Statistical analysis

Non-parametric procedures were employed for the analysis of background characteristics and laboratory data among patients in each stage, including Mann–Whitney *U*-test, Kruskal–Wallis test and χ^2-test.

The normality of the distribution of the data was evaluated by Kolmogorov–Smirnov one-sample test. Because certain variables partly did not conform to a normal distribution, natural logarithmic transformation of bilirubin, AST, ALT, GGT, α2-macroglobulin, hyaluronic acid, type IV collagen 7S and TIMP-2 were also analyzed in the following calculation. The natural logarithmic transformation of the results yielded a normal distribution or symmetrical distribution for all the analyzed factors. After the procedures, the following multiple regression analysis became rationally robust against deviations from normal distribution. In order to avoid introducing into the model any variables that were mutually correlated, we checked the interaction between all pairs of the variables by calculating variance of inflation factors. Of the highly correlated variables, less significant factors were removed from the viewpoint of multicollinearity.

Multivariate regression analysis was performed using 305 patient data from Toranomon Hospital (training dataset), to generate training data of predicting function. We used a stepwise method for selection of informative subsets of explanatory variables in the model. Multiple regression coefficient and coefficient of determination are also taken into account in the selection of variables. Next, we validated the obtained predictive function using the remaining 276 patient data from the other seven liver institutions (validation dataset).

A *P*-value of less than 0.05 with two-tailed test was considered to be significant. Data analysis was performed using the computer program SPSS version 19.[21]

For evaluation of the efficiency and usefulness of obtained function for estimation of fibrosis, we compared various fibrotic scores for hepatitis C, including AAR,[8] AST-to-platelet ratio index (APRI),[12] FIB-4[13] and FibroTest.[9]

RESULTS

Pathological diagnosis

FOUR PATHOLOGISTS INDEPENDENTLY judged the fibrotic stages and inflammatory activity for 581 specimens of chronic hepatitis/cirrhosis caused by HCV. A total of 328 patients (56.5%) had a fibrotic stage of F1, 153 (26.3%) F2, 73 (12.6%) F3 and 27 (4.6%) F4. In the training subgroup ($n = 305$), judgment of F1 was made in 172, F2 in 80, F3 in 37 and F4 in 16. In the validation group ($n = 276$), judgment as F1 was made in 156, F2 in 73, F3 in 36 and F4 in 11.

According to hepatitis activity classification, A0 was found in nine patients (1.52%), A1 in 350 (60.2%), A2 in 198 (34.1%) and A3 in 24 (4.1%).

Laboratory data of each hepatitis stage in training group

There were 161 males and 144 females with a median age of 54 years (range, 22–69). Laboratory data of the 305 patients in the training group are shown in Table 1. Although several individual items were well correlated with the severity of hepatic fibrosis, significant overlap values were noted among F1 to F4 stages: platelet count, GGT, γ-globulin, hyaluronic acid and type IV collagen 7S.

Regression function generated from training patient group

After stepwise variable selection, multivariate regression analysis finally obtained the following function: $z = 2.89 \times ln$ (type IV collagen 7S) (ng/mL) $- 0.011 \times$ (platelet count) ($\times 10^3$/mm^3) $+ 0.79 \times ln$ (total bilirubin) (ng/mL) $+ 0.39 \times ln$ (hyaluronic acid) (μm/L) $-$ 1.87. Median values of the fibrotic score of F1 ($n = 172$), F2 ($n = 80$), F3 ($n = 37$) and F4 stages ($n = 16$) were calculated as 1.00, 1.45, 2.82 and 3.83, respectively (Fig. 1). The multiple regression coefficient and coefficient of determination were 0.56 and 0.32, respectively.

A 55-year-old man with F1 fibrotic stage (Fig. 2a) showed serum type IV collagen concentration as 3.8 ng/mL, platelet as 152×10^3 count/mm^3, total bilirubin as 0.8 mg/dL and hyaluronic acid as 16 μg/L. The regression function provided his fibrotic score as 1.16. Another man aged 43 years had F3 fibrosis with severe hepatitis activity of A3 on histological examination (Fig. 2b). His type IV collagen was 11.0 ng/mL, platelet 162×10^3 count/mm^3, total bilirubin 0.7 mg/dL and hyaluronic acid 189 μg/L, and regression function calculated his fibrotic score as 4.98.

Validation of discriminant function

Validation data of 276 patients (Table 2) were collected from the other seven institutions in Japan. When applying the regression function for the validation set, the fibrotic score for hepatitis C (FSC) demonstrated good reproducibility, showing 1.10 in patients with chronic hepatitis of F1 ($n = 156$), 2.35 in F2 ($n = 73$), 3.16 in F3 ($n = 36$) and 3.58 in F4 ($n = 11$) (Fig. 3). Although F4

Table 1 Demography and laboratory data of 305 patients in training group

	F1 ($n = 172$)	F2 ($n = 80$)	F3 ($n = 37$)	F4 ($n = 16$)
Demography				
Males : females	97:75	38:42	20:17	6:10
Age (median, range)	51 (22–69)	55 (29–68)	55 (27–69)	56.5 (29–65)
Laboratory data (median, range)				
WBC ($\times 10^3/mm^3$)	4.7 (2.0–10.1)	4.3 (2.3–8.5)	4.5 (2.9–6.8)	4.7 (3.3–6.9)
Hemoglobin(g/dL)	14.6 (11.0–18.2)	14.4 (9.3–17.4)	14.6 (11.5–17.7)	14.55 (12.1–16.5)
Platelet ($\times 10^3/mm^3$)	183 (52–364)	161 (82–387)	131 (74–237)	124 (7.7–191)
Albumin (g/dL)	4.1 (2.3–4.9)	4.0 (3.5–4.6)	3.9 (3.1–4.6)	3.8 (3.3–4.3)
Bilirubin (mg/dL)	0.8 (0.2–1.9)	0.7 (0.3–1.7)	0.9 (0.4–7.5)	0.8 (0.5–7.4)
AST (IU/L)	42 (16–386)	61 (16–332)	63 (13–238)	71 (30–160)
ALT (IU/L)	60.5 (12–1664)	84.5 (10–647)	108 (27–415)	90.5 (36–264)
γ-GTP (IU/L)	40 (7–383)	48 (10–262)	54 (13–209)	58 (21–195)
γ-Globulin (g/dL)	1.47 (0.58–3.40)	1.61 (1.02–2.41)	1.69 (0.66–2.64)	1.79 (1.22–2.73)
γ-Globulin (%)	19.4 (10.0–40.5)	20.9 (14.0–28.3)	21.3 (8.1–30.4)	22.7 (16.5–36.9)
α2-Macroglobulin (mg/dL)	269 (123–505)	335 (154–551)	369 (183–627)	317 (207–511)
Haptoglobin (mg/dL)	94.5 (<5–265)	75.5 (<5–263)	56 (<5–2031)	75 (30–142)
Apolipoprotein A1 (mg/dL)	132 (71–209)	131 (73–207)	124 (98–166)	121 (83–153)
Hyaluronic acid (μg/L)	25 (<5–407)	41.5 (<5–263)	71 (<5–326)	89.5 (5–246)
TIMP-1 (ng/mL)	165 (73–291)	173 (97–302)	182 (126–308)	192.5 (128–260)
TIMP-2 (ng/mL)	77.5 (31–210)	80 (34–307)	76 (46–143)	78 (58–110)
Procollagen III peptide (U/mL)	0.75 (0.47–1.50)	0.805 (0.61–1.70)	0.86 (0.53–1.50)	1.05 (0.66–1.60)
Type IV collagen 7S (ng/mL)	4.0 (1.7–73)	4.3 (2.1–11.0)	5.2 (3.2–11.0)	5.8 (4.3–9.4)

γ-GTP, γ-glutamyl transpeptidase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; TIMP, tissue inhibitor of matrix metalloproteinase; WBC, white blood cell.

fibrotic stage consisted of only 11 patients and the score 3.58 was regarded as a rather low value, the scores of other stages of fibrosis were concordant with histological fibrosis.

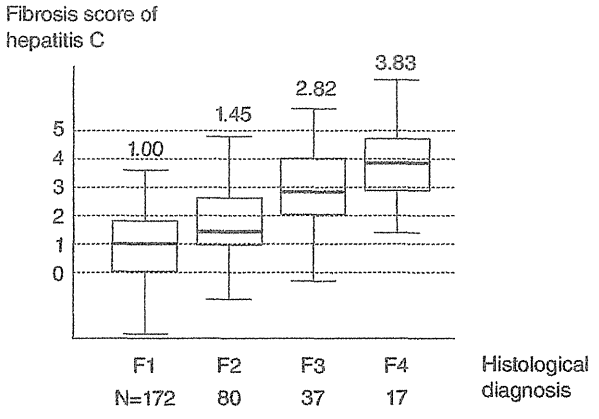

Figure 1 Box and whisker plots of fibrotic score of each group of histological fibrosis in the training dataset. Fibrotic score of hepatitis C (FSC) was generated by the function, $z = 2.89 \times ln$ (type IV collagen 7S) (ng/mL) $- 0.011 \times$ (platelet count) ($\times 10^3/mm^3$) $+ 0.79 \times ln$ (total bilirubin) (mg/dL) $+ 0.39 \times ln$ (hyaluronic acid) (μg/L) $- 1.87$.

Comparisons of efficacy with various fibrotic scores (Fig. 4)

In order to evaluate the efficacy and usefulness of the obtained FSC, we compared with previously reported fibrotic scores using training data. AAR, APRI, FIB-4 and FibroTest showed only slight correlation with actual histological stage. APRI and FIB-4 demonstrated increasing trends of the score associated with histological fibrosis, but significant overlapping scores were found through F1 to F4. Spearman's correlation coefficients of AAR, APRI, FIB-4 and FibroTest were 0.021 ($P = 0.707$), 0.462 ($P < 0.001$), 0.440 ($P < 0.001$) and 0.415 ($P < 0.001$), respectively. Our FSC showed Spearman's correlation coefficient of 0.572 ($P < 0.001$), and was of much higher value than the others.

DISCUSSION

RECOGNITION OF SEVERITY of chronic hepatitis is essential in managing patients with chronic HCV infection: estimation of length of infection, existence of any previous hepatitis activity, presumption of current fibrotic stage, and prediction of future fibrotic progression and hepatocarcinogenesis. Differential diagnosis of cirrhosis from chronic hepatitis is especially important

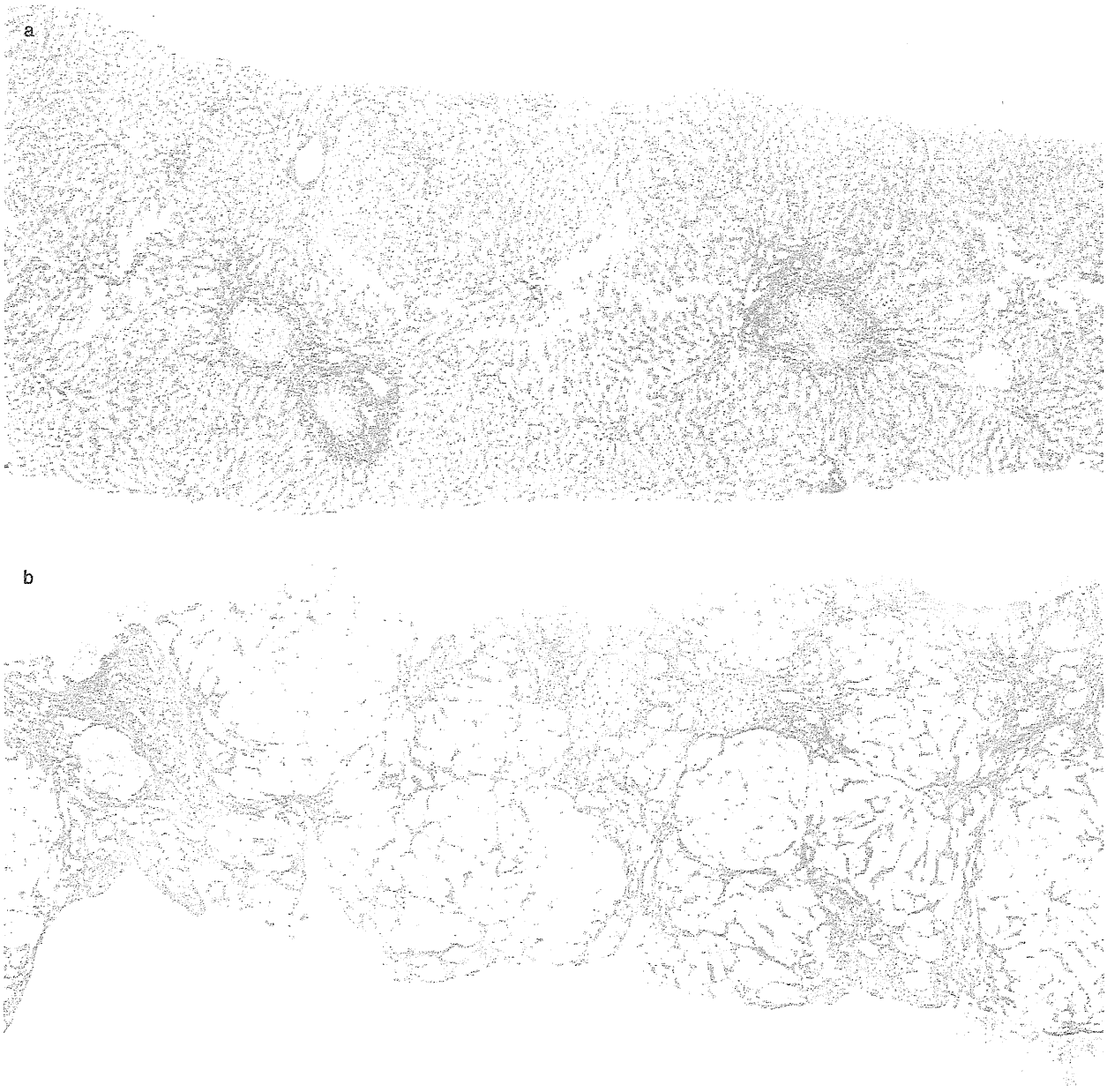

Figure 2 Case presentations of the training set. (a) A 55-year-old man with F1 fibrosis. Final regression function provided his fibrotic score as 1.16. (b) A 43-year-old man with F3 fibrosis with severe hepatitis activity. His regression coefficient was calculated as 4.98 (silver stain, ×40).

in the evaluation of chronic HCV infection. Identification of liver cirrhosis often leads to an important change in management of the patients: needs for fiberscopic examination for esophageal varices, ultrasonographic exploration for the association of liver cancer, and prediction of hepatic decompensation.

Recently, non-invasive estimation of severity of liver fibrosis has been reported in patients with HCV-related chronic hepatitis.[6–14] However, these studies were principally aimed at differentiation of advanced fibrotic stages of F3 or F4 from mild fibrotic stages of F1 or F2. Those discriminative functions were insufficient to

Table 2 Demography and laboratory data of 276 patients in validation group

	F1 ($n = 156$)	F2 ($n = 73$)	F3 ($n = 36$)	F4 ($n = 11$)
Demography				
Males : females	83:73	42:31	13:23	6:5
Age (median, range)	55 (15–74)	58 (32–77)	62.5 (30–78)	51 (38–73)
Laboratory data (median, range)				
WBC ($\times 10^3/mm^3$)	5.1 (2.1–10.5)	4.8 (2.6–9.0)	4.85 (2.3–14.2)	3.9 (3.2–6.0)
Hemoglobin (g/dL)	14.2 (8.9–17.7)	14.4 (11.8–17.4)	14.1 (10.1–16.4)	13.6 (8.9–16.3)
Platelet ($\times 10^3/mm^3$)	183 (59–440)	153 (80–265)	136 (64–348)	135 (79–153)
Albumin (g/dL)	4.3 (3.1–5.3)	4.3 (3.3–5.2)	4.05 (3.0–5.5)	3.9 (3.0–4.7)
Bilirubin (mg/dL)	0.7 (0.2–8.7)	0.7 (0.2–1.7)	0.8 (0.2–2.5)	0.8 (0.4–11.0)
AST (IU/L)	35 (11–1390)	49 (19–183)	80 (20–190)	96 (29–257)
ALT (IU/L)	49 (11–1635)	62 (12–575)	84 (14–218)	115 (29–303)
γ-GTP (IU/L)	35 (11–600)	52 (10–497)	51 (14–236)	112 (17–312)
γ-Globulin (g/dL)	1.47 (0.70–2.14)	1.60 (0.80–2.37)	1.71 (0.63–2.62)	2.19 (1.70–2.82)
γ-Globulin (%)	19.5 (9.2–26.4)	20.8 (10.8–30.8)	22.4 (9.5–29.9)	27.4 (21.8–35.3)
α2-Macroglobulin (mg/dL)	271.5 (126–572)	381 (172–573)	405.5 (196–594)	468 (242–655)
Haptoglobin (mg/dL)	95 (<5–305)	80 (<5–223)	63.5 (<5–192)	65 (<5–130)
Apolipoprotein A1 (mg/dL)	126 (45–198)	127 (63–191)	116 (46–172)	108 (62–171)
Hyaluronic acid (μg/L)	37.5 (<5–1260)	68 (5–1000)	140.5 (23–2610)	159 (33–364)
TIMP-1 (ng/mL)	157.5 (77–301)	172 (89–355)	188.5 (99–430)	192 (112–320)
TIMP-2 (ng/mL)	70 (21–294)	73 (21–207)	89 (27–280)	76 (36–120)
Procollagen III peptide (U/mL)	0.73 (0.52–8.30)	0.81 (0.53–1.60)	1.00 (0.63–1.90)	1.00 (0.68–1.60)
Type IV collagen 7S (ng/mL)	3.9 (1.2–12.0)	4.5 (2.3–9.9)	5.8 (2.8–16.0)	6.1 (4.6–10.0)

γ-GTP, γ-glutamyl transpeptidase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; TIMP, tissue inhibitor of matrix metalloproteinase; WBC, white blood cell.

recognize the stepwise progression of viral hepatitis from F1 through F4. This dichotomy (mild or severe) of chronic hepatitis C seemed less valuable in the study of disease progression, disease control abilities of antiviral drugs and estimation of histological improvement after anti-inflammatory drugs. A histology-oriented, practical and reliable formula is therefore required for the diagnosis and investigation of chronic hepatitis C.

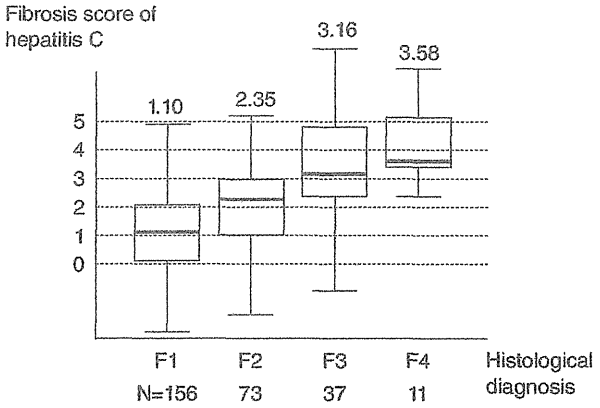

Figure 3 Box and whisker plots of fibrotic score of each group of histological fibrosis in the validation dataset. Fibrotic score of hepatitis C (FSC) was generated by the function, $z = 2.89 \times ln$ (type IV collagen 7S) (ng/mL) $- 0.011 \times$ (platelet count) ($\times 10^3/mm^3$) $+ 0.79 \times ln$ (total bilirubin) (ng/mL) $+ 0.39 \times ln$ (hyaluronic acid) (μg/L) $- 1.87$.

This study was aimed to establish non-invasive evaluation and calculation of liver fibrosis for patients with chronic HCV infection. Although it was retrospectively performed as a multicenter study of eight institutions, judgment of histological diagnosis was independently performed by four pathologists in the other hospital, informed of nothing except for the patient's age, sex and positive HCV infection. Objective judgment of the histological staging and grading in sufficient biopsy specimens could be obtained.

As many as 581 patients with chronic hepatitis C were analyzed in this study, who had been diagnosed as having chronic hepatitis or cirrhosis by liver biopsy performed in experienced liver units in Japan. To obtain the most suitable equation approximating histological fibrotic stage, multivariate analysis was performed using two demographic parameters (age and sex) and 21 hematological and biochemical markers with or without logarithmic transformation. They included many kinds of fibrotic markers: α2-macroglobulin, haptoglobin concentration, haptoglobin typing, apolipo-

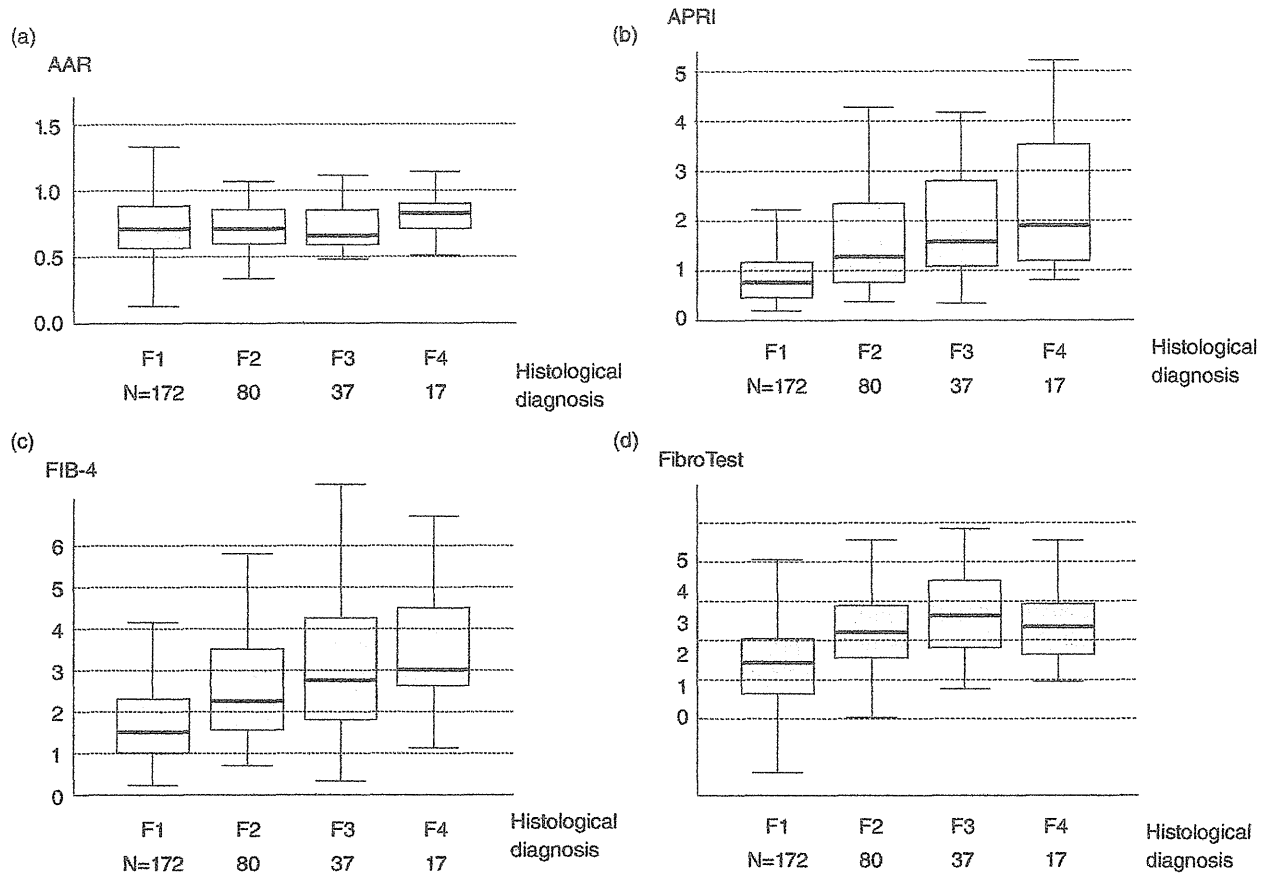

Figure 4 Previously published fibrotic scores: (a) aspartate aminotransferase (AST)/alanine aminotransferase (ALT) ratio (AAR),[8] (b) AST-to-platelet ratio index (APRI), calculated by AST / (upper limit of normal of AST) / (platelet count [$\times 10^9$/L]) $\times$ 100.[12] (c) FIB-4 score, calculated by age $\times$ AST [IU/L] / (platelet count [$\times 10^9$/L] $\times$ ALT [IU/L]$^{0.5}$).[13] (d) FibroTest score regression coefficient was: $Z = 4.467 \times \log^{10}$ (α2-macroglobulin [g/L]) $- 1.357 \times \log^{10}$ (haptoglobin [g/L]) $+ 1.017 \times \log^{10}$ [γ-glutamyltransferase [GGT] [IU/L]) $+ 0.0281 \times$ (age [years]) $+ 1.737 \times \log^{10}$ (bilirubin [μm/L]) $- 1.184 \times \log^{10}$ (apolipoprotein A1 [g/L]) $+ 0.301 \times$ (sex [female = 0, male = 1]) $- 5.54$.[9]

protein A1, hyaluronic acid, TIMP-1, TIMP-2, procollagen III peptide and type IV collagen 7S. Multiple regression analysis finally generated a first-degree polynomial function consisting of four variables: type IV collagen 7S, platelet count, bilirubin and hyaluronic acid. A constant numeral (−1.87) was finally adjusted in the regression equation in order to obtain fitted figures for fibrotic stages of F1, F2, F3 and F4. From the magnitude of the standardized partial regression coefficient of individual variable in the function, *ln* (type IV collagen 7S) demonstrated the most potent contribution toward the prediction of liver fibrosis. Platelet count and *ln* (bilirubin) proved to be the second and third distinctive power in the model, respectively.

The obtained figure of FSC was generated to imitate actual "F factor" of histological staging. FSC was sufficiently fitted to actual fibrotic stages with certain overlapping as was usually found in histological ambiguity judged by pathologists. Because judgment of fibrosis in chronic hepatitis often shows a transitional histological staging, pathological examination could not always achieve a clear-cut diagnosis discriminating F1, F2, F3 or F4. Considering the limitation of pathological difficulty in differentiation of the four continuous disease entities, the obtained regression function showed satisfactory high accuracy rates in the prediction of liver disease severity. FSC can provide one or two decimal places (e.g. 2.4 or 2.46) and the utility of the score is possibly higher

than mere histological staging of F1, F2, F3 or F4. The reproducibility was confirmed by the remaining 276 patients' data obtained from the other seven hospitals. Although the validation data were collected from different geographic area and different chronologic situation, FSC showed similar results in prediction of histological staging.

Fibrotic score for hepatitis C seemed a very useful quantitative marker in evaluating severity of fibrotic severity of hepatitis C patients without invasive procedures and without any specialized ultrasonography or magnetic resonance imaging. FSC also has an advantage of measurement, in which old blood samples are available for retrospective assessment of varied clinical settings: old sera from 20 years ago at the time of initial liver biopsy, or paired sera before and after a long-term anti-inflammatory therapy, for example. These kinds of retrospective assessments of fibrotic staging will be valuable in estimating a long-term progression of liver disease, in evaluating efficacy of a long-term medication or other medical intervention, or in making a political judgment from the viewpoint of socioeconomic efficacy.

The score can be calculated for any patients with chronic HCV infection. Although this multiple regression model dealt with appropriate logarithmic transformation for non-normal distribution parameters, the regression analysis was based on a linear regression model. Very slight fibrosis can be calculated as less than 1.00, which is commonly found with a slight degree of chronic hepatitis with a tiny fibrotic change as F0. Very severe fibrosis may be calculated as more than 4.00, which is an imaginable and nonsense number in the scoring system of fibrosis. FSC is, however, very useful and valuable in real clinical setting. Estimation of severity of liver fibrosis in outpatient clinics, evaluation of natural progression of patients' fibrosis over 10 years, and assessment of a long-term administration of interferon in patients with chronic hepatitis C from the viewpoint of fibrotic change. In this study, because certain patients actually had a history of interferon administration, regression of liver fibrosis during and after the treatment could be assessed when prior sera were available for serial evaluation of FSC. We can also expect the usefulness of evaluation of carcinogenic risk after sustained virological response, and stage progression with alcohol intake or obesity-induced steatosis. Recent development of new directly acting antiviral agents require evaluation for long-term histological advantage, for aggravation of hepatitis stage during viral and biochemical breakthrough caused by HCV mutation, estimation of future carcinogenic risk, and even for the best way of management of patients with chronic hepatitis C. FSC seems one of the ideal methods of approximation for fibrotic stage of chronic hepatitis C. Repeated measurement is quite suitable for patients with an unestablished treatment or trial, every 1 or 2 years, for example. Because the current regression function was generated from the data of HCV-related chronic liver disease, this equation would not be suitable for the recognition of HBV-related chronic liver disease,[22] alcoholic liver disease and other congenital or autoimmune liver diseases. To recognize the latter diseases, other studies about individual diseases must be performed.

We compared the usefulness of the FSC with that of other fibrotic scores.[8,9,12,13] More simple and inexpensive AAR or APRI could not well estimate fibrotic stages with poor correlation coefficients of 0.021 and 0.462, which were much lower than the coefficient of FSC of 0.572. FibroTest, which contained three costly fibrotic markers (α2-macroglobulin, haptoglobin and apolipoprotein A1), also showed a low correlation coefficient of 0.415, suggesting that the usefulness was limited in HCV positive Asian patients. Although FIB-4 demonstrated the best coefficient of 0.440 among the fibrotic scores, significant overlaps were found between neighboring stages and obtained scores were not coordinated for rea17l histological classification. Because this study also measured those special markers included in FibroTest, the ability of discrimination of fibrotic stages could be compared among the five fibrotic scoring systems.

In conclusion, FSC was a useful and reliable biomarker for prediction of liver fibrosis in patients with chronic HCV infection. FSC is expected to be introduced and utilized in varied kinds of studies and trials. Its accuracy and reproducibility require further validation using more numbers of patients in several countries other than Japan.

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Original article

A randomized trial of daclatasvir with peginterferon alfa-2b and ribavirin for HCV genotype 1 infection

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Background: Daclatasvir-containing regimens have the potential to address limitations of current regimens combining peginterferon alfa and ribavirin with first-generation protease inhibitors for treatment of chronic HCV genotype 1 infection.

Methods: In this randomized, double-blind study, 27 Japanese treatment-naive patients received once-daily daclatasvir 10 mg or 60 mg or placebo, each combined with peginterferon alfa-2b/ribavirin; 18 prior null (n=9) or partial (n=9) responders received the same daclatasvir-containing regimens without a placebo arm. Daclatasvir recipients with protocol-defined response (HCV RNA<15 IU/ml at week 4, undetectable at week 12) were treated for 24 weeks; those without protocol-defined response and placebo recipients continued treatment to week 48.

Results: Sustained virological response 24 weeks post-treatment (SVR_{24}) was achieved by 66.7%, 90.0% and 62.5% of treatment-naive patients in the daclatasvir 10 mg, 60 mg and placebo groups, respectively. Prior non-responders had more frequent virological failure; 22.2% and 33.3% of daclatasvir 10 mg and 60 mg recipients, respectively, achieved SVR_{24}. Adverse events were similar across groups and were typical of peginterferon alfa-2b/ribavirin. Pyrexia, headache, alopecia, decreased appetite and malaise were the most common adverse events; two daclatasvir recipients discontinued due to adverse events.

Conclusions: Daclatasvir 60 mg combined with peginterferon alfa-2b and ribavirin achieved a high rate of SVR_{24} in treatment-naive patients with HCV genotype 1 infection, with tolerability similar to that of peginterferon alfa-2b/ribavirin alone. However, regimens with greater antiviral potency are needed for prior non-responders.

Introduction

The advent of direct-acting antivirals (DAAs) marks a significant advance in the treatment of chronic HCV infection. Regimens containing the non-structural protein 3 (NS3) protease inhibitors telaprevir and boceprevir, as well as multiple investigational agents, have demonstrated significantly increased rates of sustained virological response (SVR) compared with peginterferon alfa/ribavirin (alfa/RBV) alone [1]. SVR rates of 68–75% have been achieved with alfa/RBV combined with boceprevir or telaprevir in treatment-naive patients with HCV genotype 1 infection [2,3], although efficacy is lower in patients who previously failed alfa/RBV therapy [4,5].

Initial experience with regimens containing telaprevir or boceprevir has, however, identified several limiting characteristics that emphasize the need for continued development of alternative DAAs. Telaprevir- and boceprevir-containing regimens have complicated

dosing schedules and are associated with frequent adverse events such as rash and anaemia [2–5]. In addition, telaprevir and boceprevir have frequent drug–drug interactions with other medications, potentially limiting utility for patients with concomitant medical conditions, and their efficacy has been established only in HCV genotype 1 infection [6,7]. Compounds from alternative mechanistic classes offer potential for greater and broader antiviral potency to include HCV genotypes 2, 3 and 4, as well as improved tolerability, more convenient dosing schedules, reduced risk of drug resistance and reduced potential for drug–drug interactions [8]. Agents of new mechanistic classes with non-overlapping resistance profiles allow development of DAA combinations that may be effective for patients resistant to current NS3 protease inhibitors.

Daclatasvir (BMS-790052) is a first-in-class non-structural protein 5A (NS5A) replication complex inhibitor with picomolar potency and activity against HCV genotypes 1 to 6 [9]. Daclatasvir has a human pharmacokinetic profile consistent with once-daily dosing and has shown potent antiviral activity in Phase I clinical studies [10]. Daclatasvir has been well tolerated in combination with alfa/RBV in clinical studies, with an adverse event profile similar to that of alfa/RBV alone [11,12]. In a previous Phase II study in patients with chronic HCV genotype 1 infection, 83% of patients achieved SVR following a 48-week regimen of daclatasvir 60 mg once daily combined with standard peginterferon alfa-2a/RBV (alfa-2a/RBV) [12].

We assessed the efficacy and safety of daclatasvir in combination with peginterferon alfa-2b (alfa-2b; PegIntron) and RBV in Japanese patients with chronic HCV genotype 1 infection, including HCV treatment-naive patients and patients who previously failed to achieve SVR following alfa/RBV therapy (null and partial responders). The response-guided design assessed whether a shorter 24-week course of therapy was sufficient for daclatasvir recipients who achieve early virological milestones.

Methods

Study design

In this five-arm, double-blind, randomized Phase IIa study (ClinicalTrials.gov identifier NCT01016912), enrolled patients were either naive to treatment with interferons and DAAs active against HCV or had prior non-response to alfa/RBV, defined as failing to achieve a 2 $\log_{10}$ reduction of HCV RNA at week 12 (null responder) or having never achieved undetectable serum HCV RNA after at least 12 weeks of therapy (partial responder) [13]. Treatment-naive patients were randomly assigned (1:1:1) to receive once-daily oral daclatasvir 10 or 60 mg or placebo, each in combination with subcutaneous alfa 60 to 150 μg once weekly and twice-daily oral RBV 600 to 1,000 mg/day. Alfa and RBV doses were determined by body weight in accordance with Japanese label recommendations. Prior non-responders were randomly assigned (1:1) to receive the same daclatasvir-containing regimens but there was no placebo arm for this group because of the known very poor responsiveness of these populations to retreatment with alfa/RBV.

Patients were treated for 24 or 48 weeks. Randomized treatment assignment was double-blind and placebo-controlled for daclatasvir in the first 24 weeks. The study was unblinded at week 24 and conducted subsequently as open label. Patients receiving daclatasvir-containing regimens stopped treatment at week 24 if they achieved a protocol-defined response (PDR), defined as HCV RNA below the assay limit of quantitation (<15 IU/ml) at week 4 and undetectable at week 12; daclatasvir recipients without PDR continued treatment to week 48. All placebo recipients were treated for 48 weeks.

The study protocol and informed consent were approved by an independent ethics committee and institutional review boards at each participating site prior to study initiation. The study was designed and conducted by the sponsor (Bristol-Myers Squibb) in collaboration with the principal investigators, and was conducted in compliance with the Declaration of Helsinki, local regulatory requirements and Good Clinical Practice, as defined by the International Conference on Harmonisation.

Patients

Patients were enrolled in six academic clinical research centers in Japan between December 2009 and February 2010. Enrolled patients were men and women, aged 20–70 years, with chronic HCV genotype 1 infection and HCV RNA≥10^5 IU/ml. Women of childbearing potential must have been using effective methods of contraception due to the contraindication of RBV for women who are pregnant or who may become pregnant.

Patients were excluded if they had evidence of liver cirrhosis by laparoscopy, imaging studies or liver biopsy within 24 months prior to screening, history or evidence of hepatocellular carcinoma or other chronic liver disease; coinfection with HBV or HIV; haemoglobinopathies or other diagnoses associated with increased risk of anaemia; or other medical, psychiatric or social reason rendering the individual inappropriate for study participation. Patients were also excluded if they had been exposed to any investigational drug or placebo within 4 weeks prior to dosing, or had any previous exposure to new or investigational HCV therapeutic agents. Exclusionary laboratory parameters included alanine aminotransferase ≥5× upper limit of normal,

total bilirubin ≥2 mg/dl, international normalized ratio ≥1.7, albumin ≤3.5 g/dl, haemoglobin <12 g/dl, white blood cell count $<4\times10^9$/l, absolute neutrophil count $<1.5\times10^9$/l, platelet count $<100\times10^9$/l or creatinine clearance <50 ml/min. Prohibited medications included proton pump inhibitors and moderate or strong inducers or inhibitors of CYP3A4.

Safety and efficacy assessments

Assessments that included HCV RNA, physical examination, adverse events, laboratory tests, pregnancy test and concomitant medications, were conducted at screening, study day 1 (baseline), weeks 1, 2, 4, 6, 8 and 12, then every 4 weeks until the end of therapy, and post-treatment weeks 4, 12 and 24. Twelve-lead electrocardiograms were recorded at screening and on-treatment weeks 4, 12, 24 and 48. Serum HCV RNA was determined centrally using the COBAS TaqMan HCV Auto assay (Roche Diagnostics KK, Tokyo, Japan), lower limit of quantitation =15 IU/ml. HCV genotype was determined at a central laboratory by PCR amplification and sequencing. *IL28B* genotype was determined by PCR amplification and sequencing of the rs12979860 single-nucleotide polymorphism.

Efficacy end points

The primary efficacy end point was the proportion of patients with HCV RNA undetectable at weeks 4 and 12 on-treatment (extended rapid virological response [eRVR]). Secondary end points included the proportions of patients with undetectable HCV RNA at week 4 (rapid virological response [RVR]), week 12 (complete early virological response [cEVR]) and post-treatment weeks 12 (SVR_{12}) and 24 (SVR_{24}).

HCV resistance testing was performed on stored specimens by PCR amplification and population sequencing of the HCV NS5A domain. Resistance testing was performed on all samples at baseline and on samples indicative of virological failure when HCV RNA was ≥1,000 IU/ml. Virological failure was defined as either <2 $\log_{10}$ HCV RNA decrease from baseline at week 12, virological rebound (HCV RNA detectable on treatment after previously undetectable or ≥1 $\log_{10}$ increase in HCV RNA from nadir) or detectable HCV RNA at end of therapy or post-treatment in patients with undetectable HCV RNA at end of therapy (relapse).

Sample size and statistical analysis

With the target sample size of eight patients per treatment group, a safety event occurring at an incident rate of 19% with 80% probability could be detected. Randomization was conducted by the sponsor at a central randomization centre. Patients were randomly allocated to treatment groups; investigators received treatment kit assignments by fax from the randomization centre for eligible screened patients. Categorical variables were summarized using counts and percentages; continuous variables were summarized with univariate statistics. CIs were two-sided with an 80% confidence level. CIs for binary end points were exact binomial, whereas the CIs for continuous end points were based on the normal distribution. All statistical analyses were conducted using SAS/STAT Version 8.02 (SAS Institute, Cary, NC, USA).

Results

Patient characteristics and disposition

A total of 51 patients were screened; 6 were excluded due to abnormal thyroid function, history of cholecystectomy, ventricular arrhythmia or white blood cell count $<4\times10^9$/l (3 patients), respectively. Twenty-seven treatment-naive patients and 18 prior non-responders met study criteria and were randomized and treated (Table 1). All patients were Japanese; other than an imbalance in gender distribution and older age in non-responders, baseline characteristics were similar across treatment groups. Although the study permitted any HCV genotype 1 subtype, all enrolled patients had genotype 1b, reflecting the high proportion of this subtype in Japan [14]. The non-responder group included nine null responders and nine partial responders, with similar distributions in the two treatment arms. Prior non-responders were primarily (16/18 patients) *IL28B* genotypes CT or TT as expected for this population; 18 of the 27 treatment-naive patients were genotype CC, consistent with the overall distribution of *IL28B* genotypes in Japan [15,16]. However, there was an imbalance of *IL28B* genotypes (CC versus CT/TT) among the three treatment-naive groups, with six, one and two patients with non-CC genotypes in the daclatasvir 10 mg, daclatasvir 60 mg and placebo groups, respectively.

The 24-week double-blind phase was completed by 38 of 45 patients. Two treatment-naive patients, one each from the daclatasvir 10 mg and 60 mg groups, discontinued due to neutropenia (week 12) and depression (week 20), respectively. Five patients discontinued due to lack of efficacy (viral breakthrough), including one treatment-naive patient (daclatasvir 10 mg group) and four non-responders (one in the daclatasvir 10 mg group and three in the daclatasvir 60 mg group).

Four patients (three in the daclatasvir 10 mg group and one in the daclatasvir 60 mg group) discontinued open-label treatment between weeks 36 and 45 due to lack of efficacy (viral breakthrough). Eleven patients had reduction of alfa-2b dose, including two, six and three patients in the placebo, daclatasvir 10 mg and daclatasvir 60 mg groups, respectively. Thirty-three patients had RBV dose reductions, including 7, 14

Table 1. Baseline demographic and disease characteristics

Baseline parameter	Treatment-naive			Non-responders	
	Placebo + alfa-2b/RBV (*n*=8)	DCV 10 mg + alfa-2b/RBV (*n*=9)	DCV 60 mg + alfa-2b/RBV (*n*=10)	DCV 10 mg + alfa-2b/RBV (*n*=9)	DCV 60 mg + alfa-2b/RBV (*n*=9)
Median age, years (range)	50 (42–66)	51 (21–68)	55 (36–66)	58 (48–67)	63 (42–70)
Male, *n* (%)	4 (50)	2 (22)	6 (60)	3 (33)	3 (33)
HCV genotype 1b, *n* (%)	8 (100)	9 (100)	10 (100)	9 (100)	9 (100)
Mean HCV RNA, $\log_{10}$ IU/ml (SD)	6.9 (0.54)	6.6 (0.44)	6.5 (0.81)	6.8 (0.54)	6.8 (0.57)
Response to prior alfa/RBV					
Null response, *n* (%)	N/A	N/A	N/A	4 (44)	5 (56)
Partial response, *n* (%)	N/A	N/A	N/A	5 (56)	4 (44)
IL28B genotype (rs12979860)					
CC, *n*	6	3	9	0	1
CT, *n*	2	6	1	7[a]	7
TT, *n*	0	0	0	1	1

[a]*IL28B* genotype not available for one non-responder recipient of daclatasvir (DCV) 10 mg. N/A, not available; alfa-2b/RBV, peginterferon alfa-2b/ribavirin.

and 12 patients in the placebo, daclatasvir 10 mg and daclatasvir 60 mg groups, respectively.

Virological response

In treatment-naive daclatasvir recipients, HCV RNA levels declined rapidly after initiation of therapy, with HCV RNA becoming undetectable by week 4 (RVR) in 77.8% and 80% of patients in the daclatasvir 10 mg and 60 mg groups, respectively, compared with none in the placebo group (Table 2). The primary efficacy end point, eRVR, was achieved by 66.7% and 80.0% of patients in the daclatasvir 10 mg and 60 mg groups, respectively, versus 0% in the placebo group.

PDR was achieved by 7 of 9 (77.8%) and 10 of 10 (100%) treatment-naive patients in the daclatasvir 10 mg and 60 mg groups, respectively; these patients completed treatment after 24 weeks. HCV RNA was undetectable at the end of therapy (week 24) in 10 of 10 (100%) daclatasvir 60 mg recipients with PDR and in 6 of 7 (85.7%) daclatasvir 10 mg recipients with PDR. SVR_{24} was achieved by 6 of 7 (85.7%) daclatasvir 10 mg recipients with PDR and by 9 of 10 (90.0%) daclatasvir 60 mg recipients with PDR. Overall, in the combined group of treatment-naive patients with PDR (24 weeks of therapy) or without PDR (48 weeks) SVR_{24} was achieved by 66.7% and 90.0% of patients receiving daclatasvir 10 mg and 60 mg, respectively, compared with five of eight (62.5%) treatment-naive placebo recipients after 48 weeks of therapy. Two treatment-naive daclatasvir 10 mg recipients failed to achieve PDR; neither achieved SVR following 48 weeks of therapy.

Viral suppression was less pronounced in prior non-responders. The primary efficacy end point, eRVR, was achieved by 55.6% and 22.2% of patients in the daclatasvir 10 mg and 60 mg groups, respectively; SVR_{24} was achieved by 22.2% and 33.3% of these patients. PDR was achieved by 55.6% and 33.3% in the daclatasvir 10 mg and 60 mg groups, respectively (Table 2). Although all eight non-responders with PDR had undetectable HCV RNA through the end of therapy (week 24), among these patients only two of five (40.0%) daclatasvir 10 mg recipients and two of three (66.7%) daclatasvir 60 mg recipients achieved SVR_{24}, with the remaining patients experiencing post-treatment relapse. PDR was not achieved by four of nine and six of nine non-responder recipients of daclatasvir 10 mg and 60 mg, respectively; at the end of post-treatment follow-up, none of four and one of six of these patients achieved SVR_{24}.

In treatment-naive patients, HCV RNA was undetectable at week 12 (cEVR) and post-treatment week 24 (SVR_{24}) in 100% of daclatasvir recipients with *IL28B* genotype CC (rs12979860; three of three and nine of nine daclatasvir 10 mg and 60 mg recipients, respectively). Response rates were lower in patients with *IL28B* genotype CT: SVR_{24} was achieved by three of six treatment-naive patients with genotype CT in the daclatasvir 10 mg group; the single daclatasvir 60 mg recipient who failed to achieve SVR_{24} was also genotype CT.

Virological failure

Virological failure of all types was less frequent in treatment-naive patients than in non-responders. Treatment-naive recipients of daclatasvir 60 mg had the lowest rate of virological failure and no on-treatment viral breakthrough. Breakthrough occurred in one treatment-naive patient receiving daclatasvir 10 mg and in one placebo recipient, and one daclatasvir 10 mg recipient had detectable HCV RNA at the end of treatment (Table 2). Four treatment-naive patients

Table 2. Virological outcomes

	Treatment-naive			Prior non-responders	
	Placebo (n=8)	DCV 10 mg (n=9)	DCV 60 mg (n=10)	DCV 10 mg (n=9)	DCV 60 mg (n=9)
All patients					
HCV RNA undetectable week 4 (RVR)	0/8 (0; 0.0, 25.0)	7/9 (77.8; 51.0, 93.9)	8/10 (80.0; 55.0, 94.5)	5/9 (55.6; 30.1, 79.0)	3/9 (33.3; 12.9, 59.9)
HCV RNA undetectable week 12 (cEVR)	5/8 (62.5; 34.5, 85.3)	7/9 (77.8; 51.0, 93.9)	10/10 (100; 79.4, 100.0)	5/9 (55.6; 30.1, 79.0)	5/9 (55.6; 30.1, 79.0)
HCV RNA undetectable weeks 4 and 12 (eRVR)	0/8 (0; 0.0, 25.0)	6/9 (66.7; 40.1, 87.1)	8/10 (80.0; 55.0, 94.5)	5/9 (55.6; 30.1, 79.0)	2/9 (22.2; 6.1, 49.0)
HCV RNA undetectable, EOT	7/8 (87.5; 59.4, 98.7)	7/9 (77.8; 51.0, 93.9)	10/10 (100; 79.4, 100.0)	5/9 (55.6; 30.1, 79.0)	5/9 (55.6; 30.1, 79.0)
SVR_{24}	5/8 (62.5; 34.5, 85.3)	6/9 (66.7; 40.1, 87.1)	9/10 (90.0; 66.3, 99.0)	2/9 (22.2; 6.1, 49.0)	3/9 (33.3; 12.9, 59.9)
Viral breakthrough[a]	1/8 (12.5)	1/9 (11.1)	0/10 (0)	4/9 (44.4)	4/9 (44.4)
Post-treatment relapse[a]	2/8 (25.0)	1/9 (11.1)	1/10 (10.0)	3/9 (33.3)	2/9 (22.2)
Patients with PDR					
HCV RNA<15 IU/ml at week 4, undetectable at week 12 (PDR)	0/8 (0; 0.0, 25.0)	7/9 (77.8; 51.0, 93.9)	10/10 (100; 79.4, 100.0)	5/9 (55.6; 30.1, 79.0)	3/9 (33.3; 12.9, 59.9)
HCV RNA undetectable, EOT[a]	–	6/7 (85.7)	10/10 (100)	5/5 (100)	3/3 (100)
SVR_{24}[a]	–	6/7 (85.7)	9/10 (90.0)	2/5 (40.0)	2/3 (66.7)
Post-treatment relapse[a]	–	1/7 (14.3)	1/10 (10.0)	3/5 (60.0)	1/3 (33.3)

Data are end point (n/total n [%; 80% CI]) unless otherwise indicated. [a]Data are end point (n/total n [%]). cEVR, complete early virological response; DCV, daclatasvir; EOT, end of treatment; eRVR, extended rapid virological response; PDR, protocol-defined response; RVR, rapid virological response; SVR_{24}, sustained virological response 24 weeks post-treatment.

relapsed post-treatment, including two placebo recipients and one in each daclatasvir group. In non-responders, four patients in each treatment group experienced viral breakthrough and five relapsed post-treatment (three receiving daclatasvir 10 mg, two receiving daclatasvir 60 mg; Table 2).

NS5A-L31M/V and/or NS5A-Y93H, which are the predominant genotype 1b NS5A polymorphisms associated with daclatasvir resistance, were detected at baseline in three of the seven daclatasvir recipients with virological failure [17]. NS5A-L31M/V–Y93H variants were detected post-failure in the four treatment-naive daclatasvir recipients with virological failure. Emerging NS5A variants were more variable in the 13 prior non-responders who failed treatment, and included L31I/M/V–Y93H, R30Q/A92K, ΔP32 and L31F–ΔP32. Most patients with virological failure had non-CC *IL28B* genotypes, including all 4 treatment-naive daclatasvir recipients, 1 of 3 treatment-naive placebo recipients and 11 of 13 non-responders (data missing for 1 patient).

Safety

The most frequent adverse events were pyrexia, headache, alopecia, decreased appetite and malaise (Table 3). There were no consistent differences in adverse events between groups receiving placebo or either dose of daclatasvir. Frequencies of grade 3 or 4 adverse events were comparable across treatment groups; the majority of events were cytopenias. There was one serious adverse event (gastroenteritis of moderate intensity) in a non-responder treated with daclatasvir 10 mg combined with alfa-2b/RBV; the event occurred during treatment week 24 and resolved within 8 days without treatment, coincident with the end of study therapy. There were no deaths.

Two adverse events led to discontinuation of study treatment in naive patients: neutropenia (daclatasvir 10 mg + alfa-2b/RBV) and depression (daclatasvir 60 mg + alfa-2b/RBV group); both events resolved post-treatment without intervention. There were no consistent differences in haematological or laboratory abnormalities between groups receiving placebo or daclatasvir (Table 4); most abnormalities were mild or moderate in intensity (grade 1 or 2).

Discussion

Clinical outcomes with current telaprevir- and boceprevir-containing regimens can be limited by frequent virological failure, poor tolerability, complicated dosing schedules and drug–drug interactions with other medications [2–5]. Our results and other clinical findings suggest that daclatasvir, in combination with alfa/RBV and/or other DAAs, may offer a viable alternative to regimens containing first-generation NS3 protease inhibitors [12,18,19].

Table 3. Adverse events occurring in >25% of patients in any treatment group

	Treatment-naive			Non-responders	
Grade 1–4 adverse event	Placebo (*n*=8)	DCV 10 mg (*n*=9)	DCV 60 mg (*n*=10)	DCV 10 mg (*n*=9)	DCV 60 mg (*n*=9)
Pyrexia	5	8	9	6	8
Headache	5	6	4	5	3
Alopecia	2	3	7	2	3
Decreased appetite	1	3	3	5	2
Malaise	2	7	2	2	1
Pruritus	3	3	2	1	5
Anaemia	5	2	2	4	0
Nasopharyngitis	2	5	3	0	2
Lymphopenia	1	2	3	3	2
Rash	3	2	5	0	1
Diarrhoea	0	3	4	3	1
Injection site pruritus	2	3	3	2	1
Fatigue	3	0	3	3	1
Neutropenia	1	4	2	2	0
Back pain	1	0	3	5	0
Stomatitis	1	3	3	1	1
Abdominal discomfort	1	2	3	1	1
Constipation	3	1	1	1	2
Nausea	3	1	1	2	0
Dysgeusia	0	0	3	3	0
Insomnia	1	2	3	0	0
Cheilitis	0	2	3	0	0
Arthralgia	3	2	0	0	0

Data are *n*. DCV, daclatasvir.

Table 4. On-treatment haematological and laboratory abnormalities

	Treatment-naive, placebo (*n*=8)		Treatment-naive, DCV 10 mg (*n*=9)		Treatment-naive, DCV 60 mg (*n*=10)		Non-responders, DCV 10 mg (*n*=9)		Non-responders, DCV 60 mg (*n*=9)	
Event	Any grade	Grade 3/4	Any grade	Grade 3/4	Any grade	Grade 3/4	Any grade	Grade 3/4	Any grade	Grade 3/4
Anaemia	5	1	5	0	6	1	6	3	3	0
Neutropenia	5	1	7	4	8	2	7	2	5	0
Leukopenia	5	0	7	1	5	0	5	2	4	0
Thrombocytopenia	3	0	4	0	5	0	1	0	5	0
Lymphopenia	2	1	3	2	4	3	3	3	4	2
Low albumin	2	0	3	0	3	0	5	0	3	0
Elevated ALT	0	0	1	1	0	0	0	0	1	0
Elevated AST	0	0	2	1	0	0	0	0	0	0
Elevated bilirubin	3	0	5	1	5	0	5	0	1	0
Elevated lipase	1	0	2	0	2	0	0	0	2	0

Data are *n*. ALT, alanine aminotransferase; AST, aspartate aminotransferase; DCV, daclatasvir.

This study demonstrates that the combination of daclatasvir and alfa-2b/RBV provides more rapid virological response than alfa-2b/RBV alone. A high proportion of treatment-naive patients receiving daclatasvir and alfa-2b/RBV achieved PDR and qualified for 24 weeks of therapy, including all 10 patients receiving daclatasvir 60 mg. PDR was generally predictive of SVR_{24}: 15 of 17 treatment-naive daclatasvir recipients with PDR subsequently achieved SVR_{24}. In the control arm, 62.5% of patients achieved SVR_{24} following 48 weeks of therapy with alfa-2b/RBV. Rates of SVR_4, SVR_{12} and SVR_{24} correlated well at the 60 mg dose of daclatasvir in both treatment-naive patients and non-responders, suggesting that late relapses are infrequent with this regimen.

The overall SVR_{24} rate of 90% in treatment-naive patients receiving the daclatasvir 60 mg regimen compares favourably with SVR rates reported for telaprevir

and boceprevir in global registration studies, and was achieved with a shorter six-month therapeutic regimen [2,3]. This 90% response rate is similar to the 83% and 90% SVR_{24} rates achieved with regimens combining telaprevir with alfa-2b/RBV in generally similar Japanese populations of patients with HCV genotype 1b infection and *IL28B* genotype TT (rs8099917) or CC (rs12979860) [20,21]. Outcomes in treatment-naive patients in the present study are comparable to the 100% SVR_{24} rate (8/8 patients) achieved in a parallel study where a similar patient population received daclatasvir 60 mg combined with alfa-2a/RBV [18]. However, cross-study comparisons and small patient numbers do not support definitive conclusions concerning outcomes with alfa-2a versus alfa-2b. The 90% SVR_{24} rate achieved with the daclatasvir 60 mg regimen in treatment-naive patients is comparable to the 83% SVR_{24} rate achieved with 48 weeks of treatment with daclatasvir 10 or 60 mg + alfa-2a/RBV in US/European patients with predominantly HCV genotype 1a infection [12]. In a recent Phase IIb study with a response-guided design similar to that applied in this study, 87% of patients with genotype 1b infection achieved SVR_{12} after 24 or 48 weeks of therapy with daclatasvir 60 mg in combination with alfa-2a/RBV; however, SVR_{12} was achieved by a lower percentage of patients (58%) with genotype 1a [19]. For daclatasvir 10 mg recipients in the present study, the overall SVR_{24} rate was 66.7%. This lower rate, compared with results achieved with daclatasvir 60 mg, was attributable to a reduced early virological response and a higher rate of virological failure, and provides additional support for selection of the 60 mg dose of daclatasvir for further evaluation in Phase III studies.

Results suggest that *IL28B* genotype may influence outcomes with this regimen, although data are limited. All 12 treatment-naive daclatasvir recipients with CC genotype achieved SVR_{24}, compared with 3 of 7 (43%) patients with non-CC genotype. However, three of the four treatment-naive patients with non-CC genotypes who failed to achieve SVR received the lower 10 mg dose of daclatasvir, which may have been a factor in non-response. Further study is needed to determine the possible influence of *IL28B* genotype on outcomes with this regimen. Data from other studies in which DAAs were combined with alfa/RBV suggest that the magnitude of *IL28B* effect is generally reduced with more potent regimens [22]. Only one patient in the non-responder cohort had CC genotype, precluding assessment of *IL28B* effects in this population.

Because of higher rates of on-treatment and post-treatment virological failure, a lower proportion of patients with prior non-response to alfa/RBV achieved PDR and SVR_{24} compared with treatment-naive patients. SVR_{24} was achieved by 33.3% of prior non-responders receiving daclatasvir 60 mg, comparable to results achieved with telaprevir- or boceprevir-containing regimens after 48 weeks of therapy [4,5]. Results suggest that virological failure in this study was predicted primarily by host alfa/RBV responsiveness. The non-responder population in this study had previously shown poor response to alfa/RBV; 50% were prior null responders. All but one prior non-responder had *IL28B* non-CC genotypes, which may have contributed to their initial failure with alfa/RBV as well as to the high virological failure rate in the present study. Together, these results suggest that alternative regimens are needed for non-responders to address their interferon non-responsiveness. In this regard, two studies in prior null responders have evaluated regimens containing two DAAs with or without alfa/RBV. SVR rates exceeding 90% were achieved in genotype-1b-infected patients with a regimen combining daclatasvir with the NS3 protease inhibitor asunaprevir [23,24], and in genotype-1a-infected patients using a quadruple regimen of daclatasvir, asunaprevir and alfa/RBV [24].

Virological failure was infrequent in treatment-naive patients and occurred primarily in patients receiving daclatasvir 10 mg. The single failure in treatment-naive patients receiving daclatasvir 60 mg was post-treatment relapse in the only patient from this group with non-CC *IL28B* genotype. As expected, virological failure was more frequent in non-responders; failure was experienced by similar proportions of patients receiving the 10 mg and 60 mg doses of daclatasvir. Daclatasvir-resistant HCV variants were detected in all patients with virological failure, most frequently the combination of NS5A-L31V-Y93H which confers high-level daclatasvir resistance *in vitro* [17]. This resistance pattern is consistent with that observed in other clinical studies of daclatasvir [12,24].

Safety profiles of the study regimens were generally similar and comparable to that typically seen with alfa/RBV [25]. There was no marked difference in the patterns of adverse events or laboratory abnormalities between treatment groups, with no evidence suggesting that daclatasvir at either dose contributed significantly to overall regimen tolerability or safety. The observed safety profile of daclatasvir is consistent with results of previous studies of daclatasvir monotherapy [10], daclatasvir combined with alfa/RBV [11,12,19] and daclatasvir combined with other DAAs [23,24,26]. The single serious adverse event (gastroenteritis) was considered treatment-related by the investigator, but the relative contributions of daclatasvir and alfa-2b/RBV to the event cannot be assessed.

Limitations of this study include the relatively small sample size, which precludes quantitative comparisons of efficacy outcomes and definitive conclusions

regarding the possible contribution of daclatasvir to low-frequency safety signals. Only Japanese research sites were included; therefore, possible effects of patient ethnicity cannot be assessed, such as potential contributions of the high prevalence of *IL28B* CC genotype in Japan. In addition, the enrolled population was exclusively HCV genotype 1b and thus not representative of genotype 1 populations in Western countries. Finally, interpretation of study results cannot be extended to cirrhotic patients, who were excluded from the study.

Together, efficacy and safety results suggest that some weaknesses of current regimens that combine an NS3 protease inhibitor with alfa/RBV can be addressed with regimens that utilize a potent DAA with an alternative mechanism of action. The combination of daclatasvir 60 mg and alfa-2b/RBV elicited rapid clearance of detectable HCV RNA and achieved SVR_{24} in a large proportion of treatment-naive patients with HCV genotype 1b infection, with safety and tolerability similar to that of alfa/RBV alone. For non-responders, the efficacy of this regimen is similar to that seen with boceprevir or telaprevir in combination with alfa/RBV, although daclatasvir may have tolerability advantages and relatively lower risk of drug–drug interactions [27–29]. For non-responders and other difficult-to-treat patients, daclatasvir is being assessed as part of more potent regimens containing one or two additional DAAs with or without alfa/RBV. Overall, the results of this study support current Phase III development of daclatasvir 60 mg combined with alfa/RBV in larger treatment-naive populations infected with a broader range of HCV genotypes.

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ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

Effect of *PNPLA3* rs738409 variant (I148 M) on hepatic steatosis, necroinflammation, and fibrosis in Japanese patients with chronic hepatitis C

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Abstract
Background Host genetic factors have been suspected to influence histological liver damage in chronic liver disease. The nonsynonymous single-nucleotide polymorphism rs738409 C > G in the patatin-like phospholipase domain-containing 3 gene (*PNPLA3*, also known as adiponutrin), encoding the I148 M protein variant, has been identified as a novel genetic marker for hepatic steatosis and fibrosis in nonalcoholic fatty liver disease and alcoholic liver disease. We aimed to determine whether the *PNPLA3* rs738409 variant was associated with hepatic steatosis, necroinflammation, and fibrosis in Japanese patients with chronic hepatitis C.
Methods In a cross-sectional study in Japan, we analyzed 276 patients with chronic hepatitis C who underwent liver biopsy. Genotyping for rs738409 was performed using the TaqMan genotyping assay.
Results The frequencies of the rs738409 CC, CG, and GG genotypes were 32.6, 46.4, and 21.0 %, respectively. Multivariate analysis revealed that the GG genotype was independently associated with the presence of steatosis [odds ratio (OR) 2.58, 95 % confidence interval (CI) 1.37–4.84, $p = 0.003$], severe necroinflammatory activity (OR 2.16, 95 % CI 1.12–4.16, $p = 0.02$), and advanced fibrosis (OR 2.10, 95 % CI 1.07–4.11, $p = 0.03$), after adjustment for age, sex, body mass index, and diabetes.
Conclusions The *PNPLA3* rs738409 variant influences histological liver damage in Japanese patients with chronic hepatitis C. The G allele homozygotes are at higher risk for hepatic steatosis, severe necroinflammation, and advanced fibrosis.

Keywords *PNPLA3* · Single-nucleotide polymorphism · Hepatitis C · Steatosis · Fibrosis

Introduction

Chronic hepatitis C (CHC) is a leading cause of liver cirrhosis and hepatocellular carcinoma in many countries. Hepatic steatosis occurs in more than half of patients infected with hepatitis C virus (HCV) and appears to be associated with a more rapid progression of liver fibrosis and a lower response to interferon-α-based therapy [1–3]. Both viral and host factors, including HCV genotype 3, older age, higher body mass index (BMI), diabetes, and alcohol consumption, are thought to contribute to HCV-related steatosis, [2, 4, 5]. HCV genotype 3, which is directly responsible for steatosis, is far less frequent in Japan than in Europe or the United States.

The rate of progression of liver fibrosis varies among patients with CHC. Known risk factors for fibrosis progression include older age, male sex, higher BMI, steatosis, insulin resistance, alcohol consumption, and co-infection with human immunodeficiency virus. However, these factors remain poor predictors of fibrosis progression [6, 7].

Host genetic factors have been suspected to influence histological liver damage in chronic liver disease. The

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nonsynonymous single-nucleotide polymorphism rs738409 C > G in the patatin-like phospholipase domain-containing 3 gene (*PNPLA3*, also known as adiponutrin), encoding an isoleucine to methionine substitution at residue 148 (I148 M), was initially associated with hepatic fat content in the first genome-wide association study (GWAS) on nonalcoholic fatty liver disease (NAFLD) [8]. The GWAS showed that the G allele of rs738409 influenced hepatic fat content independently of BMI, insulin resistance, and dyslipidemia. There has been growing evidence that the rs738409 variant (I148 M) is associated with steatosis, steatohepatitis, fibrosis, and cirrhosis both in NAFLD [9–11] and alcoholic liver disease [12–14]. Our GWAS of Japanese patients with NAFLD revealed that the G allele of rs738409 is associated only with typical nonalcoholic steatohepatitis with fibrosis (i.e., type 4 in Matteoni's classification [15]—not with simple steatosis or with steatosis with lobular inflammation or ballooning degeneration (i.e., types 1–3) [16].

Recent studies have suggested that the rs738409 variant is associated with hepatic steatosis and fibrosis in European patients with CHC [17, 18]. The influence of the rs738409 variant may be different in different populations. For example, the effect of the rs738409 variant on hepatic fat content is more evident among Hispanic-Americans, in whom the G allele of rs738409 is more frequent, when compared with European-Americans and African-Americans [8]. In the present cross-sectional study, we aimed to examine whether the *PNPLA3* rs738409 C > G variant (I148 M) is associated with hepatic steatosis, necroinflammation, and fibrosis in Japanese patients with CHC.

Methods

Patients

This study included a total of 276 Japanese patients with CHC who underwent liver biopsy between 2009 and 2012 at the Saiseikai Suita Hospital and the Hospital of Kyoto Prefectural University of Medicine. Inclusion criteria were as follows: patients older than 18 years, positive for anti-HCV, and positive for serum HCV-RNA. Exclusion criteria included consumption of more than 20 g of alcohol per day, positivity for hepatitis B virus surface antigen, the presence of other types of liver diseases (e.g., primary biliary cirrhosis, autoimmune hepatitis, Wilson's disease, or hemochromatosis), previous treatment with drugs known to produce hepatic steatosis, and a history of gastrointestinal bypass surgery. None of the patients had received any antiviral therapy before the liver biopsy although many of the patients had received ursodeoxycholic acid and herbal medicines.

The Ethics Committee of the Saiseikai Suita Hospital and the Kyoto Prefectural University of Medicine approved this study. Informed consent was obtained from each patient in accordance with the Declaration of Helsinki.

Laboratory tests

Clinical and laboratory data were collected at the time of liver biopsy. Body mass index (BMI) was calculated using the following formula: weight in kilograms/(height in meters)^2. Diabetes was defined as a fasting plasma glucose concentration of $\geq$126 mg/dl or a 2-h plasma glucose concentration of $\geq$200 mg/dl during an oral glucose (75 g) tolerance test or by the use of insulin or oral hypoglycemic agents to control blood glucose [19].

Venous blood samples were taken in the morning of the day of liver biopsy after a 12-h overnight fast. The laboratory evaluation included a blood cell count and measurement of serum aspartate aminotransferase (AST), alanine aminotransferase (ALT), γ-glutamyl transpeptidase (γ-GTP), and albumin. These parameters were measured using standard clinical chemistry techniques. HCV genotype was determined according to the classification of Simmonds et al. [20], and the serum HCV-RNA level was quantified as described previously [5].

Histopathological examination

Histopathological examination of the liver was performed as described previously [5]. The degrees of inflammation and fibrosis were evaluated according to the METAVIR scoring system [21].

Genotyping

DNA was extracted from peripheral blood mononuclear cells using the Gentra Puregene kit (Qiagen, Germantown, MD, USA) according to the manufacturer's protocol. DNA concentration and purity were measured with a NanoDrop 1000 spectrophotometer (Thermo Scientific, Waltham, MA, USA). Genotyping for rs738409 was performed using the TaqMan SNP genotyping assay (Applied Biosystems, Foster City, CA, USA).

Statistical analysis

Statistical analyses were performed using SPSS Statistics 22 (IBM, Chicago, IL, USA) or R (http://www.r-project.org/). To evaluate the association between the rs738409 genotypes and clinical parameters, we used the Jonckheere–Terpstra trend test (for continuous variables) and the Cochran–Armitage trend test (for categorical variables). Logistic regression analysis was used for multivariate

Table 1 Patient characteristics

Characteristic	Total	*PNPLA3* rs738409			P^a
		CC	CG	GG	
n	276 (100)	90 (32.6)	128 (46.4)	58 (21.0)	
Age (years)	58.2 ± 13.0	59.5 ± 11.9	57.7 ± 13.3	57.1 ± 13.7	0.29
Male sex	112 (40.6)	36 (40.0)	50 (39.1)	26 (44.8)	0.61
BMI (kg/m^2)	23.0 ± 3.4	23.0 ± 3.7	22.9 ± 3.4	23.1 ± 2.8	0.49
Diabetes	13 (4.7)	4 (4.4)	6 (4.7)	3 (5.2)	0.84
Platelet count ($\times 10^4/\mu l$)	16.0 ± 5.8	15.5 ± 5.1	16.4 ± 6.3	16.0 ± 5.7	0.52
AST (IU/l)	58.7 ± 43.6	61.7 ± 53.7	58.2 ± 38.7	55.0 ± 36.4	0.95
ALT (IU/l)	67.4 ± 58.0	69.2 ± 61.0	68.4 ± 60.1	62.3 ± 48.4	0.92
γ-GTP (IU/l)	59.8 ± 79.4	62.8 ± 94.3	57.5 ± 69.9	60.3 ± 74.8	0.53
Albumin (g/dl)	4.0 ± 0.5	4.0 ± 0.5	4.0 ± 0.5	4.1 ± 0.4	0.27
HCV genotype					0.09
1	198 (71.7)	55 (61.1)	102 (79.7)	41 (70.7)	
2	76 (27.5)	33 (36.7)	26 (20.3)	17 (29.3)	
ND	2 (0.8)	2 (2.2)	0 (0)	0 (0)	
HCV RNA level (logIU/ml)	6.0 ± 1.1	6.0 ± 1.1	6.1 ± 1.0	6.1 ± 1.3	0.37
Liver histology					
Steatosis					
<1 %	139 (50.4)	67 (74.5)	53 (41.4)	19 (32.8)	
1–10 %	100 (36.2)	17 (18.9)	58 (45.3)	25 (43.1)	
11–33 %	26 (9.4)	3 (3.3)	15 (11.7)	8 (13.8)	
>33 %	11 (4.0)	3 (3.3)	2 (1.6)	6 (10.3)	
Activity grade[b]					
0	7 (2.5)	3 (3.3)	2 (1.5)	2 (3.5)	
1	120 (43.5)	42 (46.7)	60 (46.9)	18 (31.0)	
2	120 (43.5)	35 (38.9)	56 (43.8)	29 (50.0)	
3	29 (10.5)	10 (11.1)	10 (7.8)	9 (15.5)	
Fibrosis stage[b]					
0	10 (3.6)	3 (3.3)	4 (3.1)	3 (5.2)	
1	111 (40.2)	41 (45.6)	53 (41.4)	17 (29.3)	
2	83 (30.1)	26 (28.9)	40 (31.3)	17 (29.3)	
3	45 (16.3)	10 (11.1)	20 (15.6)	15 (25.9)	
4	27 (9.8)	10 (11.1)	11 (8.6)	6 (10.3)	

Values are mean ± standard deviation or numbers (%). Where no other unit is specified, values refer to numbers (%) of patients

ALT alanine aminotransferase, *AST* aspartate aminotransferase, *BMI* body mass index, *ND* not determined, *γ-GTP* γ-glutamyl transpeptidase

[a] Jonckheere–Terpstra test or Cochran–Armitage trend test

[b] According to reference [21]

analysis. Values of $p < 0.05$ were considered significant. Post hoc power analysis was performed using nQuery Advisor (Statistical Solutions, Boston, MA, USA).

Results

The characteristics of the 276 study subjects with chronic hepatitis C and the frequency distribution of the *PNPLA3* rs738409 C > G polymorphism are summarized in Table 1. The frequencies of the rs738409 CC, CG, and GG genotypes were 32.6, 46.4, and 21.0 %, respectively, and were in Hardy–Weinberg equilibrium. The rs738409 genotype was not significantly associated with clinical or biochemical factors, including age, sex, BMI, diabetes, HCV genotype, platelet count, and levels of serum AST, ALT, γ-GTP, albumin, and HCV-RNA.

We assessed the impact of the rs738409 genotype on histological liver damage in a cross-sectional manner. The prevalence of steatosis (defined as ≥1 %), severe

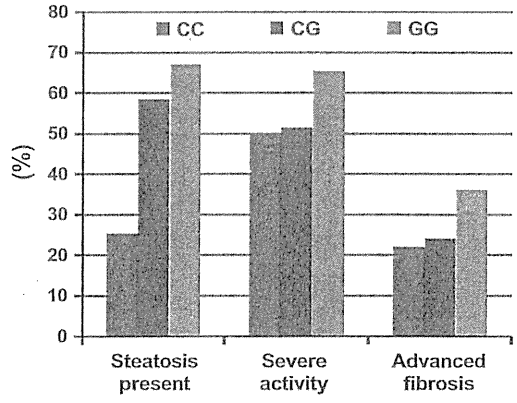

Fig. 1 The prevalence of steatosis (defined as ≥1 %), severe necroinflammatory activity (grade 2 or 3), and advanced fibrosis (stage 3 or 4) according to the *PNPLA3* rs738409 genotype

Table 2 Multivariable logistic regression analysis of the association of *PNPLA3* (rs738409 C>G), under a recessive inheritance model, with the presence of steatosis, severe necroinflammatory activity and advanced fibrosis

Variables	OR	95 % CI	*p*
Steatosis present (≥1 %)			
rs738409 (GG vs. CG + CC)	2.58	1.37–4.84	0.003
Age (years)	0.99	0.97–1.01	0.45
Sex (male vs. female)	1.19	0.70–2.02	0.51
BMI (kg/m^2)	1.19	1.10–1.30	<0.001
Diabetes (yes vs. no)	0.70	0.21–2.36	0.57
Severe necroinflammatory activity (grade 2 or 3)			
rs738409 (GG vs. CG + CC)	2.16	1.12–4.16	0.02
Age (years)	1.06	1.03–1.08	<0.001
Sex (male vs. female)	1.24	0.73–2.14	0.42
BMI (kg/m^2)	1.11	1.02–1.20	0.01
Diabetes (yes vs. no)	2.17	0.54–8.68	0.28
Advanced fibrosis (stage 3 or 4)			
rs738409 (GG vs. CG + CC)	2.10	1.07–4.11	0.03
Age (years)	1.07	1.04–1.10	<0.001
Sex (male vs. female)	1.31	0.72–2.40	0.38
BMI (kg/m^2)	1.14	1.04–1.24	0.006
Diabetes (yes vs. no)	1.77	0.52–6.05	0.37

BMI body mass index, *CI* confidence interval, *OR* odds ratio

necroinflammatory activity (grade 2 or 3), and advanced fibrosis (stage 3 or 4) according to the rs738409 genotype is shown in Fig. 1. Steatosis was present in 25.5, 58.6, and 67.2 %, severe necroinflammatory activity was found in 50.0, 51.6, and 65.5 %, and advanced fibrosis was found in 22.2, 24.2, and 36.2 % of patients with CC, CG, and GG genotypes, respectively.

To evaluate the effect of the rs738409 variant G allele, we used a recessive model of inheritance comparing G allele homozygotes (GG) with heterozygotes (CG) or C allele homozygotes (CC) (i.e., GG vs. CG + CC), according to previous reports [17, 18]. Multivariate analysis revealed that the GG genotype was independently associated with the presence of steatosis [odds ratio (OR) 2.58, 95 % confidence interval (CI) 1.37–4.84, $p = 0.003$], severe necroinflammatory activity (OR 2.16, 95 % CI 1.12–4.16, $p = 0.02$), and advanced fibrosis (OR 2.10, 95 % CI 1.07–4.11, $p = 0.03$), after adjustment for age, sex, BMI, and diabetes (Table 2). Besides the rs738409 GG genotype, a higher BMI was independently correlated with the presence of steatosis, severe necroinflammatory activity, and advanced fibrosis, and older age was independently associated with severe necroinflammatory activity and advanced fibrosis.

Discussion

In the present study, we showed that the *PNPLA3* rs738409 GG genotype was independently associated with the presence of steatosis, severe necroinflammatory activity, and advanced fibrosis in Japanese patients with CHC. Our results appear to be compatible with the following previous studies of European patients. The Swiss Hepatitis C Cohort Study [22] reported that the rs738409 G allele was associated with an increased risk of steatosis in Caucasian patients with HCV genotype non-3. The large Italian cross-sectional study [17] showed that the rs738409 GG genotype was associated with steatosis, fibrosis stage, cirrhosis, lower response to antiviral therapy, and hepatocellular carcinoma occurrence in CHC. In the cross-sectional and prospective study of Caucasian patients with CHC from Belgium, Germany, and France [18], the rs738409 GG genotype was associated with steatosis, fibrosis, and fibrosis progression.

However, to our knowledge, this is the first report to demonstrate the association of the *PNPLA3* rs738409 variant with advanced fibrosis in Japanese patients with CHC. Sato et al. [23] recently reported that the rs738409 GG genotype was associated with a higher prevalence of steatosis in Japanese patients with CHC, and Moritou et al. [24] showed that the rs738409 G allele tended to be associated with steatosis in such patients. In contrast, Nakamura et al. [25] reported that there was no correlation between the rs738409 genotype and steatosis and liver cirrhosis diagnosed by ultrasonography in Japanese patients with CHC. However, ultrasonography is a less accurate method for the diagnosis of steatosis and liver cirrhosis than the liver biopsy that was used in the present study.

Genotype frequency varies according to ethnicity. Importantly, our study, together with other studies of