Inuyama classification of chronic hepatitis: F0, no fibrosis; F1, portal fibrosis widening; F2, portal fibrosis widening with bridging fibrosis; F3, bridging fibrosis plus lobular distortion; and F4, liver cirrhosis [19]. FIB4 indices were calculated as follows: FIB4 index = age × AST level/[platelet count × (ALT level)$^{0.5}$] [20]. The median postoperative observational period was 3.3 years.

The study protocol conformed to the ethical guidelines of the 1975 Helsinki Declaration and was approved by the Ethics Committee of Hokkaido University Hospital.

Therapeutic strategy for HCC

Our therapeutic strategy for HCC and the therapeutic outcomes were described in previous reports [2, 21]. For patients with an ICG-R15 of 15 % or less, hemihepatectomy or extended hemihepatectomy was performed if the liver resection rate was less than 60 %; otherwise, the liver resection rate was decreased by percutaneous transhepatic portal embolization before surgery. For patients with ICG-R15 values of 15 20 %, 20 25 %, and 25 40 %, sectionectomy, segmentectomy, and partial resection were performed, respectively. Postoperative follow-up examinations by liver function tests, tumor marker analysis, ultrasonography, computed tomography, magnetic resonance imaging, and bone scintigraphy were performed every 3 4 months. In patients who experienced recurrence, rehepatectomy, transcatheter arterial chemoembolization, percutaneous ethanol injection therapy, and radiofrequency ablation were repeatedly performed.

FastLec-Hepa method

The FastLec-Hepa method is a sandwich immunoassay that uses lectin probes and antibody probes. In this method, WFA$^+$-M2BP molecules are first captured by WFA-coated magnetic beads, and then the bound products are detected by anti-human M2BP monoclonal antibodies linked to alkaline phosphatase. The FastLec-Hepa assay requires no pretreatment of serum samples owing to the oligomerization characteristics of M2BP and the high signal-to-noise ratio of WFA. All processes were automatically performed in 17 min using an HISCL-2000i clinical immunochemistry analyzer (Sysmex, Hyogo, Japan) [9].

To decrease the measurement variation, the WFA$^+$-M2BP level in each serum sample ([WFA$^+$-M2BP]) was converted to a cutoff index (COI) using the following equation:

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

where [WFA$^+$-M2BP]$_{NC}$ represents the WFA$^+$-M2BP level of the background buffer as a negative control and [WFA$^+$-M2BP]$_{PC}$ represents the WFA$^+$-M2BP level of the standard solution of recombinant human M2BP as a positive control. The level in the standard solution is equivalent to the mean plus 2.5 standard deviations of healthy volunteers [10].

Statistical analysis

Statistical analyses and data visualizations were performed using R version 3.0.2 (http://www.r-project.org/). The increase in serum WFA$^+$-M2BP levels according to the severity of liver fibrosis was analyzed using the Jonckheere Terpstra test, the Kruskal Wallis test, and the Wilcoxon rank-sum test. Diagnostic accuracies were evaluated using the areas under the curve and were tested by the DeLong test. The influences of clinicopathological factors on serum WFA$^+$-M2BP levels were analyzed using the Wilcoxon rank-sum test and the multivariate linear regression model. In the analysis of the effects of liver function on serum WFA$^+$-M2BP levels, the severity of liver dysfunction was represented by the first principal component of normalized liver function tests as z scores. Recurrence and overall survival rates were estimated by the Kaplan Meier method and were tested by log-rank tests and Cox proportional hazard analyses.

Results

Patient characteristics and clinical courses

The study population consisted of 306 males and 70 females with a median age of 64 years. Among the total of 376 patients, 152 patients were positive for HBV surface antigen alone, 110 patients were positive for HCV antibody alone, nine patients were positive for both, and 105 patients were not infected with HBV or HCV. Preoperative liver function was maintained at sufficient levels for all patients to undergo hepatectomy. The histological stage of liver fibrosis was F0 in 29 patients, F1 in 42 patients, F2 in 97 patients, F3 in 78 patients, and F4 in 130 patients. According to our therapeutic strategy, we performed partial resection in 86 patients, segmentectomy in 63 patients, sectionectomy in 99 patients, hemihepatectomy in 120 patients, and extended hemihepatectomy in eight patients (Table 1).

The 1-, 3-, and 5-year overall survival rates of all patients were 90.6, 74.1, and 63.5 %, respectively, and the recurrence rates were 34.9, 65.5, and 75.3 %, respectively.

Performance of serum WFA$^+$-M2BP levels as a serum fibrosis marker

The COI values of serum WFA$^+$-M2BP levels ranged from 0.28 to 23.55. Serum WFA$^+$-M2BP levels showed a

Table 1 Baseline characteristics of the 376 patients

	Median/Number	Range/Percentage
Gender		
Male	306	81.4 %
Female	70	18.6 %
Age (years)	64	35 90
HBV		
Negative	215	57.2 %
Positive	161	42.8 %
HCV		
Negative	257	68.4 %
Positive	119	31.6 %
ICG R15 (%)	14.5	2.5 94.2
PT (%)	92.5	10.2 178.0
Platelets (10^3/μl)	154	17.4 484
Albumin (g/ml)	4.0	2.9 5.2
ALT (IU/l)	39	7 312
Total bilirubin (mg/dl)	0.7	0.0 2.4
AFP (ng/ml)	18.4	0 1,089,163
AFP L3 (%)	2.4	0.0 90.1
PIVKA II (AU/ml)	218.5	2.3 664,680
Tumor number		
1	253	67.3 %
2	70	18.6 %
3	19	5.1 %
>3	34	9.0 %
Tumor size (cm)	4.5	0.7 20.0
LN metastasis		
No	374	99.5 %
Yes	2	0.5 %
Vascular invasion		
No	276	73.4 %
Yes	100	26.6 %
Differentiation		
Well	13	3.5 %
Moderate	205	54.5 %
Poor	158	42.0 %
Operation		
Partial resection	86	22.9 %
Segmentectomy	63	16.8 %
Sectionectomy	99	26.3 %
Hemihepatectomy	120	31.9 %
Extended hemihepatectomy	8	2.1 %
Fibrosis stage		
F0	29	7.7 %
F1	42	11.2 %
F2	97	25.8 %
F3	78	20.7 %
F4	130	34.6 %
FIB4 index	2.82	0.05 43.32
WFA^+ M2BP COI	1.34	0.28 23.55

AFP alpha fetoprotein, *AFP L3 Lens culinaris* agglutinin reactive fraction of alpha fetoprotein, *ALT* alanine aminotransferase, *COI* cutoff index, *HBV* hepatitis B virus, *HCV* hepatitis C virus, *ICG R15* indocyanine green retention rate at 15 min, *LN* lymph node, *PIVKA II* protein induced by vitamin K absence II, *PT* prothrombin time

significant monotonic increasing tendency with an increase in the severity of liver fibrosis ($p < 0.001$). The median COI values were 0.76, 0.90, 1.17, 1.39, and 1.87 in F0 stage, F1 stage, F2 stage, F3 stage, and F4 stage patients, respectively. The COI values stratified by the stage of liver fibrosis were significantly different according to the Kruskal Wallis test ($p < 0.001$), and the differences between adjacent stages were considerable between stages F0 and F1 ($p = 0.062$) and stages F1 and F2 ($p = 0.079$) and significant between stages F2 and F3 ($p = 0.047$) and stages F3 and F4 ($p = 0.005$) in the Wilcoxon rank-sum test (Fig. 1a, b).

The areas under the curve for serum WFA^+-M2BP levels and the FIB4 index were 0.741 and 0.636 for the diagnosis of significant fibrosis (F2 stage or greater), and 0.701 and 0.706 for the diagnosis of cirrhosis (F4 stage), respectively. The diagnostic accuracies of serum WFA^+-M2BP levels and the FIB4 index were significantly different in the diagnosis of significant fibrosis ($p = 0.008$), and were of the same level in the diagnosis of cirrhosis ($p = 0.883$; Fig. 1c, d).

Clinicopathological determinants of serum WFA^+-M2BP levels

In addition to the progression of liver fibrosis, gender ($p = 0.003$), HCV infection ($p < 0.001$), ICG-R15 ($p < 0.001$), prothrombin time ($p = 0.005$), platelet counts ($p < 0.001$), albumin levels ($p < 0.001$), ALT levels ($p < 0.001$), total bilirubin levels ($p = 0.044$), tumor number ($p = 0.006$), and tumor size ($p = 0.040$) influenced serum WFA^+-M2BP levels significantly (Table 2). Among these clinicopathological factors, cirrhosis ($p = 0.003$), female gender ($p = 0.001$), HCV infection ($p < 0.001$), ICG-R15 ($p = 0.008$), platelet counts ($p = 0.004$), albumin levels ($p < 0.001$), ALT levels ($p = 0.001$), and total bilirubin levels ($p = 0.019$) were significant independent determinants of serum WFA^+-M2BP levels in a multiple linear regression analysis where the COI values of serum WFA^+-M2BP levels were assigned as the response variable and the 11 significant influencing factors were assigned as the covariates (Table 3).

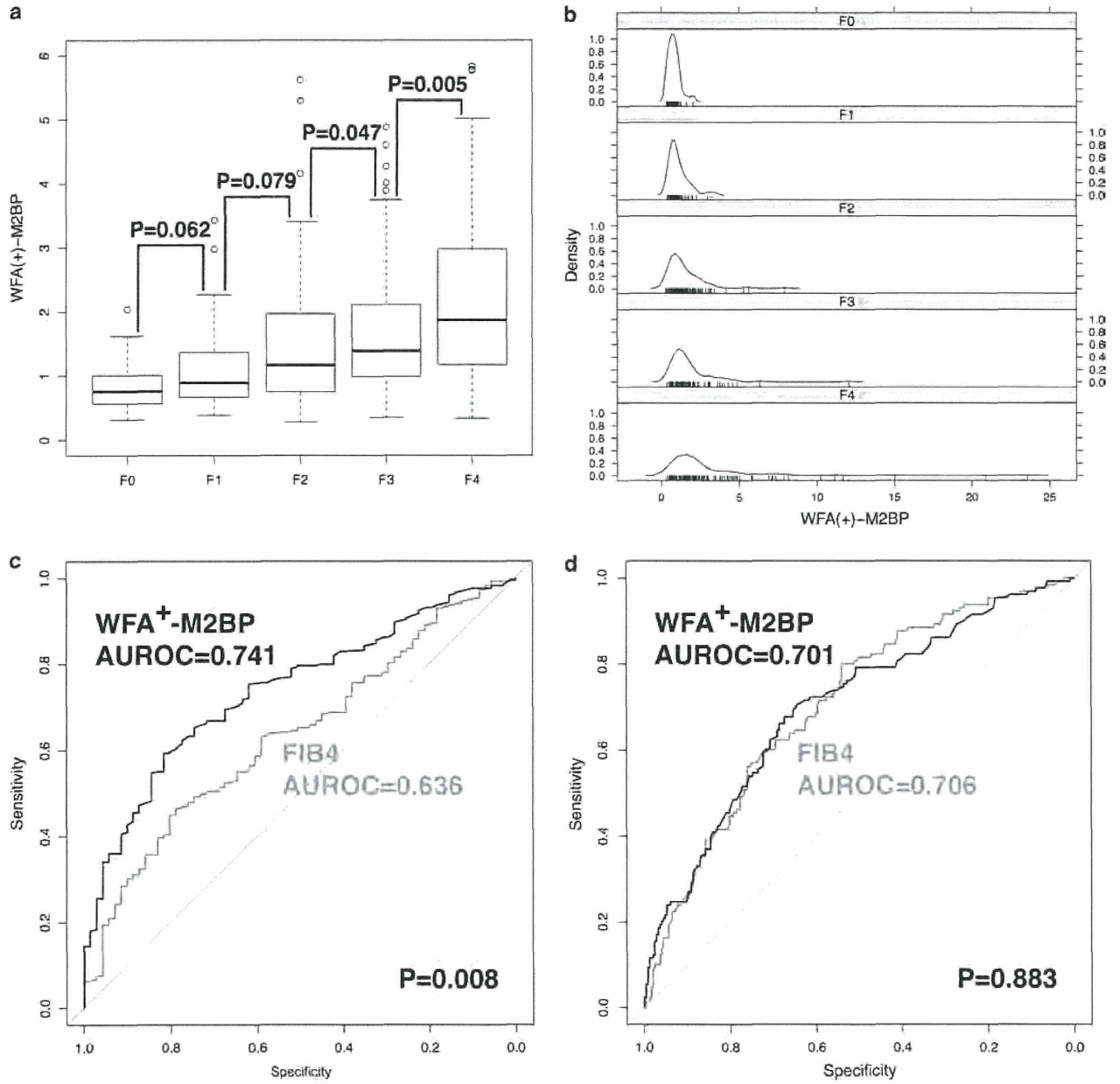

Fig. 1 Distributions and diagnostic accuracies of serum *Wisteria floribunda* agglutinin positive Mac 2 binding protein (*WFA*$^+$ *M2BP*) levels. Box plots (**a**) and distribution curves (**b**) of the cutoff index values for serum WFA$^+$ M2BP levels stratified by the stage of liver fibrosis. Receiver operating characteristic curves and areas under the receiver operating characteristic curves (*AUROC*) for serum WFA$^+$ M2BP levels and the FIB4 index for the diagnosis of F2 F4 stage liver fibrosis (**c**) and F4 stage liver fibrosis (**d**)

Clinicopathological characterization of serum WFA$^+$-M2BP

Among the determinants of serum WFA$^+$-M2BP levels, ICG-R15 levels, platelet counts, albumin levels, ALT levels, and total bilirubin levels were associated with liver dysfunction. We used the first principal component as the representative of these five factors to decrease the dimensions of the data, and classified the patients into mild ($N = 126$), moderate ($N = 125$), and severe ($N = 125$) liver dysfunction groups according to the values of the first principal components (Fig. S1).

Serum WFA$^+$-M2BP levels showed an obvious increasing tendency along with both pathological and functional progression of liver fibrosis. The mean COI values of WFA$^+$-M2BP were 0.84, 1.22, 1.39, 1.20, 1.53, 1.98, 1.43, 2.46, and 4.00 in the F0 F1/mild, F2 F3/mild, F4/mild, F0 F1/moderate, F2 F3/moderate, F4/moderate, F0

Table 2 Serum *Wisteria floribunda* agglutinin positive Mac 2 binding protein (*WFA$^+$ M2BP*) levels stratified by clinicopathological factors

	Number	WFA$^+$ M2BP[a]	p[b]
Gender			
Male	306	1.25 (0.28 20.87)	0.003
Female	70	1.78 (0.42 23.55)	
Age (years)			
<60	135	1.39 (0.28 20.87)	0.950
≥60	241	1.33 (0.32 23.55)	
HBV			
Negative	215	1.36 (0.32 23.55)	0.150
Positive	161	1.22 (0.28 20.87)	
HCV			
Negative	257	1.10 (0.28 20.87)	<0.001
Positive	119	2.13 (0.34 23.55)	
ICG R15 (%)			
≤15	198	1.07 (0.28 11.12)	<0.001
>15	178	1.73 (0.34 23.55)	
PT (%)			
≥75	333	1.25 (0.28 23.55)	0.005
<75	43	1.62 (0.42 8.14)	
Platelets			
≥100 × 10^3/μl	323	1.22 (0.28 20.87)	<0.001
<100 × 10^3/μl	53	2.37 (0.34 23.55)	
Albumin (g/ml)			
≥3.5	335	1.33 (0.28 23.55)	<0.001
<3.5	41	2.09 (1.41 7.07)	
ALT (IU/l)			
≤50	247	1.15 (0.32 15)	<0.001
>50	129	1.78 (0.28 23.55)	
Total bilirubin (mg/dl)			
≤1.5	358	1.33 (0.28 23.55	0.044
>1.5	18	2.20 (0.65 20.87)	
AFP (ng/ml)			
≤25	205	1.25 (0.34 20.87)	0.270
>25	171	1.39 (0.28 23.55)	
AFP L3 (%)			
≤15	259	1.33 (0.32 23.55)	0.793
>15	117	1.38 (0.28 7.90)	
PIVKA II (AU/ml)			
≤40	118	1.39 (0.32 15.00)	0.820
>40	258	1.30 (0.28 23.55)	
Tumor number			
Single	253	1.24 (0.32 23.55)	0.006
Multiple	123	1.56 (0.28 20.87)	
Tumor size cm			
≤5	223	1.40 (0.32 23.55)	0.040
>5	153	1.17 (0.28 20.87)	
LN metastasis			
No	374	1.34 (0.28 23.55)	0.307
Yes	2	2.14 (1.56 2.72)	
Vascular invasion			
No	276	1.33 (0.28 23.55)	0.945
Yes	100	1.39 (0.35 20.87)	
Differentiation			
Well to moderate	218	1.37 (0.34 23.55)	0.175
Poor	158	1.26 (0.28 20.87)	

AFP alpha fetoprotein, *AFP L3 Lens culinaris* agglutinin reactive fraction of alpha fetoprotein, *ALT* alanine aminotransferase, *HBV* hepatitis B virus, *HCV* hepatitis C virus, *ICG R15* indocyanine green retention rate at 15 min, *LN* lymph node, *PIVKA II* protein induced by vitamin K absence II, *PT* prothrombin time

[a] Median, with the range in *parentheses*

[b] Wilcoxon rank sum test

Table 3 Clinicopathological determinants of serum *Wisteria floribunda* agglutinin positive Mac 2 binding protein (WFA$^+$ M2BP) levels

	Coefficient	SE	p[a]
Intercept	6.37	1.309	<0.001
Female gender	0.82	0.255	0.001
HCV positive	1.039	0.223	<0.001
ICG R15 (%)	0.027	0.01	0.008
PT (%)	0.008	0.007	0.252
Platelets (10^3/μl)	0.005	0.002	0.004
Albumin (g/ml)	1.665	0.268	<0.001
ALT (IU/l)	0.009	0.003	0.001
Total bilirubin (mg/dl)	0.74	0.315	0.019
Tumor number multiple	0.125	0.208	0.548
Tumor size (cm)	0.027	0.03	0.365
Cirrhosis (F4 stage)	0.71	0.234	0.003

ALT alanine aminotransferase, *HCV* hepatitis C virus, *ICG R15* indocyanine green retention rate at 15 min, *PT* prothrombin time, *SE* standard error of regression coefficient

[a] Multiple linear regression analysis

F1/severe, F2 F3/severe, and F4/severe groups, respectively (Fig. 2).

Serum WFA$^+$-M2BP levels were also dependent on gender and HCV infection. The COI values were significantly higher in HCV-positive patients than in HCV-negative F2 stage ($p < 0.001$), F3 stage ($p < 0.001$), and F4 stage ($p < 0.001$) patients (Fig. 3a). Even when the patients were stratified by both pathological and functional severities of liver fibrosis, the differences in the COI values

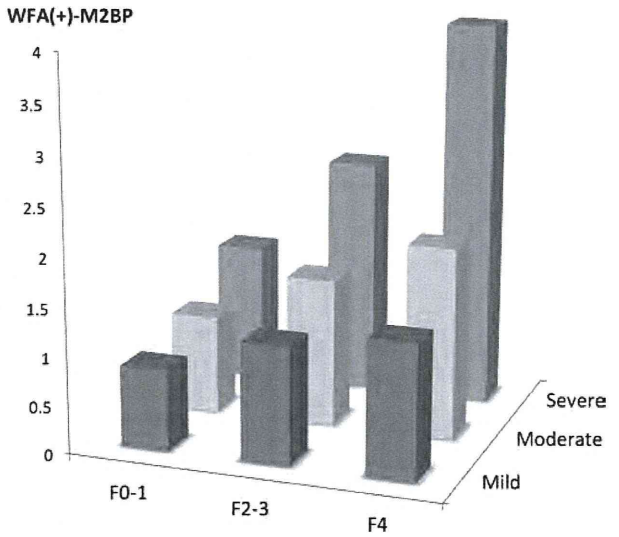

Fig. 2 Increase in serum *Wisteria floribunda* agglutinin positive Mac 2 binding protein (WFA^+ M2BP) levels with pathological and functional progression of liver fibrosis. Each *bar* represents the mean cutoff index value of serum WFA^+ M2BP levels in subgroups of patients classified on the basis of pathological fibrosis stage (F0 F1, F2 F3, or F4) and extent of liver dysfunction (mild, moderate, or severe)

between HCV-positive and HCV-negative patients were significant in the F2 F3/mild ($p = 0.017$), F2 F3/moderate ($p < 0.001$), F4/moderate ($p = 0.009$), F2 F3/severe ($p = 0.004$), and F4/severe ($p = 0.001$) groups (Fig. 3b d).

Gender affected serum WFA^+-M2BP levels in a manner different from HCV infection. The COI values were significantly higher in female patients than in male patients only in patients with F4 disease severity ($p < 0.001$), and more specifically, in the F4/severe ($p = 0.003$) group (Fig. S2).

Significance of serum WFA^+-M2BP levels in liver surgery

According to the results obtained, we performed further analyses separately in HCV-negative and HCV-positive patients. The diagnostic thresholds of the COI values to distinguish F4 stage patients from F3 stage patients, which maximized the sum of the sensitivity and specificity, were 1.435 in HCV-negative patients and 4.615 in HCV-positive patients.

When these COI values were applied, the sensitivity and the specificity were 58.8 % (47/80) and 81.4 % (144/177), respectively, in HCV-negative patients, and 32.0 % (16/50) and 94.2 % (65/69), respectively, in HCV-positive patients. In HCV-negative patients, serum WFA^+-M2BP levels at the time of the operations were significant risk factors for HCC recurrence ($p = 0.018$) and decreased overall survival ($p = 0.018$) after liver surgery. The 3-year recurrence rate and the 5-year overall survival rate were 58.9 % and 69.4 %, respectively, in patients with low serum WFA^+-M2BP levels (COI $\leq$ 1.435), and 75.3 % and 57.2 %, respectively, in patients with high serum WFA^+-M2BP levels (COI $>$ 1.435) (Fig. S3a, b). In HCV-positive patients, an elevated serum WFA^+-M2BP level was also a significant risk factor for HCC recurrence ($p = 0.001$) and decreased overall survival ($p = 0.013$). The 3-year recurrence rate and the 5-year overall survival rate were 64.0 % and 64.5 %, respectively, in patients with low serum WFA^+-M2BP levels (COI $\leq$ 4.615), and 86.8 % and 36.5 %, respectively, in patients with high serum WFA^+-M2BP levels (COI $>$ 4.615) (Fig. S3c, d).

To evaluate the significance of serum WFA^+-M2BP levels compared with other liver function tests and HCC stages, univariate and multivariate analyses for overall survival and tumor recurrence were performed using HBV infection, HCV infection, ICG-R15, serum albumin levels, serum total bilirubin levels, the FIB4 index, AFP levels, AFP L3 levels, PIVKA II levels, TNM stage (Union for International Cancer Control), and serum WFA^+-M2BP levels as explanatory variables (Table 4). Serum WFA^+-M2BP level was a significant independent risk factor for tumor recurrence ($p = 0.033$). Serum WFA^+-M2BP level as an independent risk factor for decreased overall survival, however, was not statistically significant in the multivariate analysis ($p = 0.520$).

Discussion

WFA^+-M2BP is a promising serum marker of liver fibrosis. Similarly to other reports [17], we found serum WFA^+-M2BP levels demonstrated diagnostic accuracies superior to those of other fibrosis markers, such as serum hyaluronic acid and serum type IV collagen (Fig. S4a c).

WFA^+-M2BP was identified as a fibrosis-specific glycoform of a serum protein marker of liver fibrosis using a combination of proteomic and glycomic approaches [9, 12]. M2BP is a ligand of Mac-2 that is highly expressed in activated macrophage-lineage cells, including Kupffer cells, and M2BP is associated with the antiviral host defense [22, 23]. M2BP also interacts with a number of fibrosis-associated extracellular proteins, such as collagens IV VI, fibronectin, and nidogen [14]. The analyses of the glycan structure identified by WFA and the relationship between WFA-associated glycosylation and liver diseases are still under way. However, the diagnostic potential of WFA was also confirmed with another liver fibrosis marker, WFA-positive colony stimulating factor, and two markers of cholangiocellular carcinoma, WFA-positive mucin 1 and WFA-positive L1 cell adhesion molecule [24 26].

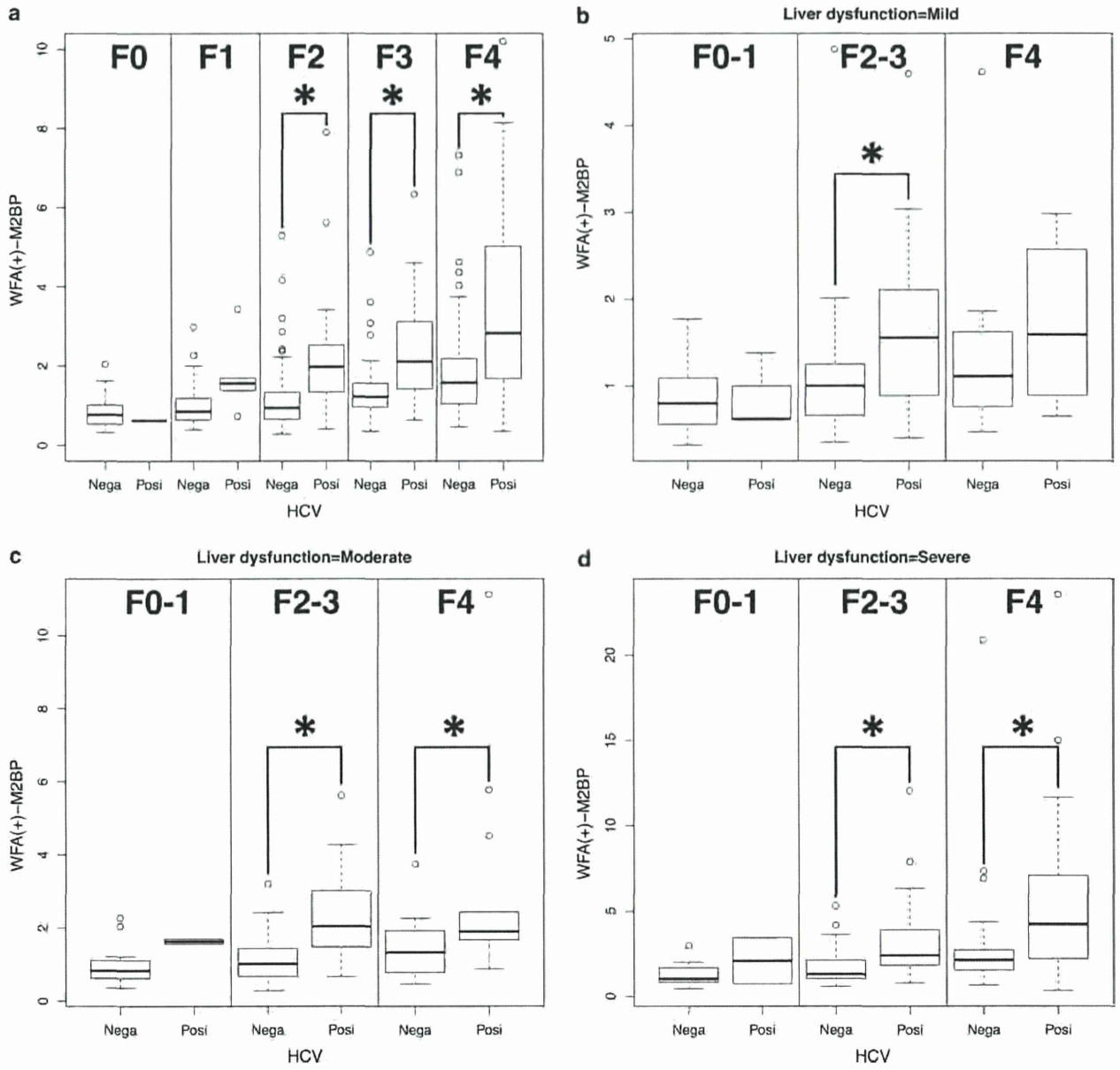

Fig. 3 Comparisons of serum *Wisteria floribunda* agglutinin positive Mac 2 binding protein (WFA^+ M2BP) levels between hepatitis C virus (*HCV*) negative and HCV positive patients. Box plots of the cutoff index (*COI*) values of HCV negative and HCV positive patients in the F0, F1, F2, F3, and F4 groups (**a**). Box plots of the COI values stratified by HCV infection and fibrosis stage in patients with mild (**b**), moderate (**c**), and severe (**d**) liver dysfunction

A series of clinical studies revealed that WFA^+-M2BP is an accurate serum marker of liver fibrosis and is related to the therapeutic effect of poly(ethylene glycol) interferon alpha and ribavirin therapy and the development of HCC in chronic hepatitis C patients [9, 16 18]. However, the clinicopathological characteristics of serum WFA^+-M2BP levels have not yet been elucidated sufficiently for their appropriate clinical utilization as a biomarker. In the current study, we characterized the behavior of serum WFA^+-M2BP levels according to clinicopathological conditions and assessed the diagnostic ability of serum WFA^+-M2BP levels comprehensively.

All of the patients included in this study were HCC patients who underwent liver surgery. Because most of the patients had developed HCC as a result of prolonged hepatitis and liver fibrosis, the proportion of the patients with severe fibrosis in this study was considerably higher than that in other clinical studies on WFA^+-M2BP. The proportions of F0 F1 stage and F4 stage patients were 18.9 % and 34.6 %, respectively, in this study versus

Table 4 Risk analysis of clinicopathological factors for overall survival and tumor recurrence

	Number	Overall survival				Tumor recurrence			
		5 year OS rate[a] (%)	p^b	HR	p^c	3 year tumor recurrence rate[a] (%)	p^b	HR	p^c
HBV									
Negative	215	65.1 (57.7 73.5)	0.502			65.1 (57.2 71.5)	0.735		
Positive	161	61.4 (53.4 70.7)				66.3 (57.5 73.3)			
HCV									
Negative	257	65.6 (59.0 72.9)	0.519			64.2 (57.2 70.1)	0.396		
Positive	119	59.5 (49.9 70.9)				67.9 (57.3 75.9)			
ICG R15 (%)									
≤15	198	64.9 (57.6 73.3)	0.635			61.9 (53.8 68.6)	0.073		
>15	178	62.1 (54.1 71.3)				69.5 (61.0 76.1)			
Albumin (g/ml)									
≤3.5	51	66.7 (60.8 73.1)	<0.001	2.04 (1.28 3.26)	0.003	83.3 (63.9 92.3)	<0.001	1.75 (1.23 2.50)	0.002
>3.5	325	40.1 (26.8 60.1)		Reference		62.9 (56.8 68.2)		Reference	
Total bilirubin (mg/dl)									
≤1.5	358	64.6 (58.9 70.8)	0.008	Reference	0.120	65.1 (59.2 70.0)	0.089		
>1.5	18	32.1 (13.0 79.4)		1.83 (0.85 3.98)		72.5 (26.0 89.8)			
FIB4 index									
≤3.25	228	69.7 (62.9 77.2)	0.005	Reference	0.009	61.4 (53.9 67.6)	0.137		
>3.25	148	54.0 (45.1 64.7)		1.64 (1.13 2.38)		71.7 (62.5 78.7)			
AFP (ng/ml)									
≤25	205	74.7 (67.9 82.1)	<0.001	Reference	0.074	56.9 (49.1 63.6)	<0.001	Reference	0.200
>25	171	49.6 (41.2 59.7)		1.47 (0.96 2.25)		76.6 (67.9 82.9)		1.22 (0.90 1.66)	
AFP L3 (%)									
≤15	259	72.7 (66.6 79.4)	<0.001	Reference	0.001	61.9 (54.9 67.8)	<0.001	Reference	0.170
>15	117	41.6 (31.6 54.7)		2.00 (1.32 3.02)		73.6 (62.7 81.3)		1.25 (0.91 1.73)	
PIVKA II (AU/ml)									
≤40	118	75.8 (67.1 85.7)	<0.001	Reference	0.140	58.4 (47.7 66.9)	0.028	Reference	0.720
>40	258	57.5 (50.6 65.3)		1.40 (0.89 2.20)		68.8 (61.9 74.5)		1.05 (0.80 1.39)	
TNM stage (UICC)									
I, II	297	70.8 (64.9 77.2)	<0.001	Reference	<0.001	60.3 (53.7 65.2)	<0.001	Reference	<0.001
III, IV	79	33.8 (22.6 50.4)		2.19 (1.47 3.26)		86.4 (74.1 92.9)		1.83 (1.34 2.51)	
WFA^+ M2BP									
Low	276	76.9 (71.7 82.5)	0.002	Reference	0.520	61.0 (54.1 66.8)	0.001	Reference	0.033
High	100	66.5 (57.3 77.1)		1.14 (0.77 1.69)		77.7 (66.8 85.0)		1.34 (1.02 1.77)	

AFP alpha fetoprotein, *AFP L3 Lens culinaris* agglutinin reactive fraction of alpha fetoprotein, *HBV* hepatitis B virus, *HCV* hepatitis B virus, *HR* hazard ratio, *ICG R15* indocyanine green retention rate at 15 min, *PIVKA II* protein induced by vitamin K absence II, *UICC* Union for International Cancer Control, *WFA^+ M2BP* Wisteria floribunda agglutinin positive Mac 2 binding protein

[a] The 95 % confidence interval is given in *parentheses*

[b] Log rank test

[c] Cox proportional hazard regression analysis

38.8 64.5 % and 12.5 17.6 %, respectively, in other studies [9, 16 18]. Furthermore, serum WFA^+-M2BP levels in patients with severe fibrosis were relatively low for their fibrosis severity in this study, because liver function in all patients was kept at the required level for surgery. Moreover, the liver functions correlated with serum WFA^+-M2BP levels. The mean COI value for M2BP in F4 stage patients was 1.87 in this study, versus 2.96 3.67 in other studies. Such a characteristic explains the reason for the lower-than-expected diagnostic accuracy of serum WFA^+-M2BP levels in this study, especially for diagnosis of severe fibrosis.

In addition to the pathological diagnosis of cirrhosis, serum WFA^+-M2BP levels also depended on gender, HCV infection, and liver dysfunction characteristics, as indicated by abnormal ICG-R15, platelet counts, albumin levels, ALT levels, and total bilirubin levels. These results imply that WFA^+-M2BP is a serum marker of both pathological and functional severities of liver fibrosis. To analyze the quantitative relationships of liver fibrosis stage and liver

function test results with serum WFA^+-M2BP levels, we used the first principal component of ICG-R15, platelet counts, albumin levels, ALT levels, and total bilirubin levels as an integrated index of liver dysfunction (Fig. S1). The independent contributions of liver fibrosis stage and liver dysfunction to serum WFA^+-M2BP levels are clearly demonstrated in the three-dimensional histogram (Fig. 2). Serum WFA^+-M2BP levels reflect both pathological and functional progression of liver fibrosis comprehensively and continuously.

Serum WFA^+-M2BP levels also were dependent on gender and HCV infection. Serum WFA^+-M2BP levels in HCV-positive patients were similar to those of HCV-negative patients in the F0 F1 stage and were significantly higher than those of HCV-negative patients throughout the progression of liver fibrosis (F2, F3, and F4 stages). Artini et al. [11] reported that M2BP expression levels are also higher in HCV-positive patients than in HCV-negative patients throughout the progression of liver fibrosis. The mean serum M2BP levels in asymptomatic carriers, chronic hepatitis patients, and cirrhotic patients were 10.5, 14.3, and 13.4 U/ml, respectively, in the HBV-positive group and 8.4, 20.8, and 46.3 U/ml, respectively, in the HCV-positive group. HCV infection was considered to elevate the increasing rate of serum WFA^+-M2BP levels at least in part by promoting M2BP expression. On the basis of these results, we applied different diagnostic criteria for serum WFA^+-M2BP in HCV-negative and HCV-positive patients. In contrast to HCV infection, the differences in serum WFA^+-M2BP levels between male and female patients were significant only in F4 stage patients with severe liver dysfunction. Therefore, we applied the same criteria to both male and female patients in this study.

In the analysis of clinicopathological determinants of serum WFA^+-M2BP, we also investigated eight tumor-associated factors: AFP, AFP L3, PIVKA II, tumor number, tumor size, lymph node metastasis, vascular invasion, and tumor differentiation. However, none of the tumor-associated factors affected the COI values for serum WFA^+-M2BP levels significantly and independently. Serum WFA^+-M2BP levels were demonstrated to be independent of tumor progression.

In conclusion, WFA^+-M2BP is a newly developed serum marker of liver fibrosis that can be measured quickly and easily in clinical practice. Serum WFA^+-M2BP levels reflect both pathological and functional progression of liver fibrosis comprehensively and continuously. The increase in rates along with the severity of liver fibrosis and appropriate diagnostic COI values for serum WFA^+-M2BP levels were different between HCV-negative and HCV-positive patients. High serum WFA^+-M2BP levels were a significant risk factor for tumor recurrence and decreased overall survival after liver surgery in both HCV-negative and HCV-positive patients.

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Conflict of interest The authors declare that they have no conflict of interest.

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Development and Applications of the Lectin Microarray

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Abstract The lectin microarray is an emerging technology for glycomics. It has already found maximum use in diverse fields of glycobiology by providing simple procedures for differential glycan profiling in a rapid and high-throughput manner. Since its first appearance in the literature in 2005, many application methods have been developed essentially on the same platform, comprising a series of glycan-binding proteins immobilized on an appropriate substrate such as a glass slide. Because the lectin microarray strategy does not require prior liberation of glycans from the core protein in glycoprotein analysis, it should encourage researchers not familiar with glycotechnology to use glycan analysis in future work. This feasibility should provide a broader range of experimental scientists with good opportunities to investigate novel aspects of glycoscience. Applications of the technology include not only basic sciences but also the growing fields of bio-industry. This chapter describes first the essence of glycan profiling and the basic fabrication of the lectin microarray for this purpose. In the latter part the focus is on diverse applications to both structural and functional glycomics, with emphasis on the wide applicability now available with this new technology. Finally, the importance of developing advanced lectin engineering is discussed.

Keywords Bio-affinity • Biomarker • Functional glycomics • Glycan profiling • Lectin microarray

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