTH. Among patients who received LMV and maintained an ALT level of under 40 IU/L, the rate of YMDD motif mutant and BTH occurrences were 11%, 3% (1 year), 42%, 13% (3 years) and 61%, 19% (5 years).[32] The rate of YMDD motif mutant and BTH were low after three or more years of LMV treatment. In our data, once a BTH was experienced, FibroScan values were less likely to show improvement, which may indicate the emergence of HCC. Development of HBV resistance to LMV is typically indicated by an increase in HBV DNA followed by an increase in serum ALT level. With the advent of ETV, the frequency of viral breakthrough has been dramatically reduced;[33] however, the viral kinetics must be carefully determined.

In an analysis of the relationship between FibroScan values and the development of HCC, higher FibroScan values were shown to be associated with the development of HCC in a large prospective study of patients with chronic liver disease.[34] FibroScan values of over 10 kPa were associated with a significantly increased risk of subsequent HCC development and mortality for both chronic hepatitis B and C, irrespective of virological response owing to antiviral treatment.[35,36] Foucher *et al.*[34] suggested that the cutoff value established with a negative predictive value of greater than 90% was 53.7 kPa for HCC in patients with chronic liver diseases. Masuzaki *et al.*[35] reported that patients with chronic hepatitis C with higher FibroScan values had a significantly higher risk of HCC, with a hazard risk of 16.7 with 10.1–15 kPa, 20.9 with 15.1–20 kPa, 25.6 with 20.1–25 kPa and 45.5 with over 25 kPa, as compared to under 10 kPa. In the present study, 6 (13.3%) of 45 patients developed HCC. For those who underwent NA treatment and achieved virological response; however, the FibroScan values of most (66.7%) were consistently over 10 kPa during the follow-up period. Therefore, patients with a high FibroScan value need careful attention to prevent the development of HCC, even after achieving virological response by NA treatment.

Some limitations of FibroScan must be taken into account, such as whether or not the results are reliable. FibroScan values may be influenced by ALT flares, with a risk of overestimating liver stiffness because ALT flares reflect liver cell inflammation, edema and swelling.[36] In fact, FibroScan values of our patients transiently increased with the onset of BTH, thus FibroScan was done only after ALT level had returned to normal owing to the addition of ADV. Although the machine provides no feedback when FibroScan measurement is unsuccessful, Castéra *et al.*[37] recommended that successful measurements be validated by use of the following criteria: success rate of under 60%, IQR/M of more than 30% of median values, obesity, particularly BMI greater than 30 kg/m^2 and limited operator experience (fewer than 500 examinations) are the main determinants of unreliable FibroScan measurement. Another limitation is the small size of the study population; therefore, the clinical correlations with BTH or HCC development cannot be made appropriately.

In conclusion, transient elastography is a useful tool for the evaluation of liver stiffness and follow-up assessment of NA treatment of patients with chronic HBV infection. Liver stiffness, measured by transient elastog-

raphy, of chronic hepatitis B patients treated with NA showed a rapid decline in the first 3 years followed by a more steady transition from years three to five irrespective of the long term virological effect.

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ORIGINAL ARTICLE

Pegylated interferon α-2b plus ribavirin for older patients with chronic hepatitis C

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Abstract

AIM: To analyze the efficacy and safety of a combination therapy of pegylated interferon (PEG-IFN) α-2b plus ribavirin (RBV) in older Japanese patients (65 years or older) infected with hepatitis C virus (HCV).

METHODS: This multicenter study included 938 patients with HCV genotype 1 who received 1.5 μg/kg per week PEG-IFN α-2b plus RBV 600-1000 mg/d for 48 wk and 313 HCV genotype 2 patients who received this treatment for 24 wk.

RESULTS: At 24 wk after the end of combination therapy, the overall sustained virological response (SVR) for genotypes 1 and 2 were 40.7% and 79.6%, respectively. The SVR rate decreased significantly with age in each genotype, and was markedly reduced in genotype 1 ($P < 0.001$). Moreover, the SVR was significantly higher in patients with genotype 1 who were less than 65 years (47.3% of 685) than in those 65 years or older (22.9% of 253) ($P < 0.001$) and was higher in patients with genotype 2 who were less than 65 years (82.9% of 252) than in those 65 years or older (65.6% of 61) ($P = 0.004$). When patients received a dosage at least 80% or more of the target dosage of PEG-IFN α-2b and 60% or more of the target dosage of RBV, the SVR rate significantly increased to 66.5% in patients less than 65 years and to 45.2% in those 65 years or older ($P <$

0.001). Adverse effects resulted in treatment discontinuation more often in patients with genotype 1 (14.4%) than in patients with genotype 2 (7.3%), especially by patients 65 years or older (24.1%).

CONCLUSION: PEG-IFN α-2b plus RBV treatment was effective in chronic hepatitis C patients 65 years or older who completed treatment with at least the minimum acceptable treatment dosage.

Key words: Hepatitis C virus; Gerontology; Pegylated interferon; Ribavirin

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INTRODUCTION

Hepatitis C virus (HCV) infection is a major cause of chronic liver disease, affecting 170 million individuals worldwide[1]. It is well known that patients with chronic hepatitis C eventually develop hepatocellular carcinoma (HCC)[2]. Previous studies have made clear that interferon (IFN) treatment is effective for eliminating HCV[3,4] and that it significantly reduces the progression of liver fibrosis and the risk of HCC[5,6]. Antiviral treatment for chronic hepatitis C has greatly improved, and the combination treatment of pegylated (PEG)-IFN α-2b plus ribavirin (RBV) has been approved and recommended in Japan since 2004, as the first choice for chronic hepatitis C. This combination treatment attained a sustained virological response (SVR) rate of 50%-60% for genotype 1 in the United States and Europe[7]. However, SVR was relatively low (42.4%) in Japan[8], where chronic hepatitis C patients are older, indicating that older patients did not respond well to IFN treatment[9]. Moreover, the combination treatment was associated with more adverse effects than IFN monotherapy[7,10]. Older patients who have decreased cardiovascular, pulmonary and renal function have a higher incidence of adverse effects than younger patients. The rate of discontinuation due to adverse effects was reported to be significantly higher in patients aged 65 years or more than in those less than 65 years[11]. Older patients with HCV infection are at risk for progressive liver disease. It was reported that clearance of HCV after IFN therapy significantly reduces the incidence of HCC and death in older chronic hepatitis C patients[6,12]. Ikeda *et al*[13] demonstrated that IFN treatment is needed for 65-70-year-old patients with chronic hepatitis C to prevent the occurrence of HCC. We also consider older patients to be acceptable candidates for antiviral treatment to prevent the development of HCC, and previously reported that monotherapy with natural IFN α was not effective in older patients[9]. Therefore, in an attempt to ameliorate these problems, we decided to treat older patients with a combination of PEG-IFN plus RBV therapy.

Little data concerning the response and safety of this combination treatment in a large number of older patients with chronic HCV infection has been published. A multicenter study of the efficacy and safety of antiviral treatments for Japanese patients with chronic liver disease, the Kyushu University Liver Disease Study (KULDS), was launched in 2003[8,14]. The present prospective study was carried out to analyze the efficacy and safety of the combination treatment of PEG-IFN α-2b plus RBV in older patients.

MATERIALS AND METHODS

Patients

Treatment of chronic hepatitis C with a combination of PEG-IFN α-2b plus RBV was accepted by the Japanese Ministry of Health in October, 2004. We used this combination treatment from December 2004 to July 2008, and enrolled chronic hepatitis C patients with exclusion criteria which included: (1) clinical or biochemical evidence of hepatic decompensation, advanced cirrhosis identified by bleeding, high-risk esophageal varices, history of gastrointestinal bleeding, ascites, encephalopathy, or HCC; (2) hemoglobin level < 11.5 g/L, white blood cell count < 3×10^9/L, and platelet count < 50×10^9/L; (3) concomitant liver disease other than hepatitis C (hepatitis B surface antigen positive or HIV positive); (4) excessive active alcohol consumption > 60 g/d or drug abuse; (5) severe psychiatric disease; or (6) antiviral or corticosteroid treatment within 12 mo prior to enrollment. Patients who fulfilled the above criteria were recruited at Kyushu University Hospital and 32 affiliated hospitals in the northern Kyushu area of Japan. We have treated 2270 Japanese patients aged 18 years or older with PEG-IFN α-2b plus RBV. All patients who were positive for both antibody to HCV and HCV RNA for over 6 mo were enrolled in KULDS. Three months before the start of treatment and every 3 mo during the treatment period, each patient was tested for α-fetoprotein (AFP) and had an abdominal ultrasonographic examination. If an abnormal AFP level of 40 ng/mL and/or focal lesions on ultrasonographic examination were found at any testing, further testing for HCC was carried out, which included dynamic computed tomography, and angiography. Patients confirmed to have HCC within 3 mo after starting treatment were excluded from this study (n = 14). Of 2270 patients, 1021 were currently under combination treatment or we were not yet able to judge the effect of the combination treatment. This left the data of 1251 patients (938 with genotype 1 and 313 with genotype 2) available for analysis.