INTRODUCTION

APPROXIMATELY 1.5–2 MILLION Japanese people are infected with hepatitis C virus (HCV), with the majority being infected with HCV genotype 1b.[1] Chronic infection with HCV is a major cause of liver disease,[2] and is estimated to account for more than 70% of hepatocellular carcinoma cases, one of the most common causes of cancer death in Japan.[3]

Combination therapy with weekly peginterferon-α (PEG IFN-α) injections and twice-daily oral ribavirin (RBV) for 48–72 weeks has been standard care for HCV genotype 1 infection for many years.[4–6] However, treatment discontinuations and dose reductions are common owing to the wide range of adverse events (AE) associated with PEG IFN-α/RBV therapy, including influenza-like symptoms, anemia and depression.[7–9]

Novel direct-acting antiviral agents, including protease inhibitors (PI) that target the HCV NS3/4A serine protease, have recently become available and are recommended for use in combination with PEG IFN-α/RBV.[6] The addition of PI to PEG IFN-α/RBV has improved treatment outcomes substantially in both treatment-naïve and treatment-experienced patients.[6,10–16] Sustained virologic response (SVR) rates of 60–88% have been reported for the first-generation PI in combination with PEG IFN-α/RBV in untreated and previously treated relapsed HCV infection,[10–16] compared with rates of 40–50% with PEG IFN-α/RBV alone.[7–9,17,18] This has enabled the use of shorter courses of PEG IFN-α/RBV than the standard 48 weeks.[13,19] However, currently available PI in combination with PEG IFN-α/RBV are associated with higher incidences of anemia, dysgeusia, rash and nausea than PEG IFN-α/RBV alone,[10–13,15,16] and high rates of patient discontinuation.[11,14] In addition, currently available PI require multiple daily dosing. Patients infected with HCV would benefit from novel agents with improved tolerability and more convenient dosing schedules.

Simeprevir (TMC435) is a once-daily, oral HCV NS3/4A PI, with potent antiviral activity against HCV genotype 1,[20] as well as against isolates of genotypes 2 and 4–6.[21] Simeprevir combined with PEG IFN-α-2a/RBV has demonstrated good tolerability and high SVR rates in both treatment-naïve and treatment-experienced patients infected with HCV genotype 1 in international studies[22–24] and in phase III studies in Japan (CONCERTO-1,-2 and -3).[25,26]

We report the results of a phase III, open-label, non-comparative study (CONCERTO-4) conducted in Japan to investigate the efficacy and safety of simeprevir in combination with PEG IFN-α-2b/RBV in patients infected with HCV genotype 1 who were treatment-naïve or had previously received interferon (IFN)-based therapy.

METHODS

Patients

ELIGIBLE PATIENTS WERE aged 20–70 years with chronic HCV genotype 1 infection and plasma HCV RNA of 5.0 $\log_{10}$ IU/mL or more at screening. Treatment-naïve patients must not have received prior treatment with any approved or investigational HCV drug (including IFN). Patients who had previously received IFN-based therapy for 24 weeks or more were eligible provided their last treatment was administered 60 days or more before the study start. Treatment-experienced patients were classified as prior relapsers (i.e. patients who had undetectable levels of HCV RNA at the last assessment while on IFN-based therapy and subsequent detectable levels of HCV RNA within 12 months from their last treatment), or prior non-responders (i.e. patients who did not achieve undetectable HCV RNA on prior IFN-based therapy or who had discontinued IFN-based therapy within 24 weeks of treatment initiation due to <2 $\log_{10}$ IU/mL reduction from baseline in HCV RNA at week 12 of treatment). All patients provided written informed consent.

Exclusion criteria included liver cirrhosis or hepatic failure, liver disease of non-HCV etiology, infection/co-infection with non-genotype 1 HCV, hepatitis B virus, or HIV-1 or HIV-2, any condition that required caution with PEG IFN-α-2b or RBV therapy, and any other clinically significant disease, organ transplant or defined laboratory abnormalities at screening. In addition, treatment-experienced patients were not eligible if they had received treatment with any HCV therapy other than IFN, PEG IFN or RBV, or if they had discontinued previous therapy due to an AE considered likely to be treatment-limiting during PEG IFN-α-2b/RBV therapy.

Study design

This was an open-label, non-comparative, multicenter study to assess the efficacy and safety of simeprevir (TMC435) combined with PEG IFN-α-2b/RBV in treatment-naïve and treatment-experienced (prior relapsers or non-responders to IFN-based therapy) patients with chronic HCV genotype 1 (NCT01366638). The study was conducted at 14 sites in Japan from 1 April 2011 to 20 November 2012. The study was

approved by the relevant institutional review boards and was conducted in accordance with the Declaration of Helsinki and Good Clinical Practice Guidelines.

Eligible patients received oral simeprevir 100 mg once daily (Q.D.) plus PEG IFN-α-2b/RBV for 12 weeks. In treatment-naïve patients and prior relapsers, this was followed by response-guided therapy (RGT) with PEG IFN-α-2b/RBV until week 24 or 48. Treatment-naïve patients and prior relapsers who achieved HCV RNA of less than 1.2 $\log_{10}$ IU/mL detectable or undetectable levels at week 4, with undetectable levels at week 12, stopped PEG IFN-α-2b/RBV therapy at week 24, while all others continued to week 48. All prior non-responders received PEG IFN-α-2b/RBV until week 48. All patients were followed for 72 weeks after treatment initiation.

Patients had to discontinue simeprevir but could continue with PEG IFN-α-2b/RBV if they experienced grade 4 elevations of aspartate aminotransferase (AST) or alanine aminotransferase (ALT) and the value was more than 2 times baseline, or if they experienced grade 4 blood bilirubin elevations and bilirubin values were the same or higher at retesting. All study medications were stopped if patients experienced grade 4 AE or laboratory abnormalities that were not considered to be related to simeprevir specifically or were not expected toxicities of PEG IFN-α-2b/RBV or HCV infection, or if patients experienced grade 3/4 skin events/allergic reactions, or worsening of hepatic disease. Additionally, all study medications were stopped if the following predefined virologic stopping criteria were met: less than 2 $\log_{10}$ IU/mL reduction in HCV RNA at week 12 relative to baseline (treatment-naïve patients and prior relapsers); HCV RNA levels of more than 2 $\log_{10}$ IU/mL at week 12 (prior non-responders); and confirmed detectable HCV RNA of 1.2 $\log_{10}$ IU/mL or more at weeks 24 or 36 (all patients). Patients who discontinued therapy proceeded immediately into follow-up.

The major efficacy end-point was the proportion of patients with undetectable HCV RNA at the end of treatment and 12 weeks after the last treatment (SVR12). Other efficacy end-points included the proportion of patients with: undetectable HCV RNA at end of treatment and 24 weeks after the last treatment (SVR24); undetectable HCV RNA at end of treatment and 4 weeks after the last treatment (SVR4); rapid virologic response (RVR; undetectable HCV RNA at week 4); complete early virologic response (cEVR; undetectable HCV RNA at week 12); undetectable HCV RNA at the end of treatment; viral breakthrough (increase of >1 $\log_{10}$ IU/mL in plasma HCV RNA level from the lowest level reached or plasma HCV RNA level >2.0 $\log_{10}$ IU/mL in patients whose plasma HCV RNA level had previously been <1.2 $\log_{10}$ IU/mL detectable or undetectable); viral relapse (detectable or quantifiable plasma HCV RNA during the post-treatment follow-up period in patients who had undetectable plasma HCV RNA at the end of treatment); and normalization of ALT. Tolerability and safety (AE, clinical laboratory parameters and vital signs) were secondary end-points.

Treatment administration

Simeprevir 100 mg was administered orally Q.D. as a single capsule. No simeprevir dose adjustments were permitted but, at the investigator's discretion, dosing could be interrupted for 4 days or less due to AE. PEG IFN-α-2b (PegIntron®; Merck Sharp & Dohme, Whitehouse Station, NJ, USA) was administered weekly as an s.c. injection (1.5 μg/kg body weight), and RBV (Rebetol®, Merck Sharp & Dohme) was administered as oral capsules (600–1000 mg total daily dose, according to body weight). Dose change, temporary interruption or discontinuation of PEG IFN-α-2b and RBV had to be conducted in accordance with the manufacturer's prescribing information. Patients were hospitalized for at least 1 week, starting on the first day of treatment. Use of erythropoiesis-stimulating agents and medications acting on the immune system was not permitted during treatment.

Study assessments

Plasma HCV RNA was quantified at screening, at baseline, on day 3, and at weeks 1, 2, 3, 4, 8, 12, 16, 20 and 24 (all patients), and weeks 28, 36, 48, 60 and 72 (patients receiving PEG IFN-α-2b/RBV until week 24), or weeks 28, 36, 42, 48, 52, 60 and 72 (patients receiving PEG IFN-α-2b/RBV until week 48). Levels were determined at a central laboratory using Roche COBAS® TaqMan® HCV Auto (Roche Molecular Diagnostics, Pleasanton, CA, USA) with a lower limit of quantification of 1.2 $\log_{10}$ IU/mL.

Sequence analysis of the HCV NS3 protease domain was performed at baseline and in patients with simeprevir treatment failure (viral breakthrough, meeting virologic stopping rule, detectable HCV RNA at end of treatment or viral relapse). The analysis of baseline polymorphisms focused on detecting previously characterized HCV genotype 1 amino acid substitutions in the NS3 region at positions 36, 43, 54, 80, 122, 138, 155, 156, 168 and 170 that have been associated with reduced susceptibility to simeprevir and other HCV NS3 PI *in vitro*.[27,28]

Safety and tolerability were evaluated throughout the entire treatment period, from first study medication intake until 28 days after the last dose. Severity of AE was graded by investigators according to the World Health Organization (WHO) grading scale. Vital sign monitoring, electrocardiograms, physical examinations and clinical laboratory tests were performed at regular intervals during the study period. Severity of laboratory abnormalities was classified according to the WHO grading scale.

Statistical analysis

A sample size of 70 patients was deemed sufficient to give a 97% probability of detecting an AE of special interest with 5% or more incidence.

Efficacy analyses were performed on the full analysis set (i.e. all patients who received the study medication and had post-baseline efficacy assessment data). The safety population included all patients who received at least one dose of simeprevir.

Ninety-five percent confidence intervals (CI) around the SVR12, SVR24 and SVR4 rates were calculated for each group. Descriptive statistics and tabulation were used to summarize baseline characteristics. All statistical analyses were performed using SAS® version 9.2 (SAS Institute, Cary, NC, USA).

RESULTS

Patients

IN TOTAL, 97 patients were screened and 79 received treatment (24 treatment-naïve patients, 29 prior relapsers and 26 prior non-responders) (Fig. 1). All 79 patients who received treatment were included in the full analysis set and safety populations. All study medications were completed by 65 patients (82.3%). The rate of treatment completion was lowest among prior non-responders (57.7% vs 91.7% for treatment-naïve patients and 96.6% for prior relapsers). Of the 14 patients who discontinued medications, one patient discontinued simeprevir and subsequently discontinued PEG IFN-α-2b/RBV, nine patients discontinued PEG IFN-α-2b/RBV after completing simeprevir treatment and four patients discontinued all study medications at the same time. The main reason for treatment discon-

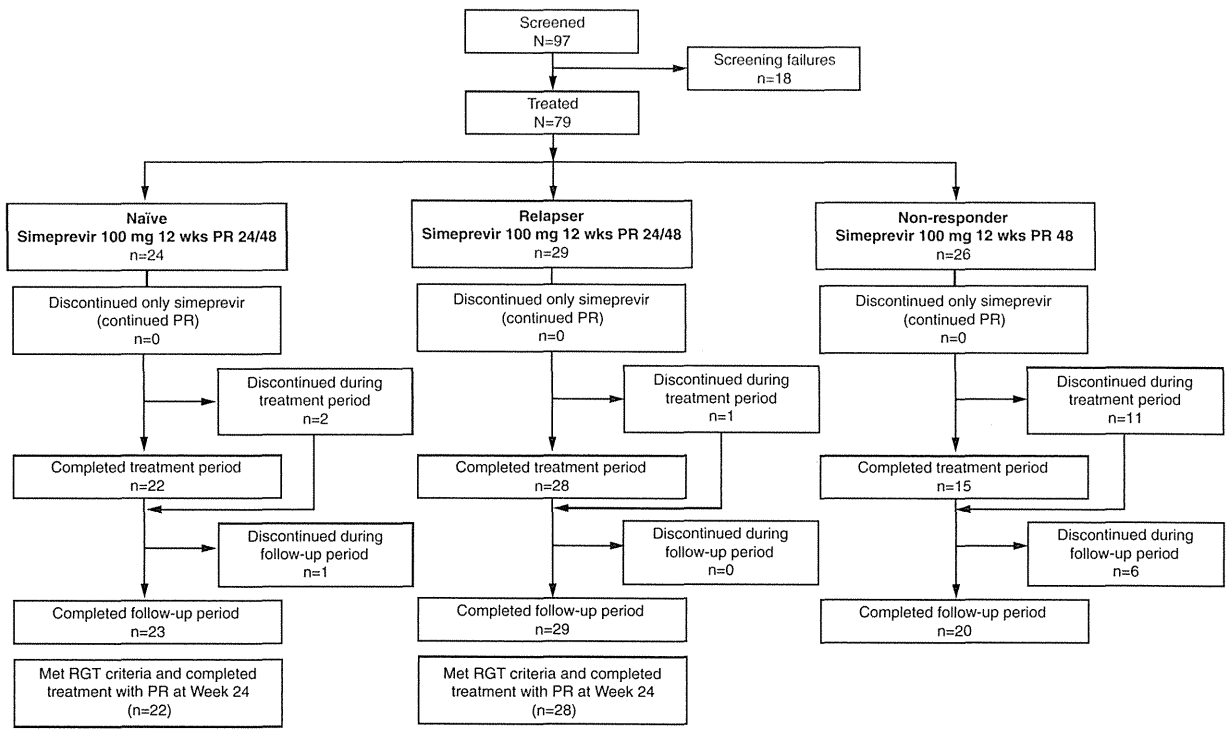

Figure 1 Patient disposition. PR, peginterferon-α-2b and ribavirin; RGT, response-guided treatment; wks, weeks.

Table 1 Patient demographics and baseline characteristics (full analysis set)

Characteristic	Treatment-naïve ($n = 24$)	Prior relapsers ($n = 29$)	Prior non-responders ($n = 26$)
Male, *n* (%)	8 (33.3)	16 (55.2)	13 (50.0)
Age, years, median (range)	60 (37–68)	60 (38–70)	53 (45–69)
Age <65 years, *n* (%)	19 (79.2)	20 (69.0)	22 (84.6)
BMI, kg/m^2, median (range)	22.95 (18.1–30.2)	22.5 (18.1–31.9)	22.4 (16.9–34.3)
IL28B genotype (SNP rs8099917)			
TT, n (%)	16 (66.7)	26 (89.7)	2 (7.7)
TG/GG, *n* (%)	8 (33.3)	3 (10.3)	24 (92.3)
IL28B genotype (SNP rs12979860)			
CC, *n* (%)	16 (66.7)	26 (89.7)	2 (7.7)
CT/TT, *n* (%)	8 (33.3)	3 (10.3)	24 (92.3)
Genotype 1b, *n* (%)	24 (100.0)	29 (100.0)	25 (96.2)
Baseline HCV RNA, $\log_{10}$ IU/mL, median (range)	6.6 (5.4–7.0)	6.6 (4.9–7.4)	6.5 (5.1–7.4)
METAVIR score, category, *n* (%)†	$n = 6$	$n = 6$	$n = 7$
F0	0	0	0
F1	5 (83.3)	4 (66.7)	5 (71.4)
F2	1 (16.7)	1 (16.7)	2 (28.6)
F3	0	1 (16.7)	0
F4	0	0	0
Platelets (×10^9/L), *n* (%)			
<150	5 (20.8)	9 (31.0)	11 (42.3)
≥150	19 (79.2)	20 (69.0)	15 (57.7)
Prior therapy, *n* (%)			
IFN only	N/A	1 (3.4)	0
IFN/RBV	N/A	0	3 (11.5)
PEG IFN only	N/A	0	0
PEG IFN/RBV	N/A	28 (96.6)	23 (88.5)
ALT			
<50 IU/mL	16 (66.7)	20 (69.0)	13 (50.0)
≥50 IU/mL	8 (33.3)	9 (31.0)	13 (50.0)
Total bilirubin (mg/dL), median (range)	0.7 (0.3–1.8)	0.8 (0.4–2.2)	0.8 (0.3–1.1)
Hemoglobin (g/dL), median (range)	14.2 (12.4–16.3)	14.4 (11.5–17.0)	13.9 (12.2–16.6)
Neutrophils (×10^2/μL), median (range)	25.4 (12.1–51.2)	25.4 (10.1–48.1)	22.2 (9.6–35.8)
Platelets (×10^4/μL), median (range)	17.1 (12.2–27.5)	16.3 (9.6–33.3)	15.4 (11.0–20.5)

†Available for patients who had a liver biopsy within two years prior to informed consent or during the screening period.
ALT, alanine aminotransferase; BMI, body mass index; HCV, hepatitis C virus; IFN, interferon; N/A, not applicable; PEG IFN, peginterferon; RBV, ribavirin; SNP, single nucleotide polymorphism.

tinuation was meeting the virologic stopping criteria (eight patients, all prior non-responders).

Demographic and disease characteristics at baseline were generally comparable across the three patient groups (Table 1), with a few notable exceptions for sex, *IL28B* genotype and baseline platelet counts. Median age was 60 years (range, 37–70), with 22.8% of patients aged 65 years or more. Most treatment-naïve patients and prior relapsers had major allele TT and CC genotypes for *IL28B* rs9088817 and rs12979860 polymorphisms, respectively. By contrast, most prior non-responders had minor alleles TG/GG and CT/TT at these loci. All but one patient (prior non-responder) had HCV genotype 1b; median HCV RNA at baseline was 6.5 $\log_{10}$ IU/mL. Most prior relapsers and prior non-responders had previously been treated with PEG IFN plus RBV. Platelet counts at baseline were slightly lower in prior non-responders, with 42.3% having counts of less than 150×10^9/L versus 31.0% or less of patients in the other groups.

Efficacy

SVR

The proportion of patients achieving SVR4, SVR12 (major efficacy end-point), and SVR24 is shown in Fig. 2. The proportion of patients achieving SVR12 was

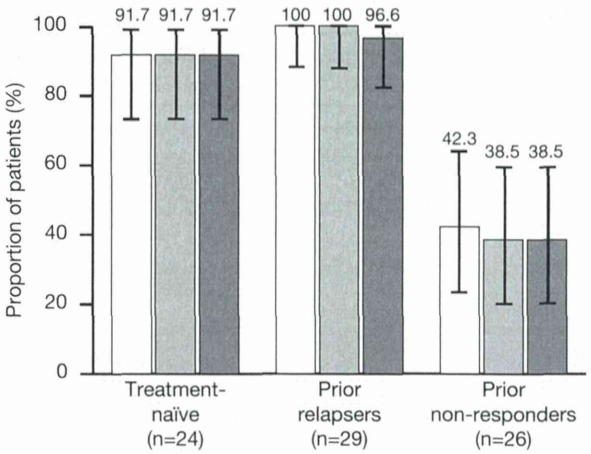

Figure 2 Sustained virologic response at 4, 12 (major end-point) and 24 weeks after the end of treatment. Bars indicate 95% confidence intervals. †$n = 12$. SVR, sustained virologic response (undetectable hepatitis C virus RNA); SVR4, SVR at end of actual treatment and at 4 weeks after the last treatment; SVR12, SVR at end of actual treatment and at 12 weeks after the last treatment (major efficacy end-point); SVR24, SVR at end of actual treatment and at 24 weeks after the last treatment. □, SVR4; ▨, SVR12; ■, SVR24.

highest among treatment-naïve patients (91.7%; 95% CI, 73.0–99.0%) and prior relapsers (100%; 95% CI, 88.1–100%). Only two patients in the treatment-naïve group did not achieve SVR12; both had undetectable HCV RNA at end of treatment, but had viral relapse at the SVR4 time point. Among prior non-responders, 10 patients (38.5%; 95% CI, 20.2–59.4%) achieved SVR12, 11 patients had detectable HCV RNA at end of treatment, four patients had viral relapse at the SVR4 time point, and one patient had discontinued follow-up before the SVR12 time point.

The SVR24 rate was 91.7% (95% CI, 73.0–99.0%) among treatment-naïve patients and 96.6% (95% CI, 82.2–99.9%) for prior relapsers (Fig. 2). All treatment-naïve patients and prior non-responders who achieved SVR12 also achieved SVR24, while 28 of 29 prior relapsers achieved both end-points (one patient experienced viral relapse at week 24 of follow-up).

Twenty-two (91.7%) treatment-naïve patients and 28 (96.6%) prior relapsers met RGT criteria and completed PEG IFN-α-2b/RBV treatment at week 24. The remaining three patients had discontinued treatment before the week 24 assessment. Rates of SVR12 and SVR24 for patients stopping treatment at week 24 were 90.9% (20/22) for treatment-naïve patients and 100% (28/28) for prior relapsers.

Virologic response

A rapid decline in mean plasma HCV RNA levels was evident in all patient groups up to week 2 (Fig. 3), by which time most patients had achieved levels below the lower limit of quantification.

Most patients in all three groups achieved RVR (60.0–86.2%; Table 2) and cEVR (79.2–100%; Table 2). All treatment-naïve patients and prior relapsers had undetectable levels of HCV RNA at the end of treatment (Table 2). In prior non-responders, 57.7% of patients had undetectable HCV RNA at end of treatment; all patients in this group had a reduction in HCV RNA from baseline of 1 $\log_{10}$ IU/mL or more at week 4.

Viral breakthrough and viral relapse

No viral breakthrough was observed in treatment-naïve patients or prior relapsers. Six prior non-responders (23.1%) had viral breakthrough (Table 2). Two of these six patients experienced breakthrough at week 8 during the simeprevir treatment period. One patient had viral breakthrough at week 8 after discontinuing simeprevir at week 5 upon meeting virologic stopping criteria. The remaining three patients experienced viral breakthrough during PEG IFN-α-2b/RBV-only treatment (weeks 12–24).

Two treatment-naïve patients experienced viral relapse at week 4 of follow-up. One prior relapser experienced viral relapse at week 24 of follow-up. Four of 15 prior non-responders with undetectable HCV RNA at

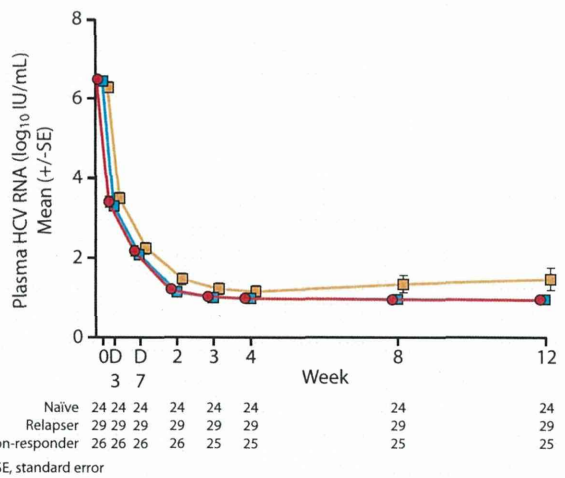

Figure 3 Mean (±SE) change in hepatitis C virus (HCV) RNA levels from baseline to week 12. D, day. —●—, naïve; —■—, relapser; —■—, non-responder.

Table 2 Virologic response rates

Response, *n*/N (%)	Treatment-naïve (*n* = 24)	Prior relapsers (*n* = 29)	Prior non-responders (*n* = 26)
RVR,† *n* (%)	19/24 (79.2)	25/29 (86.2)	15/25 (60.0)
cEVR,‡ *n* (%)	23/23 (100.0)	28/28 (100.0)	19/24 (79.2)
End of treatment response,§ *n* (%)	24/24 (100.0)	29/29 (100.0)	15/26 (57.7)
Viral breakthrough,¶ *n* (%)	0/24 (0)	0/29 (0)	6/26 (23.1)
Viral relapse,†† *n* (%)	2/24 (8.3)	1/29 (3.4)	4/15 (26.7)

Note: RVR and cEVR are assessed while on treatment. If a subject discontinues all study medications prior to the time point of the parameter of interest, then the subject is not included in the denominator.
†Undetectable HCV RNA at week 4 (i.e. while on treatment). ‡Undetectable HCV RNA at week 12. §Undetectable HCV RNA at end of treatment. ¶An increase of >1.0 $\log_{10}$ IU/mL in HCV RNA level from the lowest level reached, or HCV RNA level of >2.0 $\log_{10}$ IU/mL in patients whose HCV RNA had previously been <1.2 $\log_{10}$ IU/mL detectable or undetectable. ††Detectable HCV RNA during the post-treatment follow-up period of sustained viral response assessment in patients who had undetectable plasma HCV RNA at end of treatment. The incidence of viral relapse was calculated only for patients with undetectable HCV RNA levels at end of treatment and with ≥1 follow-up HCV RNA measurement. The denominator for prior non-responders was *n* = 15.
cEVR, complete early virologic response; HCV, hepatitis C virus; N, number of patients with data at specific time point assessed; *n*, number of patients with observation; RVR, rapid virologic response.

end of treatment had documented viral relapse at the week 4 follow-up visit.

Emerging mutations in treatment failures

Sequencing analysis of the NS3 protease domain of HCV was available for 17 (two treatment-naïve patients, one prior relapser and 14 non-responders) of the 18 simeprevir-treated patients classified as treatment failures (i.e. met predefined virologic stopping criteria, detectable HCV RNA at end of treatment, viral breakthrough or viral relapse). Emerging mutations were identified for 16 patients at the time of failure, including six patients with viral breakthrough. D168V was the most frequent emerging mutation, accounting for eight single mutations, followed by Q80R+D168E (three patients), D168E (two patients), and R155K, D168T and Q80K+D168E (one patient each).

SVR according to selected demographic and baseline disease characteristics

A summary of SVR12 rate by selected demographic and baseline disease characteristics is presented in Table 3. Most treatment-naïve patients and all prior relapsers achieved SVR12; therefore, no apparent trend was noted

Table 3 SVR12 rates by selected demographic and baseline disease characteristics

Characteristic	SVR12 rate, *n*/N (%)		
	Treatment-naïve (*n* = 24)	Prior relapsers (*n* = 29)	Prior non-responders (*n* = 26)
Sex			
Male	7/8 (87.5)	16/16 (100.0)	5/13 (38.5)
Female	15/16 (93.8)	13/13 (100.0)	5/13 (38.5)
Age			
<65 years	19/19 (100.0)	20/20 (100.0)	7/22 (31.8)
≥65 years	3/5 (60.0)	9/9 (100.0)	3/4 (75.0)
IL28B genotype (rs8099917)			
TT	16/16 (100.0)	26/26 (100.0)	0/2 (0.0)
TG/GG	6/8 (75.0)	3/3 (100.0)	10/24 (41.7)
IL28B genotype (rs12979860)			
CC	16/16 (100.0)	26/26 (100.0)	0/2 (0.0)
CT/TT	6/8 (75.0)	3/3 (100.0)	10/24 (41.7)

SVR12, undetectable hepatitis C virus RNA 12 weeks after the last treatment.

in SVR12 rates for the selected demographic and baseline disease characteristics. For non-responders, the number of patients in each subgroup was too small to draw firm conclusions. One prior non-responder who was infected with HCV genotype 1a did not achieve SVR12.

Normalization of ALT

At the end of treatment, the proportion of patients for whom ALT levels were abnormal at baseline and changed to be within the normal limits (based on WHO toxicity grades) was 13/15 (86.7%) for treatment-naïve patients, 8/13 (61.5%) for prior relapsers and 8/13 (61.5%) for prior non-responders.

Safety

Adverse events and laboratory investigations reported as AE during the entire treatment period are summarized in Table 4. All patients experienced at least one AE. No deaths occurred. Two serious AE occurred during treatment – peripheral T-cell lymphoma (unspecified) and hyperbilirubinemia – of which hyperbilirubinemia was considered by the investigator likely to be related to simeprevir. The majority of AE were grade 1 or 2. Grade 3 AE mainly occurred by week 4 and were reported for 17 patients; the most frequent grade 3 AE were neutropenia (6.3%), decreased white blood cell count (5.1%), leukopenia (3.8%) and decreased neutrophil count (3.8%). One patient experienced grade 4 decreased neutrophil count at week 1, which was considered unrelated to simeprevir. No neutropenia-related AE were serious or led to permanent discontinuation of study treatment.

Three patients discontinued treatment due to AE. One patient discontinued all three study medications at week 8 owing to grade 1 anemia, which was considered very likely related to RBV. Two patients discontinued PEG IFN-α-2b/RBV after the simeprevir treatment period owing to grade 3 allergic dermatitis and grade 2 depression, considered probably related to RBV and PEG IFN-α-2b, respectively. Rates of PEG IFN-α-2b and RBV dose interruptions due to AE were 12.7% and 17.7%, respectively, with anemia being the most frequently reported AE leading to dose interruption (5.1% for PEG IFN-α-2b and 7.6% for RBV).

The most common AE reported in more than 30% of patients overall during the entire treatment period were pyrexia (84.8%), anemia (50.6%), decreased white blood cell count (58.2%), malaise (48.1%) and headache (45.6%). During the simeprevir treatment period, the most common AE were pyrexia (83.5%), decreased white blood cell count (58.2%) and malaise (48.1%). There were no differences between patient groups in the incidence of each AE (Table 4).

Rash (any type) was reported in 34 patients (43.0%) during the entire treatment period (25 patients [31.6%] during the simeprevir treatment period), which included rash ($n = 30$; 38.0%), erythema ($n = 6$; 7.6%), skin exfoliation ($n = 2$; 2.5%), erythema multiforme ($n = 1$; 1.3%) and photosensitivity reaction ($n = 1$; 1.3%). All rash AE were of grade 1 or 2 severity. None of these AE were serious or led to permanent discontinuation of the study treatment.

Median values over time for selected laboratory parameters are presented in Fig. 4. Median bilirubin values increased transiently during the first two weeks of simeprevir treatment in all patient groups, but returned to baseline levels by week 16 (i.e. within 4 weeks of the end of the simeprevir treatment period). Median levels between weeks 2 and 16 were slightly higher in prior relapsers. Elevation of bilirubin levels was not associated with increases in ALT or AST. Seven patients experienced grade 3 elevations (>2.5 mg/dL) in blood bilirubin and one patient experienced a grade 4 elevation (>5.0 mg/dL). None of the increased bilirubin-related AE led to permanent discontinuation of study treatment. No changes were noted for uric acid or creatinine.

The incidences of grade 3 or 4 treatment-emergent or worsened laboratory abnormalities were low (occurring in <5% of patients), with the exceptions of decreases in absolute neutrophil count (grade 3, 21.5%; grade 4, 2.5%) and increases in bilirubin (grade 3, 8.9%; grade 4, 1.3%).

DISCUSSION

THIS STUDY INVESTIGATED the efficacy and safety of simeprevir in combination with PEG IFN-α-2b/RBV in a mixed population of both treatment-naïve and treatment-experienced patients chronically infected with HCV genotype 1. The dose of simeprevir (100 mg Q.D. as part of triple therapy) and treatment duration (12 weeks) was chosen based on the results of a phase II, dose and duration ranging study in Japanese treatment-naïve patients infected with genotype 1 HCV (DRAGON study).[29]

In this study, simeprevir 100 mg Q.D. for 12 weeks in combination with PEG IFN-α-2b/RBV (administered for a total of 24 or 48 weeks) demonstrated high rates of SVR12 (91.7–100%) and SVR24 (91.7–96.6%) in treatment-naïve patients and prior relapsers. Although

Table 4 Summary of AE during the entire treatment period and during treatment with simeprevir plus PEG IFN-α-2b/RBV (safety population†)

No. of patients (%)	Entire treatment period				Simeprevir + PEG IFN-α-2b/RBV treatment period			
	Treatment-naïve ($n = 24$)	Prior relapsers ($n = 29$)	Prior non-responders ($n = 26$)	Total ($n = 79$)	Treatment-naïve ($n = 24$)	Prior relapsers ($n = 29$)	Prior non-responders ($n = 26$)	Total ($n = 79$)
Discontinuation of any study medication due to AE‡	1 (4.2)	1 (3.4)	1 (3.8)	3 (3.8)	0	1 (3.4)	0	1 (1.3)
Discontinuation of simeprevir alone due to AE	0	0	0	0	0	0	0	0
Temporary interruption of PEG IFN-α-2b due to AE	3 (12.5)	4 (13.8)	3 (11.5)	10 (12.7)	N/A	N/A	N/A	N/A
Dose reduction of PEG IFN-α-2b due to AE	11 (45.8)	4 (13.8)	9 (34.6)	24 (30.4)	N/A	N/A	N/A	N/A
Temporary interruption of RBV due to AE	4 (16.7)	6 (20.7)	4 (15.4)	14 (17.7)	N/A	N/A	N/A	N/A
Dose reduction of RBV due to AE	10 (41.7)	11 (37.9)	9 (34.6)	30 (38.0)	N/A	N/A	N/A	N/A
Any serious AE	1 (4.2)	0	1 (3.8)	2 (2.5)	1 (4.2)	0	0	1 (1.3)
Grade 3/4 AE	6 (25.0)	5 (17.2)	7 (26.9)	18 (22.8)	5 (20.8)	4 (13.8)	5 (19.2)	14 (17.7)
Death	0	0	0	0	0	0	0	0
Common AE§								
Pyrexia	18 (75.0)	27 (93.1)	22 (84.6)	67 (84.8)	17 (70.8)	27 (93.1)	22 (84.6)	66 (83.5)
Decreased white blood cell count	17 (70.8)	16 (55.2)	13 (50.0)	46 (58.2)	17 (70.8)	16 (55.2)	13 (50.0)	46 (58.2)
Anemia	11 (45.8)	21 (72.4)	8 (30.8)	40 (50.6)	11 (45.8)	16 (55.2)	6 (23.1)	33 (41.8)
Malaise	12 (50.0)	12 (41.4)	14 (53.8)	38 (48.1)	12 (50.0)	12 (41.4)	14 (53.8)	38 (48.1)
Headache	11 (45.8)	12 (41.4)	13 (50.0)	36 (45.6)	13 (41.7)	10 (34.5)	12 (46.2)	32 (40.5)
Decreased appetite	12 (50.0)	12 (41.4)	7 (26.9)	31 (39.2)	12 (50.0)	12 (41.4)	7 (26.9)	31 (39.2)
Injection-site reactions	11 (45.8)	8 (27.6)	12 (46.2)	31 (39.2)	10 (41.7)	7 (24.1)	11 (42.3)	28 (35.4)
Rash	12 (50.0)	8 (27.6)	10 (38.5)	30 (38.0)	10 (41.7)	7 (24.1)	8 (30.8)	25 (31.6)
Alopecia	14 (58.3)	9 (31.0)	5 (19.2)	28 (35.4)	Overall incidence <30%			
Arthralgia	11 (45.8)	10 (34.5)	6 (23.1)	27 (34.2)	10 (41.7)	10 (34.5)	6 (23.1)	26 (32.9)
Decreased neutrophil count	11 (45.8)	8 (27.6)	7 (26.9)	26 (32.9)	11 (45.8)	8 (27.6)	7 (26.9)	26 (32.9)
Decreased platelet count	11 (45.8)	6 (20.7)	8 (30.8)	25 (31.6)	11 (45.8)	6 (20.7)	7 (26.9)	24 (30.4)

†All patients who received study drugs. ‡Permanent discontinuation of all study medication (i.e. discontinuation of simeprevir and PEG IFN-α-2b/RBV at the same time, or discontinuation of PEG IFN-α-2b/RBV after completion or discontinuation of simeprevir). §Occurring in >30% patients overall.
AE, adverse events; N/A, not available; PEG IFN, peginterferon; RBV, ribavirin.

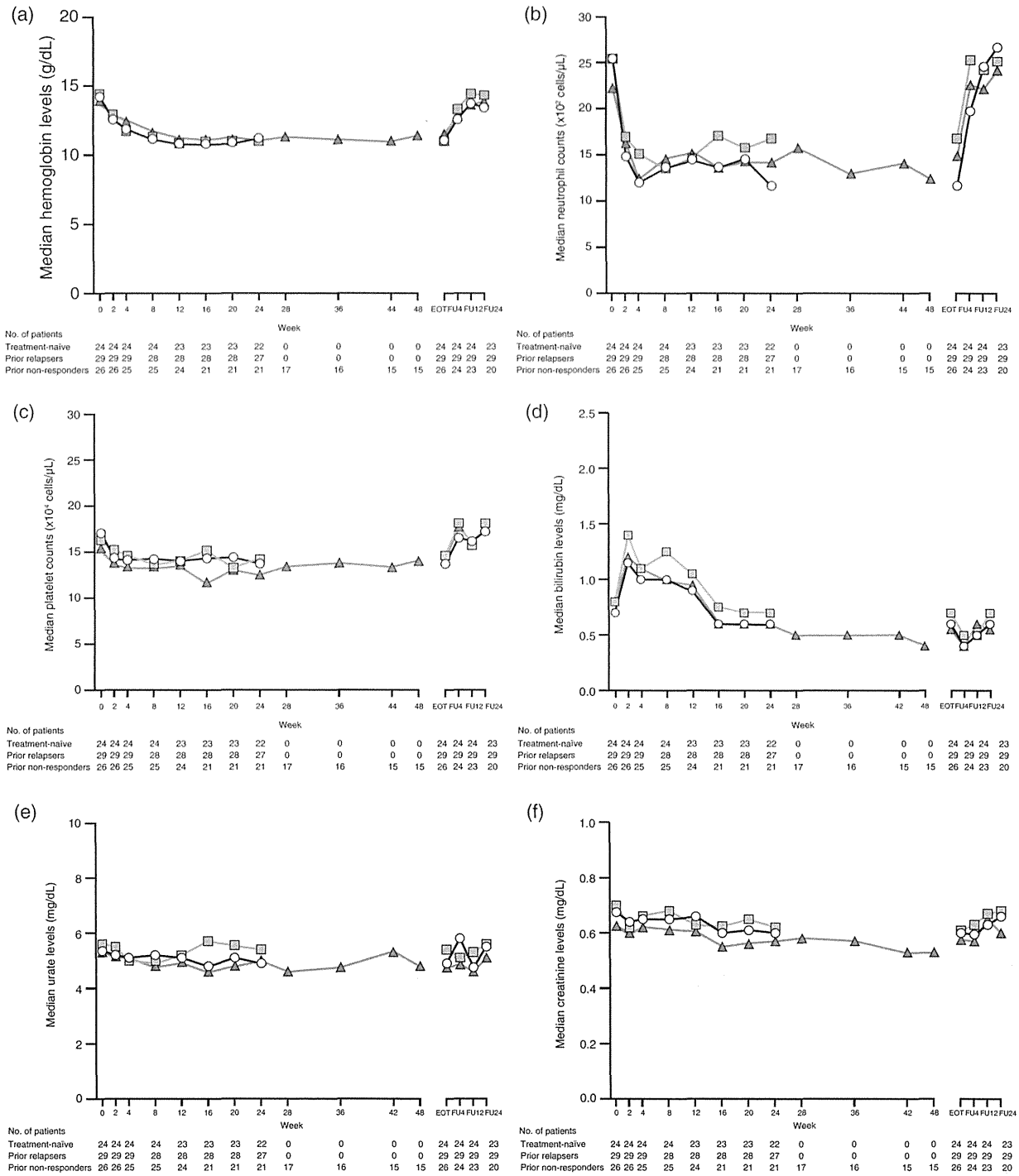

Figure 4 Changes in laboratory parameters over time. (a) Hemoglobin; (b) neutrophil count; (c) platelet count; (d) bilirubin; (e) urate; and (f) creatinine. EOT, end of treatment; FU, follow-up. —○—, treatment-naïve ($n = 24$); —□—, prior relapsers ($n = 29$); —△—, prior non-responders ($n = 26$).

our study is somewhat limited by the small patient numbers and non-comparative design, the results in this study population are consistent with the high rates of virologic response that have been reported previously for simeprevir in combination with PEG IFN-α-2a/RBV in other phase III studies in Japan[25,26] and in international studies.[22–24] In QUEST-2, a phase III study conducted outside Japan, simeprevir combined with PEG IFN-α-2a/RBV or PEG IFN-α-2b/RBV was found superior to each PEG IFN-α/RBV regimen alone in treatment-naïve HCV genotype 1 patients.[30] The response rates also compare favorably with those demonstrated in trials of telaprevir in combination with PEG IFN-α-2b/RBV in treatment-naïve patients and prior relapsers.[11,13]

The rates of SVR among prior non-responders who received 48 weeks of treatment with PEG IFN-α-2b/RBV were lower than those for treatment-naïve patients and prior relapsers, consistent with other studies of prior non-responders.[11,16,22] Most patients had received prior treatment with PEG IFN/RBV. A high proportion of the patients in this group had heterozygous TG genotypes for a polymorphism (rs8099917) in the *IL28B* locus that has been associated with poor response to therapy.[31]

All patient groups showed a rapid reduction in HCV RNA levels within the first two weeks of treatment with simeprevir, and most patients achieved RVR at week 4 (60.0–86.2%) and cEVR at week 12 (79.2–100%). All treatment-naïve patients and prior relapsers had undetectable HCV RNA at the end of treatment, with no viral breakthrough and a very low incidence of viral relapse during follow-up in these two groups. Six (23%) of 26 prior non-responders had viral breakthrough, which was documented during simeprevir treatment for three of these patients. Emerging mutations in the NS3 protease domain were identified for isolates from all six patients at the time of breakthrough and for 16 of the 17 patients classified as treatment failures in the study. All patients except for one prior non-responder were infected with HCV genotype 1b. Some of the mutations identified (D168E/T/V, Q80R/K, R155K) have been previously described in HCV genotype 1b isolates following exposure to simeprevir *in vitro* and in clinical studies[23,25,27] and are thought to confer reduced susceptibility. Data from studies of other PI suggest that poor response to IFN-based therapy increases the likelihood of the emergence of resistant isolates.[6] However, further studies are needed to evaluate emerging mutations in HCV genotype 1 and their clinical impact.

In this study, an RGT approach (based on levels of HCV RNA) was used to determine whether treatment-naïve patients or prior relapsers could reduce the duration of PEG IFN-α-2b/RBV therapy to 24 weeks. More than 90% of these patients were able to stop PEG IFN-α-2b/RBV at 24 weeks rather than continuing to 48 weeks. Those patients who stopped therapy at 24 weeks had high rates of SVR12 and SVR24 (90.9–100%), despite the shorter treatment duration. The reduction in exposure to PEG IFN-α-2b/RBV therapy following initial triple combination therapy with simeprevir could potentially limit the extent and duration of PEG IFN-α-2b/RBV-related AE.

Treatment with simeprevir 100 mg Q.D. in combination with PEG IFN-α-2b/RBV was well tolerated, with mostly grade 1/2 AE. Notably, there was a very low incidence of treatment discontinuation due to AE, and those reported were considered to be related to PEG IFN-α-2b and/or RBV. This is in contrast to studies of first-generation PI, which have reported rates of treatment discontinuation due to AE of 10–20%.[11,12,14] In addition, serious toxicities that have been widely reported with first-generation PI (e.g. anemia, cutaneous reactions, neutropenia)[6,12,13,15,32] were mainly of grade 1 or 2 severity in this study of simeprevir. In phase II studies of simeprevir, mild transient hyperbilirubinemia has been reported.[21] Although patients in this study experienced a transient elevation of blood bilirubin, levels returned to baseline values after simeprevir treatment and the elevation was not associated with increases in ALT or AST levels. Also, there were no treatment discontinuations due to increased bilirubin-related AE. Telaprevir has been associated with increases in uric acid and creatinine.[14] No changes were noted in median uric acid and creatinine values in this study of simeprevir.

In conclusion, treatment with simeprevir 100 mg Q.D. for 12 weeks in combination with PEG IFN-α-2b/RBV (for 24 or 48 weeks) demonstrated potent antiviral activity and high rates of SVR in patients who were treatment-naïve or had previously relapsed after IFN-based therapy, with most patients having a shorter treatment duration. Antiviral activity was also demonstrated in patients who had failed to respond to prior IFN-based therapy. Simeprevir was well tolerated in all patients. The present phase III CONCERTO-4 study demonstrates the efficacy and safety of simeprevir in a relatively small sample of treatment-naïve and previously treated patients with chronic HCV genotype 1 infection, while CONCERTO-1, -2 and -3 provide further data on the efficacy and safety of this regimen in a larger population.

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Elevated Serum Levels of *Wisteria floribunda* Agglutinin-Positive Human Mac-2 Binding Protein Predict the Development of Hepatocellular Carcinoma in Hepatitis C Patients

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The *Wisteria floribunda* agglutinin-positive human Mac-2-binding protein (WFA^+-M2BP) was recently shown to be a liver fibrosis glycobiomarker with a unique fibrosis-related glycoalteration. We evaluated the ability of WFA^+-M2BP to predict the development of hepatocellular carcinoma (HCC) in patients who were infected with the hepatitis C virus (HCV). A total of 707 patients who had been admitted to our hospital with chronic HCV infection without other potential risk factors were evaluated to determine the ability of WFA^+-M2BP to predict the development of HCC; factors evaluated included age, sex, viral load, genotypes, fibrosis stage, aspartate and alanine aminotransferase levels, bilirubin, albumin, platelet count, alpha-fetoprotein (AFP), WFA^+-M2BP, and the response to interferon (IFN) therapy. Serum WFA^+-M2BP levels were significantly increased according to the progression of liver fibrosis stage ($P<0.001$). In each distinctive stage of fibrosis (F0-F1, F2, F3, and F4), the risk of development of HCC was increased according to the elevation of WFA^+-M2BP. Multivariate analysis identified age >57 years, F4, AFP >20 ng/mL, WFA^+-M2BP $\geq$4, and WFA^+-M2BP 1-4 as well as the response to IFN (no therapy vs. sustained virological response) as independent risk factors for the development of HCC. The time-dependent areas under the receiver operating characteristic curve demonstrated that the WFA^+-M2BP assay predicted the development of HCC with higher diagnostic accuracy than AFP. *Conclusion:* WFA^+-M2BP can be applied as a useful surrogate marker for the risk of HCC development, in addition to liver biopsy. (HEPATOLOGY 2014;60:1563-1570)

The annual incidence of hepatocellular carcinoma (HCC) in patients with hepatitis C virus (HCV)-related cirrhosis ranges from 1% to 7%.[1,2] Therefore, reliable methods for the early identification of liver fibrosis progression and compensated liver cirrhosis are an essential part of an efficient surveillance program for the detection of HCC.[3]

Until recently, liver biopsy was considered the gold standard for assessing the severity of liver fibrosis and cirrhosis.[4,5] Although liver biopsy is generally accepted

Abbreviations: Ab, antibody; AFP, alpha-fetoprotein; AIH, autoimmune hepatitis; ALT, alanine aminotransferase; ANOVA, analysis of variance; AST, aspartate aminotransferase; AUROC, area under the ROC; CT, computed tomography; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; IFN, interferon; MRI, magnetic resonance imaging; Peg-IFN, pegylated IFN; RBV, ribavirin; ROC, receiver operating characteristic; RT-PCR, reverse-transcriptase polymerase chain reaction; SVR, sustained virological response; US, ultrasound; WFA^+-M2BP, Wisteria floribunda agglutinin-positive human Mac-2-binding protein.

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to be a safe procedure, it can cause discomfort and carries a small risk of life-threatening complications.[6,7] Recently, an assay for *Wisteria floribunda* agglutinin-positive human Mac-2-binding protein (WFA^+-M2BP) was reported as a novel, noninvasive, and rapid bedside method to assess liver fibrosis.[8] M2BP has been shown to have multibranching and sialylated N-glycans. WFA is considered to recognize the GalNAc residue of N-glycans and O-glycans or the clustered LacNAc (Gal-GlcNAc) structure. Currently, we are analyzing the glycan structures of WFA^+-M2BP in detail using mass spectrometry–based technology.[9] Glycans can reflect the differentiation stage of cells, but not necessarily the level of cellular damage, and therefore they can be very effective markers for chronic disease. In the case of hepatitis, glycans are considered to reflect the progression of fibrosis more specifically than viral load. Several reports have identified M2BP as a potential marker of fibrosis progression in proteome study.[10-13] Kuno et al. were the first to report that a rapid, simple glycan-based immunoassay for WFA^+-M2BP can quantify fibrosis.[8,14]

On the other hand, we reported that alpha-fetoprotein (AFP) is a noninvasive predictive marker for the development of HCC in patients infected with HCV, which can be used to complement the information of fibrosis stage.[15]

In this report, we evaluated the utility of WFA^+-M2BP to predict the development of HCC in patients who were infected with HCV.

Patients and Methods

Patients. Between January 1992 and December 2003, 832 patients were determined to be positive for both anti-HCV by a second- or third-generation enzyme-linked immunoadsorbent assay and HCV RNA by polymerase chain reaction (PCR). They underwent liver biopsy guided by ultrasonography at the National Hospital Organization, Nagasaki Medical Center (Ōmura, Japan). Among them, 125 (15.0%) patients were excluded from enrollment in this retrospective analysis for the following reasons: (1) positivity for hepatitis B surface antigen (n = 12); (2) a heavy habitual drinking habit defined by an average daily consumption of >100 g of ethanol (n = 26); (3) autoimmune hepatitis (AIH), primary biliary cirrhosis, or idiopathic portal hypertension (n = 8); (4) positive antinuclear antibody (Ab; defined as titer >320×) without the diagnosis of AIH (n = 8); or (5) a short follow-up period <180 days (n = 71). The remaining 707 patients were analyzed retrospectively for the incidence of HCC.

For all patients in our cohort, a blood sample was taken on the day of the liver biopsy at our hospital. All samples were preceded to separate serum and stored at −20°C. At the time of blood withdrawal, all patients underwent liver biopsy. Their medical histories had been recorded, along with the results of routine tests for blood cell counts, liver biochemical parameters, and markers for HCV infection at the time of ultrasound (US)-guided liver biopsy and at regular intervals thereafter. Complete blood cell counts and biochemical tests were performed using automated procedures in the clinical pathological laboratories of our hospital.

Staging of Hepatic Fibrosis. Liver biopsies were taken by fine-needle aspiration (16G or 18G sonopsy) guided by US. Liver tissue specimens were fixed in 10% formalin, embedded in paraffin, and stained with hematoxylin and eosin. They were evaluated for the stage of hepatic fibrosis by a pathologist according to the criteria of Desmet et al.[16]

Measurement of WFA^+-M2BP. WFA^+-M2BP quantification was measured based on a lectin-Ab sandwich immunoassay using the fully automatic immunoanalyzer, HISCL-2000i (Sysmex Co., Hyogo, Japan).[8] The measured values of WFA^+-M2BP conjugated to WFA were indexed with the obtained values using the following equation:

$$\text{Cutoff index (COI)} = \left(\left[\text{WFA}^+\text{-M2BP}\right]_{\text{sample}} - \left[\text{WFA}^+\text{-M2BP}\right]_{\text{NC}}\right) / \left(\left[\text{WFA}^+\text{-M2BP}\right]_{\text{PC}} - \left[\text{WFA}^+\text{-M2BP}\right]_{\text{NC}}\right)$$

where $[WFA^+\text{-M2BP}]_{sample}$ is the WFA^+-M2BP count of serum sample, PC is positive control, and NC is negative control. The positive control was supplied as

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a calibration solution preliminarily standardized to yield a COI value of 1.0.[14]

HCV RNA, HCV Core Antigen, and HCV Genotypes. HCV RNA was determined by reverse-transcriptase (RT)-PCR using a commercial kit (Amplicor HCV; Roche Diagnostic Systems, Basel, Switzerland). HCV core antigen was determined using the Lumispot Eiken HCV antigen assay (Eiken Chemicals, Tokyo, Japan). HCV core antigen levels were classified into low and high with a cutoff at 1,000 fmol/mL.[17] Genotypes of HCV were determined by RT-PCR with genotype-specific primers (HCV RNA core genotype; Roche Diagnostics, Tokyo, Japan).[18]

Interferon Therapy. During the observation period, 373 of the 707 (52.8%) patients received interferon (IFN) monotherapy, pegylated (Peg)-IFN monotherapy, or combination therapy with IFN plus ribavirin (RBV) or Peg-IFN plus RBV. Sustained virological response (SVR) was defined as the absence of detectable HCV RNA at the end of 6 months or more of treatment, whereas patients who failed to meet these criteria were judged as having non-SVR. There was no relapse of viremia after 6 months among the SVR patients.

Diagnosis of HCC. Patients were followed up by hematological and biochemical tests at an interval of 1-12 months. Diagnostic imaging by US, computed tomography (CT), and magnetic resonance imaging (MRI) were performed in most patients. HCC was diagnosed by typical vascular patterns on CT, MRI, and angiography or by fine-needle biopsy of space-occupying lesions detected in the liver.

Ethical Considerations. Informed consent was obtained from each patient included in the study, and the study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in the *a priori* approval by the institution's human research committee.

Statistical Analysis. Continuous variables (platelet counts, albumin, total bilirubin, aspartate aminotransferase [AST], alanine aminotransferase [ALT], AFP, HCV core antigen, and WFA^+-M2BP) were dichotomized with respect to the median value or clinically meaningful values in the multivariate analysis. To estimate the cumulative risk of developing HCC, Kaplan-Meier's method and the log-rank test were used. Cox's proportional hazards regression analysis was performed to evaluate risk factors for HCC. Regression analysis was performed to calculate Spearman's rank-correlation coefficient. Kruskal-Wallis' analysis of variance (ANOVA), followed by the Games-Howel's posthoc test, was used to assess whether there were any significant differences in terms of fibrosis stages (F0-F1, F2, F3, and F4). The diagnostic performances of WFA^+-M2BP and AFP for censored development of HCC were assessed by using time-dependent receiver operating characteristic (ROC) curves by examining the area under the ROC (AUROC).[19] Inclusion of variables was assessed using a step-wise selection method. Cochran-Armitage's test for trend was used in the categorical data analysis to assess for the presence of an association between a variable with two categories and a variable with more than three categories. A *P* value of 0.05 was considered statistically significant. Data analysis was performed with SPSS statistical software (version 22.0; (SPSS, Inc., Chicago, IL) and JMP 10 (SAS Institute Inc., Cary, NC).

Table 1. Demographic, Clinical, and Virological Characteristics of the 707 Patients Persistently Infected With HCV

Age, years	57.0 (19-79)
Male, N (%)	351 (49.6)
Observation period, years	8.2 ± 4.4*
IFN therapy	373 (52.8%)
Habitual alcohol intake	135 (19.1%)
Pathological findings	
Fibrosis (N) 0-1/2/3/4	274/193/120/120
Activity (N) 0-1/2/3	199/365/143
Platelet count, $\times 10^4/mm^3$	15.6 (3.0-39.1)
Albumin, g/dL	4.2 (2.7-5.3)
Bilirubin, mg/dL	0.7 (0.1-2.5)
AST, IU/mL	53 (11-422)
ALT, IU/mL	82 (1-1,057)
AFP, ng/mL	6 (0.7-510)
HCV core antigen ≥1,000 fmol/L (%)	539 (76.2)
HCV genotype, N (%) 1b	510 (72.1)
2a/2b	195 (27.6)
Unknown	2 (0.3)
WFA^+-M2BP	1.9 (0.2-19.2)

Values are the medians with ranges in parentheses.
*Results are expressed as the mean ± standard deviation.

Results

Characteristics at Enrollment. The baseline characteristics of the 707 patients at enrollment are summarized in Table 1. Median age was 57.0 years; 120 (17.0%) patients were diagnosed histologically with liver cirrhosis (fibrosis stage F4) and the remaining 587 had chronic hepatitis (fibrosis stage F0, F1, F2, or F3). The median value of AFP was 6 ng/mL. The median value of WFA^+-M2BP was 1.9 (range, 0.2-19.2). The average follow-up period was 8.2 years.

WFA^+-M2BP Value and Fibrosis Stage. The average values (mean ± 1 standard error) for each fibrosis stage were 1.3 ± 0.1 in F0-F1 (n = 274), 2.2 ± 0.1 in F2 (n = 193), 3.3 ± 0.2 in F3 (n = 120),

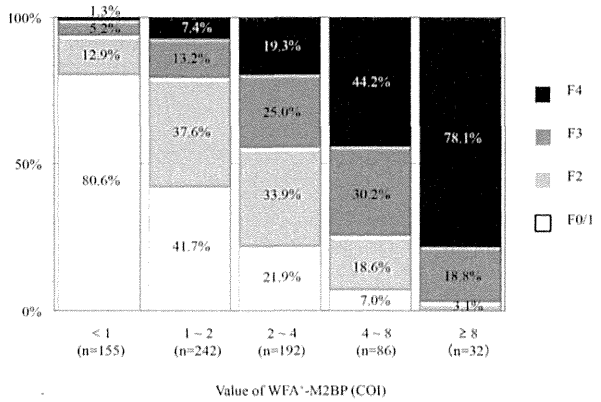

Fig. 1. Proportions of patients with different WFA$^+$-M2BP levels stratified by the fibrosis stage. The proportion of patients with F1 was diminished across increasing quintiles of WFA$^+$-M2BP level ($P < 0.0001$; Cochran-Armitage's trend test), whereas that with F4 was increased ($P < 0.0001$; Cochran-Armitage's trend test).

Table 2. Step-wise Multiple Linear Regression Model to Identify Significant Independent Factors Affecting Serum WFA$^+$-M2BP Level

Final Fitted Model	Adjusted R^2	Standardized Coefficient β	P Value
Fibrosis stage		0.258	<0.001
AFP		0.187	<0.001
Albumin		−0.202	<0.001
AST (1: <53 IU/L; ≥2: 53 IU/L)		0.186	<0.001
Platelet	0.501	−0.147	<0.001
Sex (1: male; 2: female)		0.111	<0.001
HCV core antigen		−0.098	<0.001
Total bilirubin		0.091	0.001
Age		0.071	0.014

and 5.2 ± 0.3 in F4 (n = 120). The degree of fibrosis was positively correlated with the median value of WFA$^+$-M2BP ($P < 0.001$) by a nonparametric method (Kruskal-Wallis' one-way ANOVA). Games-Howel's test confirmed that the WFA$^+$-M2BP value increased significantly with increasing stage of liver fibrosis: $P < 0.0001$ (F0-F1, compared with F2, F3, and F4); $P < 0.0001$ (F2, compared with F3 and F4); and $P < 0.0001$ (F3, compared with F4).

We estimated the diagnostic accuracy of WFA$^+$-M2BP for detecting stage F3-F4 disease. The AUROC in the prediction of ≥F3 was 0.815 (range, 0.782-0.842). The desired specificity level of 95% was achieved for a 4.0 threshold, and the sensitivity was 40.0%.

We analyzed the proportions of the patients with different WFA$^+$-M2BP levels stratified by the fibrosis stage (Fig. 1). The proportion of patients with F1 was 125 cases (80.7%) in WFA$^+$-M2BP <1 (n = 155), 101 cases (41.7%) in WFA$^+$-M2BP ≤1 and <2 (n = 242), 42 cases (21.9%) in WFA$^+$-M2BP ≤2 and <4 (n = 192), 6 cases (7.0%) in WFA$^+$-M2BP ≤4 and <8 (n = 86), and 0 cases (0.0%) in WFA$^+$-M2BP ≥8 (n = 32). The proportion of patients with F1 was diminished across increasing quintiles of WFA$^+$-M2BP level ($P < 0.0001$; Cochran-Armitage's trend test). Conversely, the proportion of patients with F4 was 2 cases (1.3%) in WFA$^+$-M2BP <1 (n = 155), 18 cases (7.4%) in WFA$^+$-M2BP ≤1, and <2 (n = 242), 37 cases (19.3%) in WFA$^+$-M2BP ≤2 and <4 (n = 192), 38 cases (44.2%) in WFA$^+$-M2BP ≤4 and <8 (n = 86), and 25 cases (78.1%) in WFA$^+$-M2BP ≥8 (n = 32). The proportion of patients with F4 was increased with increasing quintiles of WFA$^+$-M2BP level ($P < 0.0001$; Cochran-Armitage's trend test).

Relationship Between the WFA$^+$-M2BP Value and Baseline Biochemical Markers. To determine whether the WFA$^+$-M2BP value was associated with fibrosis stage, age, gender, platelet count, albumin, bilirubin, AST, ALT, AFP, HCV core antigen, HCV genotype, or histological grading, a step-wise multiple linear regression analysis was performed. Our results showed that independent variables, except for ALT, genotype, and histological grading, remained in the final equation (Table 2), suggesting that fibrosis stage was most closely associated with serum WFA$^+$-M2BP value (coefficient β, 0.258; $P < 0.001$).

Risk Factors for HCC. Cox's regression analysis was performed on several variables, including age, sex, alcohol consumption, IFN therapy during the observation period, biochemical and virological parameters, and serum WFA$^+$-M2BP level. The following factors were identified as posing an increased risk for HCC by the univariate analysis: age; response to IFN therapy (no therapy vs. SVR; $P < 0.001$); fibrosis stage (F3 and F4 vs. F0-F1; $P < 0.001$); platelet count (<15 × 10^4/mm^3 vs. ≥15 × 10^4/mm^3; $P < 0.001$); albumin (<4.2 vs. ≥4.2 g/mL; $P < 0.001$); AST (<53 vs. ≥53 IU/mL; $P < 0.001$), ALT (<82 vs. ≥82 IU/mL; $P = 0.035$), and AFP levels (≥20 and 6-20 vs. <6 ng/mL; $P < 0.001$); HCV genotype (1b vs. non-1b; $P = 0.025$); and serum WFA$^+$-M2BP level (≥4 and 1-4 vs. <1; $P < 0.001$). Multivariate analysis was performed on these factors (Table 3) and the following were identified as independent risk factors: fibrosis stage (F4); AFP (≥20 ng/mL); age (≥57 years); response to IFN therapy (no therapy vs. SVR); and WFA$^+$-M2BP (1-4 and ≥4).

Development of HCC. During the follow-up period, HCC developed in 110 (15.6%) patients. Of

Table 3. Factors Associated With Risk for HCC*

Features		HR (95% CI)	*P* Value
Fibrosis	F0-F1	1	
	F2	0.883 (0.411-1.897)	0.749
	F3	1.347 (0.624-2.906)	0.448
	F4	3.133 (1.536-6.390)	0.002
AFP	<6 ng/mL	1	
	6-20 ng/mL	1.710 (0.963-3.038)	0.067
	≥20 ng/mL	3.417 (1.807-6.460)	<0.001
Age	<57 years	1	
	≥57 years	2.039 (1.278-3.252)	0.003
IFN therapy	No therapy	1	
	Non-SVR	0.729 (0.467-1.137)	0.163
	SVR	0.089 (0.027-0.288)	<0.001
WFA^+-M2BP	<1	1	
	1-4	5.155 (1.180 – 22.500)	0.029
	≥4	8.318 (1.784 – 38.791)	0.007

Abbreviations: HR, hazard ratio; CI, confidence interval.
*Determined by multivariate analysis.

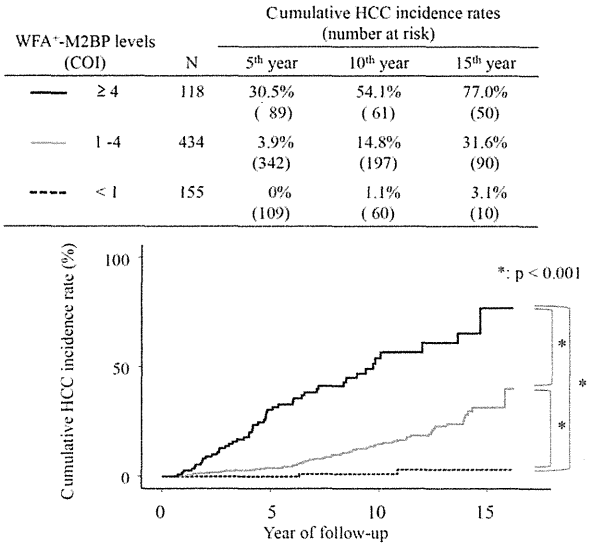

Fig. 2. Cumulative incidence of HCC according to WFA^+-M2BP level. Cumulative incidences of HCC according to the WFA^+-M2BP level were analyzed using Kaplan-Meier's method. Black solid, gray solid, and dotted lines indicate stratified WFA^+-M2BP level, ≥4, 1-4, and <1, respectively. Incidence rate differed significantly among the three groups ($P < 0.001$, by the log-rank test), increasing in accord with WFA^+-M2BP level.

the 110 patients with HCC, 58 (52.7%) were diagnosed with the disease by histological examination of biopsy-obtained or resected liver specimens. Of these 58 patients, 24 (41.3%) had hypovascular HCC.

Figure 2 shows the relation between Kaplan-Meier's estimates of the cumulative risk of HCC and the different WFA^+-M2BP levels at entry. The 10-year cumulative risk of HCC was 1.1% in the patients with WFA^+-M2BP <1 at entry, 14.8% among the patients with WFA^+-M2BP 1-4, and 54.1% in patients with WFA^+-M2BP >4. The incidence rate differed significantly among the three groups ($P < 0.001$, by the log-rank test), increasing in accord with WFA^+-M2BP level.

Figure 3 shows the relation between the cumulative incidence of HCC and WFA^+-M2BP levels, stratified by the fibrosis stage. In patients with fibrosis stage F0-F1, there were significant differences in HCC incidence between those with WFA^+-M2BP levels of 1-4 and those with levels of <1 ($P < 0.01$) and between those with WFA^+-M2BP levels of ≥4 and those with levels of <1 ($P < 0.01$). In patients with fibrosis stage F2-F3, there were significant differences in HCC incidence between those with WFA^+-M2BP levels of ≤1 and those with levels of >4 ($P < 0.01$) and between those with WFA^+-M2BP levels of 1-4 and those with levels of >4 ($P < 0.001$). In patients with fibrosis stage F4, there were significant differences in HCC incidence between those with WFA^+-M2BP levels of 1-4 and those with levels of >4 ($P < 0.05$). As with WFA^+-M2BP levels, incidence rates increased with fibrosis stage, and the change in incidence was significant for each fibrosis stage.

Predictive Accuracy of Cumulative Incidence of HCC Compared With WFA^+-M2BP and AFP. AUROC analyses for prediction of the development of HCC at 1, 2, 3, 5, 7, and 10 years (range) were 0.762 (0.553-0.971), 0.792 (0.669-0.915), 0.832 (0.751-0.914), 0.858 (0.805-0.911), 0.821 (0.767-0.876), and 0.800 (0.745-0.855) in WFA^+-M2BP and 0.791 (0.684-0.898), 0.790 (0.723-0.857), 0.772 (0.693-0.850), 0.800 (0.741-0.858), 0.796 (0.745-0.848), and 0.821 (0.773-0.868) in AFP, respectively. The WFA^+-M2BP assay was superior to AFP for predicting the development of HCC at 3, 5, and 7 years.

Discussion

Liver biopsy has long been considered the gold standard for assessment of hepatic fibrosis,[20-23] and the Metavir[24] and Desmet et al.[16] staging systems are most commonly used. A higher degree of liver fibrosis is known to be the strongest risk factor for hepatocarcinogenesis in hepatitis C patients.[1,20] However, it also has its limitations for the staging of fibrosis because of the heterogeneous distribution of fibrosis in the liver,[25] and liver biopsy is an invasive procedure with

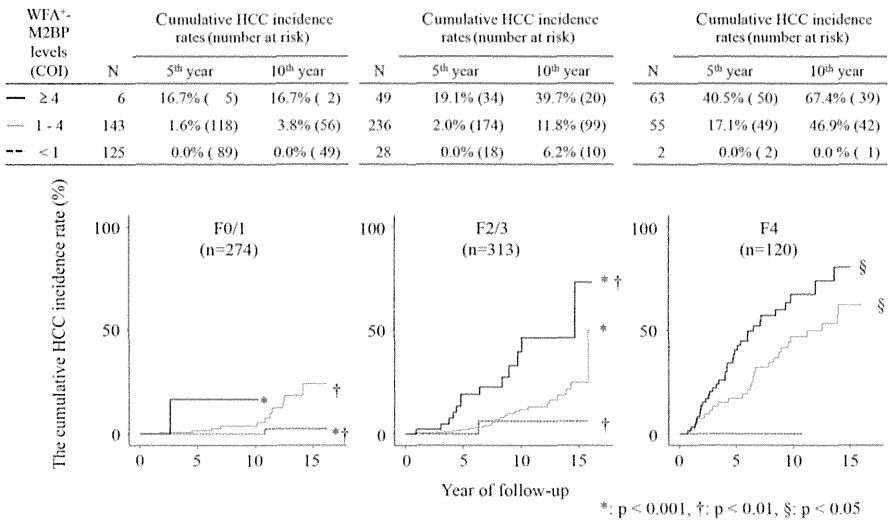

Fig. 3. Cumulative incidence of HCC according to WFA$^+$-M2BP levels, stratified by the fibrosis stage. Cumulative incidences of HCC, according to the WFA$^+$-M2BP level, stratified by the fibrosis stage were analyzed using Kaplan-Meier's method. Black solid, gray solid, and dotted lines indicate stratified WFA$^+$-M2BP level, ≥4, 1-4, and <1, respectively. Incidence rates increased in accord with WFA$^+$-M2BP level.

associated morbidity (pain, bleeding, or hemobilia).[26] For these reasons, patients are often reluctant to undergo this invasive procedure and instead choose one of several noninvasive methods available for assessing the degree of liver fibrosis.

Nevertheless, in the past, no significant progress was made in the development of noninvasive biomarkers to guide clinical usage. WFA$^+$-M2BP was recently validated as a liver fibrosis glycobiomarker with a fully automated immunoassay.[8] In the present study, we assessed the performance of the WFA$^+$-M2BP assay in comparison with liver fibrosis stage and several serum markers, and, based on the results, we estimated whether WFA$^+$-M2BP is a useful predictor of the development of HCC as well as liver biopsy stage.

The first main finding of our study was that there was a significant correlation between the WFA$^+$-M2BP value and the fibrosis stage (Fig. 1). Moreover, step-wise multiple linear regression analysis showed that liver fibrosis stage was most closely associated with serum WFA$^+$-M2BP level. In addition, the degree of necroinflammation had no apparent effect on the WFA$^+$-M2BP value. Based on these results, we proposed a clinical management algorithm using a WFA$^+$-M2BP assay to predict the fibrosis stage. This approach could be used reliably for the first-line pretherapeutic evaluation of fibrosis in HCV-infected patients. On the other hand, the most widely used noninvasive techniques have recently shifted to physical measurements, such as FibroScan,[27-30] acoustic radiation force impulse, and real-time strain elastography. FibroScan has the advantages of being rapid and technically simple; however, operator skill affects its diagnostic success rate. Also, stiffness measurements can be difficult to obtain in obese patients and impossible in patients who have ascites. This is regarded as a limitation of transient elastography.[27,28] Therefore, we suggest that FibroScan, in conjunction with an assay of serum fibrosis biomarkers, would improve the diagnostic accuracy.

The second main finding of our study was the significant association between the WFA$^+$-M2BP level and the risk of HCC development in hepatitis C patients (Figs. 2 and 3). The diagnostic performance of WFA$^+$-M2BP, based on the AUROC values, was superior to that of AFP for predicting the development of HCC at 3, 5, and 7 years. The WFA$^+$-M2BP value can be used as a noninvasive predictor of HCC development and can be considered a surrogate marker for liver fibrosis. Various risk factors have been reported for HCC development among patients with HCV, including older age,[1] male sex,[1] heavy alcohol consumption,[31] obesity,[32] cirrhosis,[1,33] lower platelet count,[34-36] high serum AFP level,[15,36-44] low serum albumin level,[31] and high serum ALT and AST level.[45-47] Our results were consistent with these findings. Among them, liver fibrosis stage was the strongest prognostic indicator of chronic hepatitis. However, liver biopsy has several disadvantages. In our study, we have shown that the WFA$^+$-M2BP value is also a significant risk factor of HCC development independent of these factors. However, even though WFA$^+$-M2BP can be considered a surrogate marker for liver fibrosis, a distinct advantage of WFA$^+$-M2BP over liver biopsy is its wider dynamic range for the evaluation of liver cirrhosis. In the Metavir and Desmet et al. scoring systems, cirrhosis is represented by a single category (F4). However, the degree of fibrosis may vary widely

among patients in this category, and the risk of HCC may not be uniform. In our study, the risk of HCC development increased with increasing WFA^+-M2BP level as well as with increasing fibrotic stage. According to the elevation of WFA^+-M2BP value, the risk of development of HCC was increased (Fig. 3). In other words, each fibrosis stage can be further stratified with clinical relevance based on the WFA^+-M2BP level.

In our study, multivariate analysis identified fibrosis stage, high AFP level, older age, SVR to IFN therapy (no therapy vs. SVR), and high WFA^+-M2BP value as independent predictors of HCC development. The stratified WFA^+-M2BP value was independently associated with HCC development. These results indicate that the correlation between high WFA^+-M2BP and HCC development remains significant, even if HCC develops from a noncirrhotic background. Tateyama et al.[15] reported that AFP was a noninvasive predictive marker for the development of HCC in this same cohort; furthermore, not only high AFP levels ($\geq$20 ng/mL), but also slightly elevated AFP levels of between 6 and 20 ng/mL could indicate substantial risks for the development of HCC, complementing the fibrosis stage. Our present study was redesigned by the addition of one parameter (WFA^+-M2BP). Multivariate analysis did not identify slightly elevated AFP levels (6-20 ng/mL) as an independent risk factor, but did identify both stratified WFA^+-M2BP levels (1-4 and $\geq$4) as independent risk factors. Also, the time-dependent AUROC analysis suggested that WFA^+-M2BP is superior to AFP as a predictor for the development of HCC. These results mean that the WFA^+-M2BP level is the most reliable noninvasive predictive marker for the development of HCC in patients infected with HCV.

One of the limitations of the present study is that this cohort of 707 patients was analyzed retrospectively. There is thus need of a future study to prospectively analyze the efficacy of WFA^+-M2BP as a predictor of HCC development.

Another limitation is that the hepatocarcinogenesis of the patients who underwent IFN therapy was not evaluated. In this study, among the patients who achieved SVR (n = 139), 3 cases developed HCC during the follow-up period. The WFA^+-M2BP titers were 6.4, 5.6, and 1.5, respectively, in the 3 patients. All 3 cases obtained titers higher than 1, and 2 cases obtained titers higher than 4. This result suggests that patients with a high WFA^+-M2BP value should be monitored for the development of HCC even after achieving SVR. However, future assessments of the WFA^+-M2BP values at IFN administration and at posttreatment will be needed to verify this recommendation.

In conclusion, this study revealed an association between WFA^+-M2BP and the risk of HCC development in chronic hepatitis C patients. The results suggested that the WFA^+-M2BP assay should not be limited to use as a surrogate for liver biopsy, but rather could be applied as dynamic indicator of the risk of HCC development.

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