Table 1. Characteristics of Patients Enrolled in the Present Study.

Factors	Value
Patients, n	238
Age, year	55.0 (18–75)
Male, n (%)	147 (61.8)
BMI (kg/m^2)	23.20 (16.7–34.9)
Alcohol intake (> 20g/day), n (%)	64 (26.9)
Fibrosis stage, n (%) F 1/2/3/4	104 (43.7)/68 (28.6)/42 (17.6)/24 (10.1)
Steatosis (≥ 10%), n (%)	25(10.5)
Pre-Tx platelet counts (×10^3/μL)	16.0 (6.4–33.2)
Post-Tx platelet counts (×10^3/μL)	16.8 (6.5–36.3)
Albumin (g/dL)	4.30 (2.9–5.5)
Pre-Tx AST (IU/mL)	60.0 (12–365)
Post-Tx AST (IU/mL)	20.0 (10–54)
Pre-Tx ALT (IU/mL)	100.0 (12–519)
Post-Tx ALT (IU/mL)	17.0 (7–64)
γ-GTP (IU/L)	37.0 (7–1790)
T. Bilirubin (mg/dL)	0.70 (0.3–1.9)
HbA1c (%)	5.70 (4.4–8.1)
Pre-Tx AFP (ng/mL)	5.0 (1–200)
Post-Tx AFP (ng/mL)	3.0 (1–46)
Pre-Tx WFA$^+$-M2BP (COI)	1.70 (0.28–12.04)
Post-Tx WFA$^+$-M2BP (COI)	0.80 (0.17–5.29)
HCV serogroup, n (%)	
1	111 (46.6)
2	101 (42.4)
Unknown	26 (11.0)
IFN regimen, n (%)	
IFN monotherapy	123 (51.6)
PEG-IFN monotherapy	28 (11.8)
IFN/PEG-IFN+RBV	87 (36.6)
Observation period, years	9.1 (5.6) *

Data are given as the medians with ranges.

*Results are expressed as the means ± standard deviation. Unless otherwise indicated, data were collected at pre-treatment (before administration of IFN therapy; pre-Tx). Several biochemical measurements were made at both pre-Tx and post-treatment (24 weeks after completion of IFN therapy; post-Tx).

Abbreviations: AST, aspartate aminotransferase; ALT, alanine aminotransferase; γ-GTP, γ-glutamyl transpeptidase; HbA1c, glycated hemoglobin; BMI, body mass index; AFP, α-fetoprotein; HCV, hepatitis C virus; PEG-IFN, pegylated interferon; RBV, ribavirin.

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Risk factors for HCC

Univariate analysis demonstrated factors that increase the risk for HCC development after SVR. Cox regression analysis was performed on 20 variables: age, sex, BMI, alcohol intake, fibrosis stage, degree of steatosis, pre-Tx platelet counts, post-Tx platelet counts, albumin, pre-Tx AST, post-Tx AST, pre-Tx ALT, post-Tx ALT, γ-GTP, T.bilirubin, HbA1c, pre-Tx AFP, post-Tx AFP, pre-Tx WFA$^+$-M2BP, post-Tx WFA$^+$-M2BP. Cutoff values for AFP and WFA$^+$-M2BP were determined by time-dependent ROC analysis as 5 ng/ml and 2.0 COI, respectively.

The following seven factors were identified as posing an increased risk for HCC by the univariate analysis: age, fibrosis stage, albumin, pre-Tx platelet count, post-Tx platelet count, post-Tx AFP, and post-Tx WFA$^+$-M2BP (Table 2).

Multivariate analysis was performed on these seven factors, and the following four factors were identified as independent risk factors: age (> 60 years, HR 5.42, 95% CI = 1.59–18.47, $P = 0.007$), sex (male, HR 4.71, 95% CI = 1.23–17.92, $P = 0.023$), pre-Tx platelet count (< 15.0×10^3/μL, HR 4.72, 95% CI = 1.45–15.30, $P = 0.010$), and post-Tx WFA$^+$-M2BP values (> 2.0 COI, HR 5.71, 95% CI = 1.66–19.57, $P = 0.006$).

Development of HCC

To evaluate the relation between post-Tx WFA$^+$-M2BP values and development of HCC, we characterized 238 patients who achieved SVR with respect to their post-Tx WFA$^+$-M2BP values. Fig 1 shows the cumulative risk of HCC and the post-Tx WFA$^+$-M2BP values. The 5 and 10-year cumulative risks of HCC were 1.9% and 5.0% in the 18 patients with post-Tx WFA$^+$-M2BP > 2.0 COI (post-Tx WFA$^+$-M2BP > 2.0 COI group), and 22.6% and 38.1% in the 220 patients with post-Tx WFA$^+$-M2BP ≤ 2.0 COI (post-Tx WFA$^+$-M2BP ≤ 2.0 COI group). The incidence rates were significantly higher in the post-Tx WFA$^+$-M2BP > 2.0 COI group ($P < 0.0001$ by the log-rank test).

Fig 2 shows the relation between the cumulative incidence of HCC and the post-Tx WFA$^+$-M2BP values, stratified by age. In the patients with age > 60 years, the 5- and 10-year cumulative risks of HCC were 27.3% and 45.5% for the post-Tx WFA$^+$-M2BP > 2.0 COI group. On the other hand, in the patients with age ≤ 60 years, the 5- and 10-year cumulative risks of HCC were 1.3% and 2.6% for the post-Tx WFA$^+$-M2BP ≤ 2.0 COI group. There were significant differences in HCC incidence between the post-Tx WFA$^+$-M2BP > 2.0 COI group and post-Tx WFA$^+$-M2BP ≤ 2.0 COI group for both age categories ($P < 0.0001$ by the log-rank test).

Fig 3 shows the relation between the cumulative incidence of HCC and the post-Tx WFA$^+$-M2BP values, stratified by stage of fibrosis. In the patients with F3/4, the 5- and 10-year cumulative risks of HCC were 25.9% and 62.9% for the post-Tx WFA$^+$-M2BP > 2.0 COI group. On the other hand, in the patients with F1/2, the 5- and 10-year cumulative risks of HCC were 1.3% and 3.9% for the post-Tx WFA$^+$-M2BP ≤ 2.0 COI group. There were significant differences in HCC incidence between the post-Tx WFA$^+$-M2BP > 2.0 COI group and post-Tx WFA$^+$-M2BP ≤ 2.0 COI group with advanced fibrosis (F3/4) patients ($P < 0.01$ by the log-rank test).

Predictive value of HCC incidence versus WFA$^+$-M2BP and AFP

Table 3 shows the AUROC analyses for prediction of the development of HCC at 3, 5 and 10 years with AFP and WFA$^+$-M2BP. The post-Tx WFA$^+$-M2BP was superior to the post-Tx AFP for predicting the development of HCC at each of 3, 5 and 10 years.

Chronological changes in the WFA$^+$-M2BP and AFP values after IFN treatment

In the 238 patients with SVR, the median values of the chronological change in WFA$^+$-M2BP at pre-Tx and post-Tx were 1.70 (range: 0.28 to 12.04 COI) and 0.80 (range: 0.17 to 5.29 COI). The post-Tx WFA$^+$-M2BP values were significantly decreased relative to the pre-Tx WFA$^+$-M2BP values ($P < 0.001$).

Next, we analyzed the WFA$^+$-M2BP and AFP values in the 16 patients who developed HCC. Fig 4A shows the chronological changes in WFA$^+$-M2BP and AFP values at pre-Tx, post-Tx, and the time of HCC development for the 16 patients. The median WFA$^+$-M2BP

Table 2. Factors Associated with Hepatocellular Carcinoma.

Univariate analysis		Hazard Ratio (95% CI)	*P*	Hazard Ratio (95% CI) Multivariate analysis	*P*
Age (year)	≤ 60	1		1	
	> 60	6.09 (2.03–18.26)	0.001	5.42 (1.59–18.47)	0.007
Sex	Female	1		1	
	Male	1.12	0.290	4.71 (1.23–17.92)	0.023
BMI (kg/m^2)	≤ 23.0	1			
	> 23.0	2.09 (0.71–6.11)	0.167		
Alcohol intake (g/day)	≤ 20	1			
	> 20	1.60 (0.58–4.42)	0.364		
Fibrosis stage	F1/2	1			
	F3/4	4.62 (1.67–12.81)	0.003		
Steatosis (%)	≤ 10	1			
	> 10	1.12 (0.30–5.69)	0.561		
Pre-Tx Platelet counts	≥ 15.0	1		1	
(×10^3/μL)	< 15.0	4.75 (1.52–14.79)	0.007	4.72 (1.45–15.30)	0.010
Post-Tx Platelet counts	≥ 15.0	1			
(×10^3/μL)	< 15.0	3.21 (1.20–12.96)	0.011		
Albumin (g/dL)	≥ 4.0	1			
	< 4.0	3.40 (1.22–9.45)	0.018		
Pre-Tx AST (IU/mL)	≤ 60	1			
	> 60	2.13 (0.74–6.14)	0.146		
Post-Tx AST (IU/mL)	≤ 20	1			
	> 20	2.33 (0.30–18.29)	0.473		
Pre-Tx ALT (IU/mL)	≤ 80	1			
	> 80	2.16 (0.79–5.83)	0.128		
Post-Tx ALT (IU/mL)	≤ 15	1			
	> 15	2.44 (0.72–8.31)	0.180		
γ-GTP (IU/L)	≤ 40	1			
	> 40	1.73 (0.60–4.99)	0.297		
T. Bilirubin (mg/dL)	≤ 1.0	1			
	> 1.0	1.66 (0.37–7.35)	0.481		
HbA1c (%)	≤ 5.5	1			
	> 5.5	1.07 (0.26–4.46)	0.929		
Pre-Tx AFP (ng/mL)	≤ 5.0	1			
	> 5.0	2.50 (0.89–7.06)	0.079		
Post-Tx AFP (ng/mL)	≤ 5	1			
	> 5	4.60 (1.53–13.84)	0.006		
Pre-Tx WFA$^+$-M2BP	≤ 2.0	1			
(COI)	> 2.0	1.37 (0.49–3.77)	0.551		
Post-Tx WFA$^+$-M2BP	≤ 2.0	1		1	
(COI)	> 2.0	7.30 (2.20–24.17)	0.001	5.71 (1.66–19.57)	0.006

Hazard ratios for the development of hepatocellular carcinoma were calculated by Cox proportional hazards analysis.

Abbreviations: AST, aspartate aminotransferase; ALT, alanine aminotransferase; γ-GTP, γ-glutamyl transpeptidase; HbA1c, glycated hemoglobin; BMI, body mass index; AFP, α-fetoprotein; HCV, hepatitis C virus; WFA$^+$-M2BP, *Wisteria floribunda* agglutinin-positive human Mac-2 binding protein.

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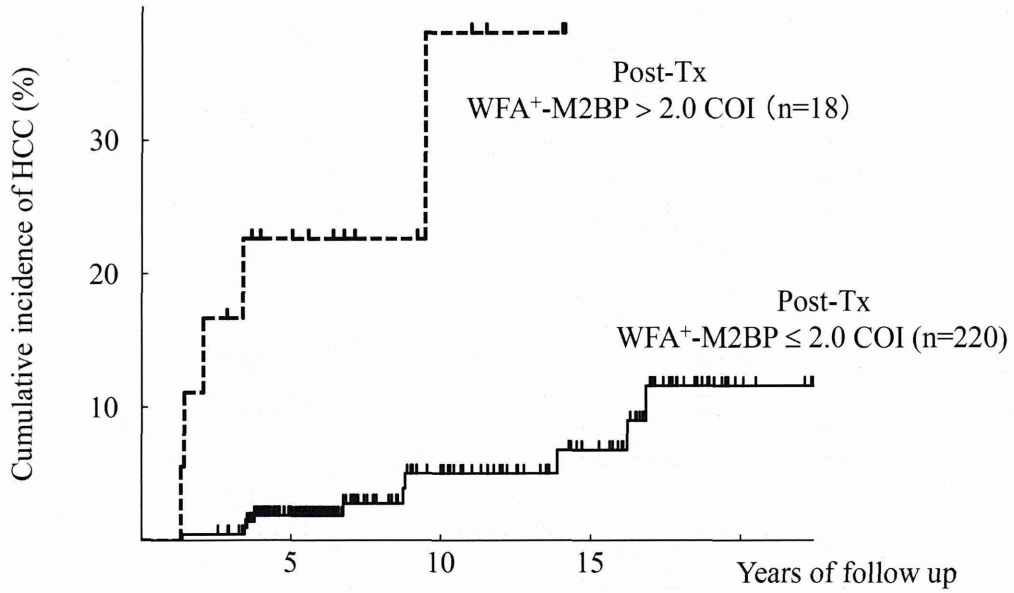

Post-Tx WFA+-M2BP > 2.0 COI (n=18, patient at risk)	22.6% (11)	38.1% (4)
Post-Tx WFA+-M2BP ≤ 2.0 COI (n=220, patient at risk)	1.9% (168)	5.0% (77)

Fig 1. Cumulative incidence of hepatocellular carcinoma (HCC) according to post-treatment WFA+-M2BP values. Cumulative incidences of HCC according to post-treatment WFA+-M2BP values were analyzed using the Kaplan-Meier method. The black solid and dotted lines indicate the stratified post-treatment WFA+-M2BP values with a COI ≤ 2.0 and a COI > 2.0, respectively. The incidence rate differed significantly between the two groups ($P < 0.0001$ by the log-rank test). The numbers of patients at risk at each time point are shown below the graphs.

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values of the 16 patients who developed HCC at pre-Tx, post-Tx and the time of HCC development were 2.07 (range: 0.99 to 8.04 COI), 1.24 (range: 0.42 to 4.44 COI) and 0.79 (range: 0.41 to 2.79 COI). The median AFP values of 16 patients at pre-Tx, post-Tx and the time of HCC development were 8 (range: 2 to 63 ng/mL), 5 (range: 1 to 7 ng/mL) and 7 (range: 3 to 5463 ng/mL). The WFA+-M2BP values at the time of post-Tx were significantly lower than those at the time of pre-Tx ($P < 0.001$). Additionally, the WFA+-M2BP values at the time of HCC development were significantly lower than those at the time of post-Tx ($P < 0.001$). The AFP values at the time of post-Tx were significantly lower than those at the time of pre-Tx ($P < 0.001$). In contrast, the AFP values at the time of HCC development were significantly higher than those at the time of post-Tx ($P < 0.001$).

We also analyzed the WFA+-M2BP and AFP values in the 222 patients who did not develop HCC. The median WFA+-M2BP values of the 222 patients who did not develop HCC at pre-Tx, post-Tx and the last clinical visit were 1.69 (range: 0.28 to 12.04 COI), 0.79 (range: 0.17 to 5.29 COI) and 0.74 (range: 0.14 to 7.24 COI). The median AFP values of the 222 patients at pre-Tx, post-Tx and the last clinical visit were 5 (range: 1 to 200 ng/mL), 3 (range: 1 to 46 ng/mL) and 3 (range: 1 to 11 ng/mL). The WFA+-M2BP values at the time of post-Tx were significantly lower than those at the time of pre-Tx ($P < 0.001$). In addition, the WFA+-M2BP values at the time of last clinical visit were significantly lower than those at the time of post-Tx ($P < 0.001$). The AFP values at the time of post-Tx were significantly lower than those at the

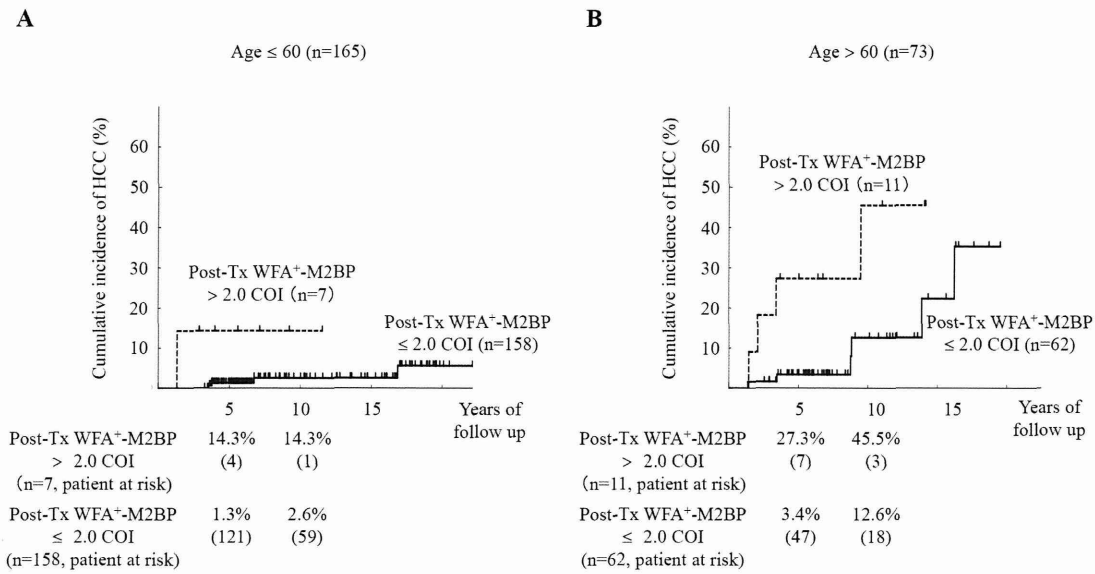

Fig 2. Cumulative incidence of hepatocellular carcinoma (HCC) according to post-treatment WFA$^+$-M2BP values, stratified by age. (A): Age ≤ 60 years (n = 165). (B): Age > 60 years (n = 73). Cumulative incidences of HCC according to post-treatment WFA$^+$-M2BP values were analyzed using the Kaplan-Meier method. The black solid and dotted lines indicate the stratified post-treatment WFA$^+$-M2BP values with a COI ≤ 2.0 and a COI > 2.0, respectively. The incidence rate differed significantly between the two groups ($P < 0.0001$ by the log-rank test). The numbers of patients at risk at each time point are shown below the graphs.

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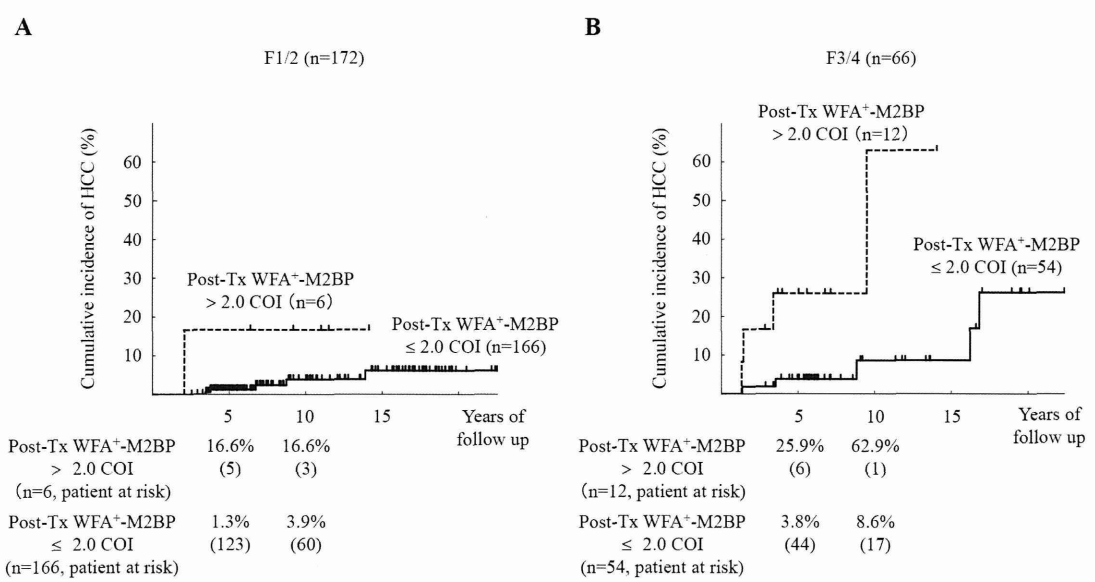

Fig 3. Cumulative incidence of hepatocellular carcinoma (HCC) according to post-treatment WFA$^+$-M2BP values, stratified by stage of fibrosis. (A): F1/2 (n = 172). (B): F3/4 (n = 66). Cumulative incidences of HCC according to post-treatment WFA$^+$-M2BP values were analyzed using the Kaplan-Meier method. The black solid and dotted lines indicate the stratified post-treatment WFA$^+$-M2BP values with a COI ≤ 2.0 and a COI > 2.0, respectively. There were no significant differences in HCC incidence with F1/2 group ($P = 0.09$ by the log-rank test). On the other hand, the incidence rate differed significantly with F3/4 group ($P < 0.01$ by the log-rank test). The numbers of patients at risk at each time point are shown below the graphs.

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Table 3. Areas under the Curve for Censored Development of HCC at 3, 5, and 10 Years in the WFA+-M2BP and AFP Group.

Factors	3 years	5 years	10 years
Pre-Tx AFP (ng/mL)	0.678 (0.508–0.849)	0.710 (0.596–0.825)	0.620 (0.482–0.758)
Post-Tx AFP (ng/mL)	0.884 (0.833–0.934)	0.782 (0.644–0.919)	0.631 (0.445–0.816)
Pre-Tx WFA+-M2BP (COI)	0.603 (0.331–0.874)	0.621 (0.410–0.831)	0.604 (0.443–0.764)
Post-Tx WFA+-M2BP (COI)	0.909 (0.788–1.000)	0.812 (0.670–0.955)	0.707 (0.545–0.868)

Abbreviations: AFP, α-fetoprotein; WFA+-M2BP, *Wisteria floribunda* agglutinin-positive human Mac-2 binding protein.

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time of pre-Tx ($P < 0.001$). Similarly, the AFP values at the time of last clinical visit were significantly higher than those at the time of post-Tx ($P < 0.001$) (Fig 4B).

Fig 5 shows the distribution of post-Tx WFA+-M2BP values. Among the 238 patients who achieved SVR, only 18 (7.6%) patients had post-Tx WFA+-M2BP > 2.0 COI. During the follow-up period, 5 patients (27.8%) developed HCC in the post-Tx WFA+-M2BP > 2.0 COI group (n = 18), and 4 of these 5 cases developed HCC within 5 years after IFN treatment. In contrast, 11 patients (5.0%) developed HCC in the post-Tx WFA+-M2BP ≤ 2.0 COI group (n = 220) ($P < 0.001$).

Discussion

Previous studies have shown various risk factors for HCC development among patients with SVR: older age [14,15,16,18,19,33], male gender [19], heavy alcohol consumption [15,16], steatosis [33], advanced fibrosis [15,16,19], and lower platelet count [18]. In addition, recent studies reported that AFP values were significantly associated with HCC [18,27,34], and were also valuable for predicting future HCC risk after IFN treatment [35]. Asahina reported that post-IFN treatment AFP values were significantly associated with hepatocarcinogenesis [36]. In the present study, we specifically analyzed whether high WFA+-M2BP values might also be risk factors for HCC in patients with SVR.

In our study, the cumulative 5- and 10-year incidences of HCC were 3.4% and 7.5%. These results were consistent with previous studies, which reported that the cumulative incidences of HCC after SVR were 1.1–5.8% and 5.5–11.1% at 5 and 10 years [14,15,18,36–39], and the annual incidences were 0.37–1.21%/year [13,40,41].

The first main finding of our study was that the post-Tx WFA+-M2BP was selected as a new predictive marker for development of HCC among patients with SVR (Table 2). The values of WFA+-M2BP for predicting the development of HCC were determined to have a COI of 2.0 by time-dependent receiver operator characteristics (ROC) analysis. The cumulative incidence was significantly higher in the post-Tx WFA+-M2BP > 2.0 COI group. We were able to stratify the patients into different risk groups using the post-Tx WFA+-M2BP values and another simple risk factor, for example, age (Fig 2). Older age has been reported to confer a risk for hepatocellular carcinoma [14], which is an important association in Japan due to the aging of the population. Moreover, the post-Tx WFA+-M2BP values were significant predictor for HCC among patients with F3/4. Cumulative incidence of HCC was significantly higher in patients with higher post-Tx WFA+-M2BP values when patients were stratified by the stage of fibrosis (Fig 3). The post-Tx WFA+-M2BP values are not just a marker for liver fibrosis. Elevation of post-Tx WFA+-M2BP values as a potential risk for hepatocarcinogenesis with advanced fibrosis. And the time-dependent AUROC analysis suggested that WFA+-M2BP is superior to AFP as a predictor for the development of HCC.

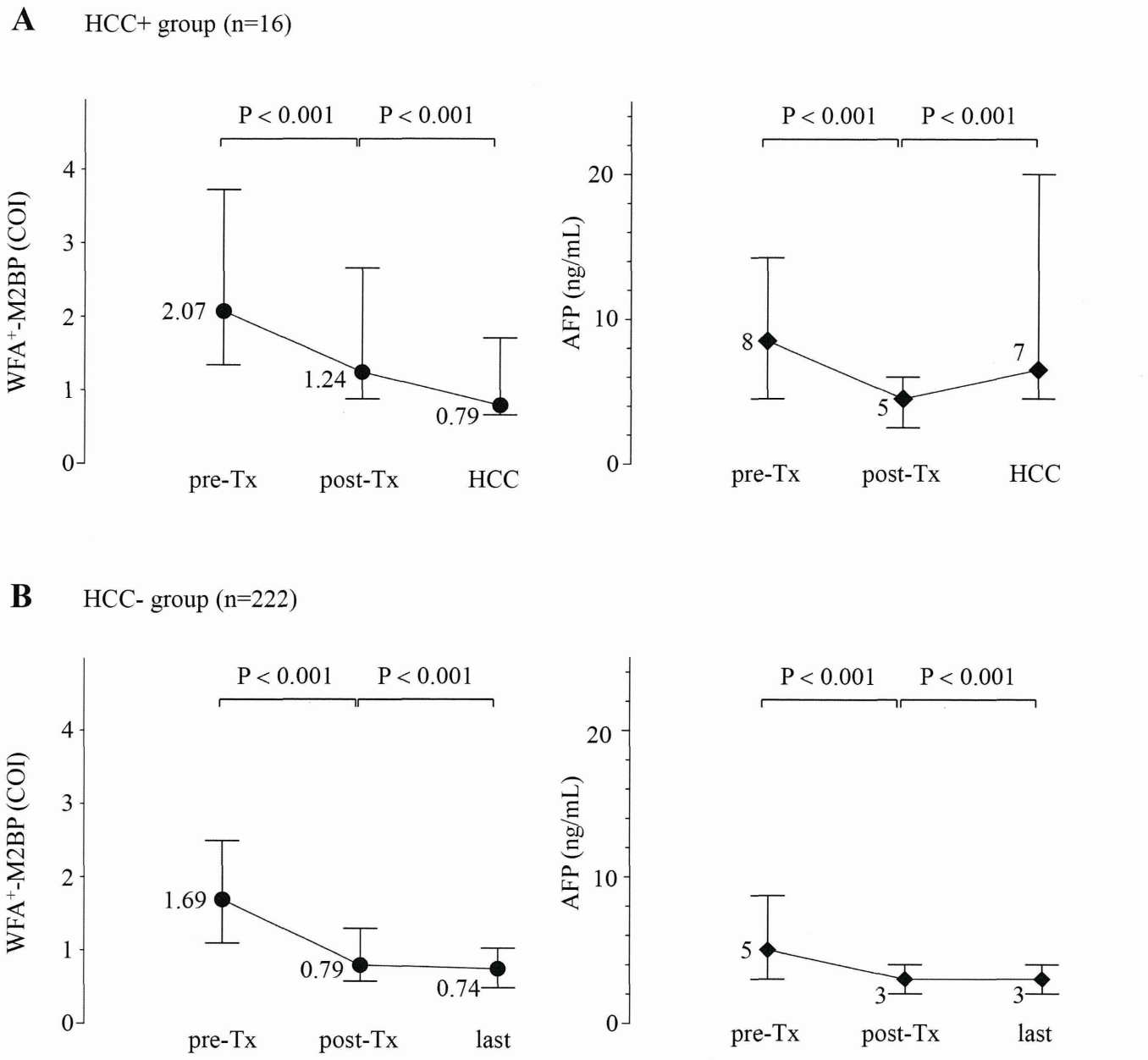

Fig 4. Chronological changes in the WFA$^+$-M2BP and AFP values at pre-treatment, post-treatment, the time of HCC development, and the last visit of the 238 patients with sustained virological response. Dots represent the median serum WFA$^+$-M2BP values at each time point, and the error bar represents the interquartile range. Diamonds represent the median serum AFP values at each time point, and the error bar represents the interquartile range. (A): Patients who developed HCC (n = 16). WFA$^+$-M2BP values were decreased at post-treatment and increased at HCC development. And AFP values were decreased at post-treatment and increased at HCC development. (B): Patients who did not developed HCC (n = 222). WFA$^+$-M2BP values were decreased at post-treatment and last clinical visit. And AFP values were decreased at post-treatment and last clinical visit.

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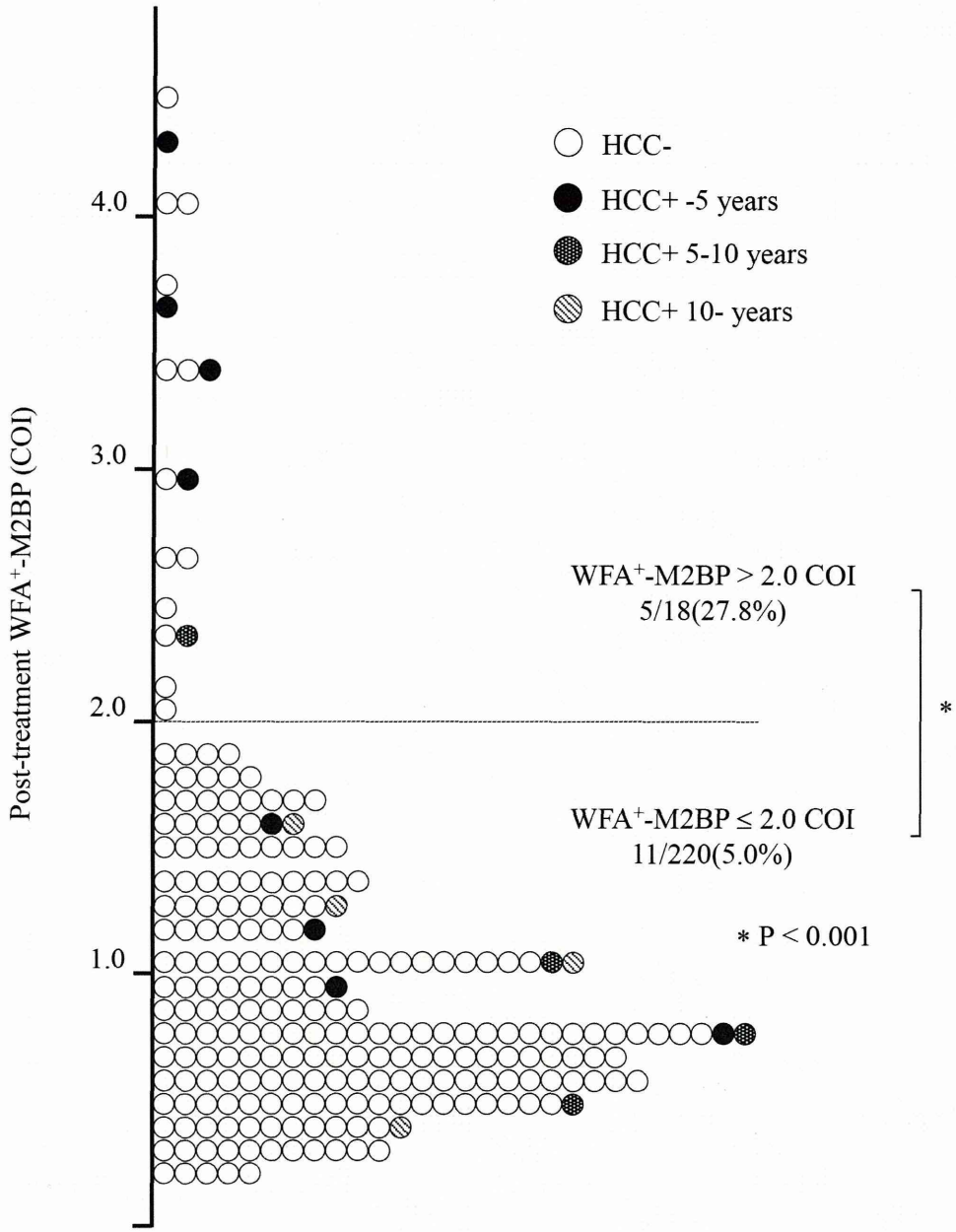

Fig 5. Relationship between post-treatment WFA$^+$-M2BP values and HCC development. The distribution of WFA$^+$-M2BP values was plotted. The dashed line indicates the 2.0 COI for WFA$^+$-M2BP. The 16 patients who developed HCC were stratified according to the duration from SVR to HCC development in 5-year increments. Each time point is designated by a distinct symbol as indicated. 222 patients did not develop HCC, and 209 of these 222 patients (94.1%) were in the post-treatment WFA$^+$-M2BP $\leq$ 2.0 COI group (white circles). During the follow-up period, 5 of 18 patients (27.8%) developed HCC in the post-treatment WFA$^+$-M2BP > 2.0 COI group, which was significantly higher than the rate in the post-treatment WFA$^+$-M2BP $\leq$ 2.0 COI group (5.0%, $P < 0.001$). In the post-treatment WFA$^+$-M2BP > 2.0 COI group, 4 of 5 cases developed HCC within 5 years after IFN treatment (black circles).

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The second main finding of our study was that the WFA^+-M2BP values were decreased in patients who achieved SVR, even in those who developed HCC. Kuno previously reported that WFA^+-M2BP values were decreased by IFN treatment [20]; to this result we added that the WFA^+-M2BP values were decreased even in our IFN-treated patients who achieved SVR. However, the post-Tx WFA^+-M2BP values were significantly higher in the patients who developed HCC than in those who did not. This finding is particularly important because the post-Tx values of WFA^+-M2BP have not been adequately evaluated. Our data are thus the first to demonstrate the distribution of WFA^+-M2BP values at post-Tx.

The third main finding of our study was that AFP and WFA^+-M2BP values manifested different behaviors between the time of post-Tx and HCC diagnosis in patients who developed HCC. Our previous paper reported a close association between AFP values and the stage of fibrosis [27], whereas another report showed an elevation in AFP values caused by necroinflammation injury and regeneration of the liver [42]. However, WFA^+-M2BP values do not always correlate with the grade of hepatic activity as defined by HAI scoring of inflammation [20,28]. A slight elevation of post-Tx AFP values (> 5ng/mL) could indicate substantial risks for the development of HCC [36]. In the 16 patients who developed HCC in our study, AFP values were elevated from post-Tx to the time of HCC development. However, the WFA^+-M2BP values decreased after SVR and decreased further at the time of HCC diagnosis (Fig 4). The Mac-2 binding protein is secreted from many cell types, including hepatocytes, and it has been shown to modulate many processes, particularly those related to cell adhesion [22,43,44]. Alterations in the quality and quantity of the Mac-2 binding protein have been observed during the progression of fibrosis [22–24]. Hepatic stellate cells are considered the main fibrogenic cell type of the liver [45,46]. Activation of hepatic stellate cells and reversal of hepatic stellate cell activation [47] might be associated with WFA^+-M2BP values. WFA^+-M2BP has been associated with changes in both the quality and quantity of the Mac-2 binding protein due to changes in glycosylation [20]. From these considerations, we think that the WFA^+-M2BP values do not reflect the results of HCC development, but rather a pre-cancer status or hepatocellular carcinogenesis.

One of the limitations of the present study was its retrospective nature. A future prospective analysis will be needed to validate the efficacy of WFA^+-M2BP as a predictor of HCC development. Another limitation is that we analyzed a relatively small number of HCC cases after SVR. Multi-center prospective registration of patients with SVR could overcome this deficiency.

Regardless of these limitations, this is the first report to describe the relationship between WFA^+-M2BP values and HCC development after SVR. The rapid progress in the development of anti-viral agents [48,49] for hepatitis C suggests that the number of patients who achieve SVR—including elderly patients or patients with advanced fibrosis, who are regarded as being at high risk for HCC—might increase in the near future, especially in Japan. Therefore, the prediction of HCC development in patients with SVR is of increasing clinical relevance.

In conclusion, this study revealed an association between WFA^+-M2BP and the risk of HCC development in patients with SVR. The results suggested that the WFA^+-M2BP should not be limited to use in fibrosis stage screening but rather could be applied as a new predictor of HCC development after SVR.

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Author Contributions

Conceived and designed the experiments: RS KY HY. Performed the experiments: RS KY SA A. Komori SN AS SH SB YK HY. Analyzed the data: RS KY HY. Contributed reagents/materials/analysis tools: RS KY SA A. Komori SN AS SH SB YK A. Kuno MK AT MO MM HN HY. Wrote the paper: RS KY MM HN TI KN HY.

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