the areas under the ROC curves was based on the theory of generalized U-statistics [21]. All of the differences were considered statistically significant at $P < 0.05$.

Results

Patients and liver specimens

Subject characteristics are summarized in Supplementary Table 1. The mean age of the 200 subjects (135 men and 65 women) was 64 ± 20 years. The 200 enrolled adults consisted of 40 healthy volunteers and 160 patients with or without hepatitis; of the latter, 106 were positive for HCVAb, 21 were positive for HBsAg, 12 had hepatitis due to alcohol, and 21 were negative for HBsAg and HCVAb (nonBnonC). Of the 200 surgical liver specimens, 84 had fibrosis grade F0; 45 were F1, 21 were F2, 16 were F3, and 34 were F4.

Liver fibrosis assessed by WFA$^+$-M2BP values

Figure 1 shows box plots of serum WFA$^+$-M2BP values for each fibrosis stage. Serum WFA$^+$-M2BP values measured by the glycan-based immunoassay ranged from 0.22 to 8.69 (COI). WFA$^+$-M2BP levels in patients with fibrosis grades F0 ($n = 84$), F1 ($n = 45$), F2 ($n = 21$), F3 ($n = 16$), and F4 ($n = 34$) had COIs of 1.62, 1.82, 3.02, 3.32, and 3.67, respectively. There were significant differences between fibrosis stages F1 and F2 ($P < 0.01$), and between fibrosis stages F2 and F3 ($P < 0.01$) (Fig. 1).

Correlation of WFA$^+$-M2BP values with VTTQ, the LMR index, and serum markers of liver fibrosis

The log of WFA$^+$-M2BP correlated with VTTQ results, a significant indicator of liver stiffness as liver fibrosis ($P = 0.0001$) (Fig. 2a). Log WFA$^+$-M2BP values were also inversely related with the LMR index, a significant indicator of liver function that varies inversely with liver fibrosis ($P = 0.0001$) (Fig. 2b), as well as with type 4 collagen concentrations ($P = 0.0080$) (Fig. 2c) and the APRI ($P = 0.0001$) (Fig. 2d), but not with hyaluronic acid (Fig. 2e) and ICG-R15 concentrations (Fig. 2f).

APRI, aspartate transaminase-to-platelet ratio index; LMR, liver-to-major psoas muscle intensity ratio; VTTQ, Virtual Touch^{TM} Tissue Quantification; WFA$^+$-M2BP, *Wisteria floribunda* agglutinin-positive Mac-2 binding protein.

Diagnostic capability of WFA$^+$-M2BP values for each type of liver fibrosis

The areas under the ROC curve for the diagnosis of types of fibrosis F1, F2, F3, and F4 with serum WFA$^+$-M2BP values were 0.686, 0.820, 0.817, and 0.806, respectively (Fig. 3). The optimal cutoff values were 1.00 m/s for $F \geq 1$, 1.86 m/s for $F \geq 2$, 2.21 m/s for $F \geq 3$, and 2.64 m/s for $F \geq 4$ (Table 1). The sensitivity of the serum WFA$^+$-M2BP cutoffs for fibrosis grades $F \geq 1$, $F \geq 2$, $F \geq 3$, and $F \geq 4$ was 84.6, 84.7, 88.2, and 82.4 %, whereas the specificity was were 47.6, 74.4, 78.7, and 71.9 %, respectively. The sensitivity of VTTQ cutoffs for these fibrosis grades was 81.9, 70.4, 72.6, and 82.4 %, respectively, while the specificity was 70.2, 82.2, 83.3, and 79.5 %, respectively (Table 1, Supplementary Table 2). Thus, WFA$^+$-M2BP cutoffs were more sensitive, but less specific, in predicting liver fibrosis in each grade than the VTTQ cutoffs.

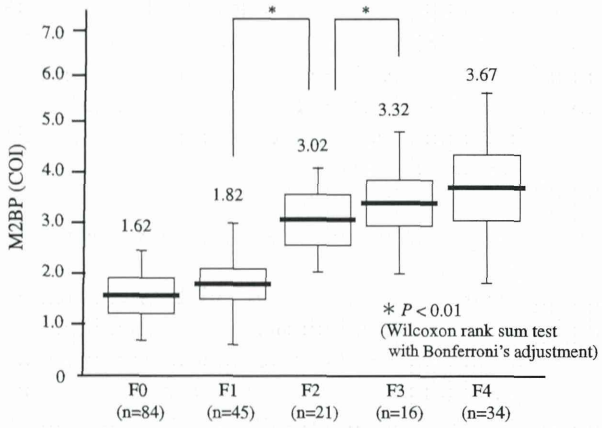

Fig. 1 Box-and-whisker plot of serum WFA$^+$-M2BP values for each fibrosis stage. The *tops* and *bottoms* of the boxes represent the first and third quartiles, respectively, with the *height* of the box represents the interquartile range, covering 50 % of the values. The *line* through the middle of each box represents the median. The *error bars* show the minimum and maximum values (range). Significant correlations were found between the stage of fibrosis and serum WFA$^+$-M2BP values, and there were significant correlations between fibrosis stages F1 and F2 ($P < 0.01$) and between fibrosis stages F2 and F3 ($P < 0.01$). *Asterisk* Statistically significant by the Wilcoxon rank sum test with Bonferroni's adjustment; $P < 0.01$. WFA$^+$-M2BP, *Wisteria floribunda* agglutinin-positive Mac-2 binding protein

Comparison of WFA$^+$-M2BP with other indicators for the diagnosis of fibrosis stage ≥3

We compared the area under the ROC curves of VTTQ, the LMR index, and serum markers (APRI, hyaluronic acid, and type 4 collagen) with that of WFA$^+$-M2BP values. The cutoff values were determined, as described above. The area under the ROC curves for the diagnosis of fibrosis ($F \geq 3$) using serum WFA$^+$-M2BP values (0.812) was comparable to that using VTTQ examination (0.814), but was significantly superior to the other surrogate markers, including LMR index (0.766), APRI (0.694), hyaluronic acid (0.683), and type 4 collagen (0.625) ($P = 0.0001$ each; Fig. 4). Serum WFA$^+$-M2BP, VTTQ, LMR index, APRI, hyaluronic acid

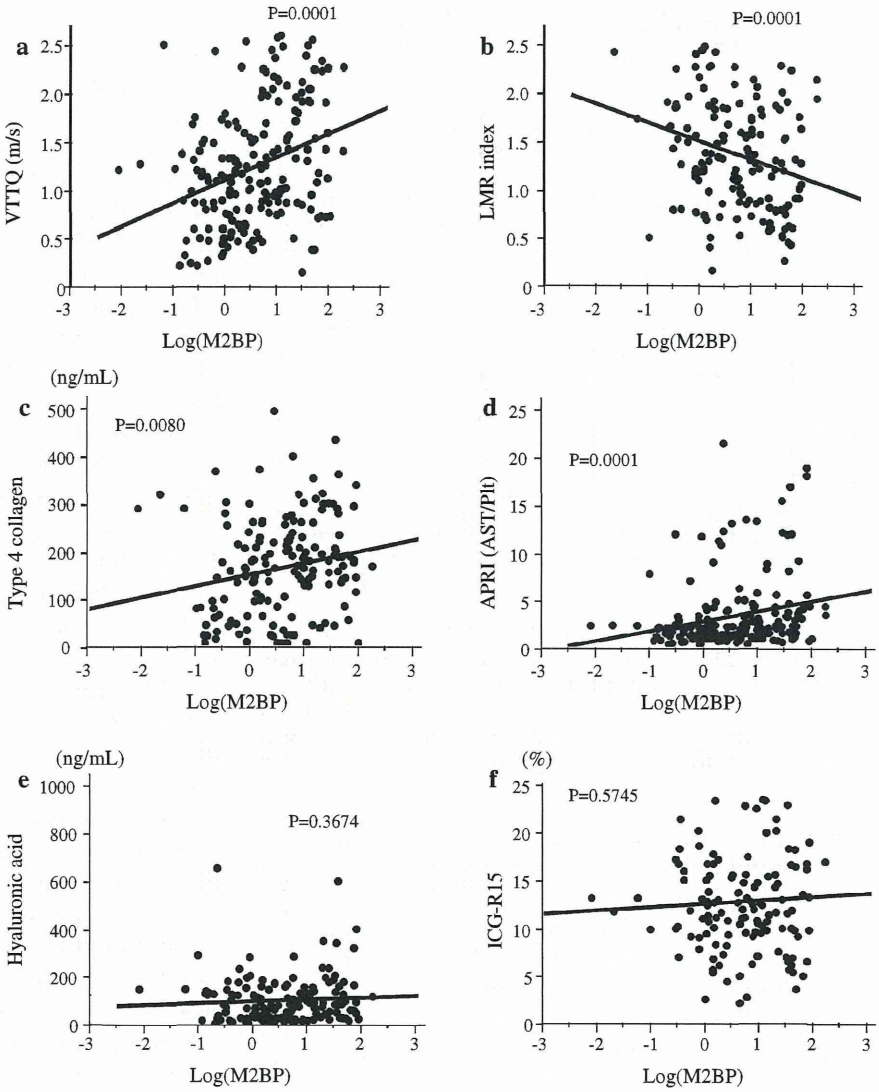

Fig. 2 Correlation of WFA^+-M2BP values with VTTQ, LMR index, and the other serum markers. The log of WFA^+-M2BP correlated with VTTQ results, a significant indicator of liver stiffness as liver fibrosis ($P = 0.0001$) (**a**). Log WFA^+-M2BP values were also inversely related with the LMR index, a significant indicator of liver function that varies inversely with liver fibrosis ($P = 0.0001$) (**b**), as well as with type 4 collagen concentrations ($P = 0.0080$) (**c**) and the APRI ($P = 0.0001$) (**d**), but not with hyaluronic acid (**e**) and ICG-R15 concentrations (**f**). *APRI* aspartate transaminase-to-platelet ratio index, *LMR* liver-to-major psoas muscle intensity ratio, *VTTQ* Virtual Touch^{TM} Tissue Quantification, *WFA^+-M2BP* *Wisteria floribunda* agglutinin-positive Mac-2 binding protein

concentration, and type 4 collagen concentration cutoffs had sensitivities for fibrosis grades $F \geq 3$ of 88.2, 72.6, 52.9, 66.7, 85.1, and 88.1 %, respectively; specificities of 78.7, 83.3, 78.7, 78.9, 40.8, and 40.5 %, respectively; PPVs of 58.9 %, 60.0 %,/ 48.2, 59.6, 34.5, and 33.0 %, respectively; and NPVs of 94.5, 89.9, 83.4, 83.5, 96.2, and 91.1 %, respectively (Table 2).

The areas under the ROC curves for the diagnosis of fibrosis ($F \geq 4$) were comparable for serum WFA^+-M2BP (0.806) and VTTQ (0.827), but significantly superior to the other surrogate markers, including LMR index (0.776), APRI (0.673), hyaluronic acid (0.657), and type 4 collagen (0.632) ($P = 0.0001$ each; Supplementary Figure 1 and Supplementary Table 3).

Diagnostic capability of WFA^+-M2BP values for liver fibrosis in each groups

The area under the ROC curves for the diagnosis of fibrosis ($F \geq 3$) using serum WFA^+-M2BP values was 0.797 in the 107 patients positive for HCVAb and 0.822 in the 21 patients negative for HBsAg and HCVAb, but was insufficient (0.620) in the 21 patients positive for HBsAg. The sensitivity, specificity, PPV, and NPV for the fibrosis grades ≥F3 in these three sets of patients using the serum WFA^+-M2BP cutoff were 96.7 %/100.0 %/61.5 %, 63.6 %/73.3 %/87.5 %, 50.9 %/60.05/88.9 %, and 98.0 %/ 100.0 %/58.3 %, respectively.

Discussion

This is the first report to quantify liver fibrosis in a large population using serum WFA^+-M2BP values by the glycan-based immunoassay, FastLec-Hepa, which was developed as a simple and accurate system for automatically detecting unique fibrosis-related glyco-alterations. The accuracy of WFA^+-M2BP values for diagnosing liver

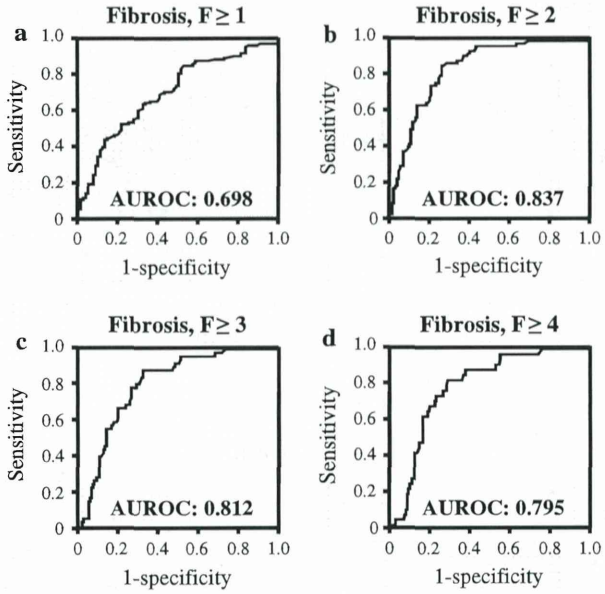

Fig. 3 Diagnostic ability of serum WFA$^+$-M2BP values to assess stages of liver fibrosis. The areas under the ROC curves of serum WFA$^+$-M2BP values for diagnosing liver fibrosis were **a** 0.698 for grade $F \geq 1$, **b** 0.837 for grade $F \geq 2$, **c** 0.812 for grade $F \geq 3$, and **d** 0.795 for grade $F \geq 4$. *ROC* receiver operating characteristic, *WFA$^+$-M2BP Wisteria floribunda* agglutinin-positive Mac-2 binding protein

Table 1 WFA$^+$-M2BP values for assessment of liver fibrosis

	Optimal cutoff (COI)	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
$F \geq 1$	1.00	84.6	47.6	69.2	69.0
$F \geq 2$	1.86	84.7	74.4	64.9	89.7
$F \geq 3$	2.21	88.2	78.7	58.9	94.5
$F \geq 4$	2.64	82.4	71.9	37.3	95.2

Optimal cutoff points gave the highest total sensitivity and specificity

APRI Aspartate transaminase-to-platelet ratio index, *COI* cutoff index, *WFA$^+$-M2BP Wisteria floribunda* agglutinin-positive Mac-2 binding protein, *m/s* meters per second *NPV* negative predictive value, *PPV* positive predictive value, *VTTQ* Virtual Touch^{TM} Tissue Quantification

fibrosis grade $F \geq 3$, measured as sensitivity, specificity, PPV, and NPV, was better than that for other surrogate markers, such as the MRI-LMR index and other serum markers of liver fibrosis, including levels of hyaluronic acid, type 4 collagen, and the APRI. WFA$^+$-M2BP could be an alternative non-invasive serum marker for liver biopsy for assessing liver fibrosis.

No reports have demonstrated the feasibility of serum WFA$^+$-M2BP values as a predictor of liver fibrosis, and the function of WFA$^+$-M2BP is unclear. Iacobelli et al. [22] identified WFA$^+$-M2BP in 1986 as a tumor-associated antigen and detected it in culture media using a monoclonal antibody from CG-5 breast cancer cell lines. WFA$^+$-M2BP is a highly glycosylated secreted protein, a plant hemagglutinin Mac-2 (galectin-3) ligand, and has 90-kDa subunits, hence, the name 90K. WFA$^+$-M2BP mainly mediates cell-to-cell and cell-to-matrix interactions, and is involved in cell proliferation and angiogenesis [23–25] by inducing the expression of cytokines, such as interleukin (IL)-1, IL-2, and IL-6 [23–25]. Indeed, endogenous WFA$^+$-M2BP ligands laminin, fibronectin, and lysosome-associated membrane protein [26, 27], and enhances cell adhesion and the extracellular matrix to promote fibrosis. Therefore, expression of WFA$^+$-M2BP levels may be proportional to the degree of liver fibrosis in patients with chronic liver diseases.

In our study, serum WFA$^+$-M2BP values had better diagnostic ability for assessment of liver fibrosis than the other serum markers, such as the APRI, hyaluronic acid, and type 4 collagen, as evaluated by the area under the ROC curves. For diagnosing fibrosis stage ≥3, the specificity and PPV using serum WFA$^+$-M2BP were 78.7 and 58.9 %, respectively compared with 78.7 and 48.2 %, respectively, using APRI; 40.8 and 34.5 %, respectively, using hyaluronic acid, and 40.5 and 33.0 %, respectively, using type 4 collagen cutoffs. These results suggest that examination of serum WFA$^+$-M2BP values is the most accurate diagnostic tool for liver fibrosis among the serum markers investigated in this study.

The diagnostic significance of surrogate markers in liver fibrosis has been evaluated distinguishing F3 from F2 fibrosis. The clinical significance of distinguishing F3 from F2 fibrosis has been widely accepted in the follow-up of patients with viral hepatitis, and also has been linked with hepatocarcinogenesis [28, 29]. The annual carcinogenesis rate was reported to be correlated with the stage of liver fibrosis in the study of 2,890 patients with hepatitis [28]. The annual incidence of hepatocellular carcinoma in patients with severe liver fibrosis of grade F3 is high (5.3 %), whereas the incidence in those with moderate liver fibrosis of grade F2 is low (1.9 %) [29]. Therefore, fibrotic change is closely correlated with hepatocarcinogenesis in patients with viral hepatitis, and it is critically important to distinguish between liver fibrosis of grades F3 and F2, which was the cause of the favorable point for the serum WFA$^+$-M2BP examination superior to the other markers in our study. In addition, we found that the measurement time for our glycan-based immunoassay was only 17 min, which has practical implications. However, a limitation is that its measurement was just applied by the glycan "sugar chain"-based immunoassay, the FastLec-Hepa system, which is currently only available in a few selected institutions. Commercial application of this system is a primary goal for evaluation of a clinically beneficial therapy through quantification of liver fibrosis in hepatitis patients. This study, however, had one important limitation, in that

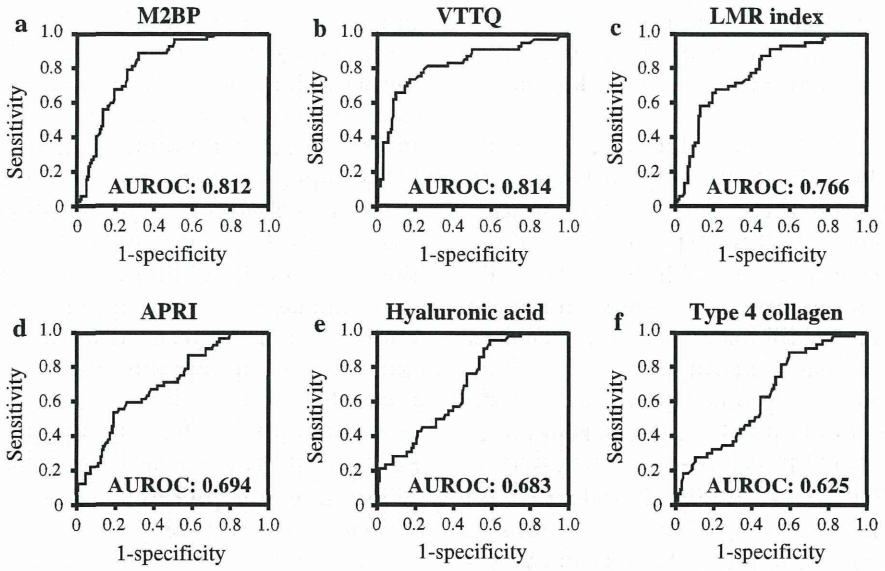

Fig. 4 Comparison of WFA$^+$-M2BP with other indicators for the diagnosis of fibrosis stage $F \geq 3$ by areas under the receiver operating curves (ROC). The areas under the ROC curves for the diagnosis of grade $F \geq 3$ fibrosis were **a** 0.812 for serum WFA$^+$-M2BP, **b** 0.814 for VTTQ, **c** 0.766 for LMR index, **d** 0.694 for APRI, **e** 0.683 for hyaluronic acid, and **f** 0.625 for type 4 collagen. *APRI* aspartate transaminase-to-platelet ratio index, *LMR* liver-to-major psoas muscle intensity ratio, *ROC* receiver operating characteristic, *VTTQ* Virtual Touch^{TM} Tissue Quantification, *WFA$^+$-M2BP Wisteria floribunda* agglutinin-positive Mac-2 binding protein

Table 2 Diagnostic performance of indicators predicting liver fibrosis ($F \geq 3$)

	Optimal cutoff	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
WFA$^+$-M2BP	2.21 (COI)	88.2	78.7	58.9	94.5
VTTQ	1.77 (m/s)	72.6	83.3	60.0	89.9
LMR index	1.15	66.7	78.9	59.6	83.5
APRI	3.33	52.9	78.7	48.2	83.4
Hyaluronic acid	58.0 (ng/mL)	85.1	40.8	34.5	96.2
Type 4 collagen	125.0 (ng/mL)	88.1	40.5	33.0	91.1

Optimal cutoff points gave the highest total sensitivity and specificity

APRI Aspartate transaminase-to-platelet ratio index, *COI* cutoff index, *WFA$^+$-M2BP Wisteria floribunda* agglutinin-positive Mac-2 binding protein, *m/s* meters per second, *NPV* negative predictive value, *PPV* positive predictive value, *VTTQ* Virtual Touch^{TM} Tissue Quantification

the number of patients with a fibrosis score of F3 was relatively low. Most patients who underwent hepatectomy had Child-Pugh grade A liver function and fibrosis scores of F0, F1, and F2, whereas most patients who underwent living donor liver transplantation had liver cirrhosis (F4). Accumulation of addition liver specimens by surgical resection may be necessary.

We have previously demonstrated that VTTQ values are correlated with liver fibrosis [9, 12, 13]. Compared with VTTQ, serum WFA$^+$-M2BP values were almost equal for predicting liver fibrosis ≥F3, with similar areas under the ROC curves (0.812 vs 0.814) and similar sensitivity (88.2 vs 72.6 %), and specificity (78.7 vs 83.3 %). However, this VTTQ method has some limitations compared with serum WFA$^+$-M2BP values. First, the diagnostic accuracy of VTTQ values in the right and left lobes of the liver was significantly different; VTTQ values in the right lobe were more accurate for diagnosing liver fibrosis than those in the left lobe, as evaluated by the area under the ROC curves and the standard deviations of each VTTQ value [9]. The other limitation is the lower diagnostic accuracy of VTTQ values in fatty liver compared with other types of hepatitis for predicting liver fibrosis [30]. Therefore, the diagnostic accuracy for assessment of liver fibrosis needs to be compared between serum WFA$^+$-M2BP and VTTQ values in these marginal clinical cases in the future. In addition, the VTTQ system installed on the ultrasound system by Siemens Medical Solutions is expensive [15–17]. Therefore, it may have the clinical significance of the WFA$^+$-M2BP assessment for the medical economy to assess the

liver fibrosis for choosing a therapeutic strategy if the serum WFA^+-M2BP examination by the glycan 'sugar chain'-based immunoassay spread more widely in the world.

With regard to the MRI-LMR index, the liver-specific contrast agent, Gd-EOB-DTPA, is widely used to improve the detectability of focal liver lesions and the characterization of liver tumors on MRI [31]. Gd-EOB-DTPA is specifically taken up by hepatocytes. Therefore, the uptake of Gd-EOB-DTPA in the liver could directly reflect the function of the liver, which varies inversely with liver fibrosis. The present study showed that serum WFA^+-M2BP values had better diagnostic ability than the LMR index in predicting of liver fibrosis ≥F3, with higher areas under the ROC curves (0.812 vs 0.766), sensitivity (88.2 vs 66.7 %), and NPV (94.5 vs 83.5 %). Considering these results, WFA^+-M2BP values may indicate liver fibrosis, as well as liver function. The specific function of WFA^+-M2BP in the progress of liver fibrosis urgently needs to be clarified by basic research.

In assessing the heterogeneity of samples, we found that the area under the ROC curves for the diagnosis of fibrosis ($F \geq 3$) using serum WFA^+-M2BP values was insufficient (0.620) only in the 21 patients positive for HBsAg. Similarly, measurements of liver stiffness, using VTTQ and transient elastography, were shown superior in patients with HCV than in those with HBV [32]. The impact of HBV infection on the function of activated WFA^+-M2BP in fibrosing liver has not been determined, suggesting the need for further research. In classifying liver inflammation in the 160 patients ($n = 160$) as A1-A4 by the METAVIR grading system, we found that 33 were classified as A0 (2.04 ± 0.39), 73 as A1 (2.75 ± 0.26), 38 as A2 (2.70 ± 0.37), 16 as A3 (2.11 ± 0.56), and none as A4 by the METAVIR grading system [33]. WFA^+-M2BP levels did not correlate significantly with hepatic inflammatory activity in the general patient cohort and in the three groups, those with HCV, with HBV, and nonBnonC. These findings are in agreement with those of previous reports [10, 11], which did not observe a correlation between WFA^+-M2BP levels and inflammatory activity. Further research is needed to clarify the molecular mechanisms of hepatic WFA^+-M2BP production in patients with different etiologies and inflammatory grades of hepatitis.

The previous report [34] analyzed the ability of serum M2BP levels to predict liver fibrosis only in patients with HCV [34]. In that study, however, M2BP concentrations could distinguish only between patients classified as F4 and those classified as F0, F1, or F2, but could not differentiate patients classified as F0 to F3. Our previous reports [10, 11] showed that a WFA-antibody sandwich ELISA (glycan-based immunoassay) was superior to screening with monoclonal anti-M2BP antibody in accelerated stability and spiking tests by using lectin microarray analysis and human embryonic kidney 293 (HEK293) cells [10, 11]. Human endogenous M2BP consists of 10–16 monomers, each weighing 1000–1500 kDa, with 70–112 *N*-glycans attached to each macromolecule [34]. Since alterations in M2BP during the progression of liver disease and fibrosis were due to changes in *N*-glycosylation, measurements of serum hyperglycosylated WFA^+-M2BP by glycan-based immunoassays, FastLec-Hepa, seemed reasonable.

In conclusion, examination of serum WFA^+-M2BP values based on a glycan-based immunoassay is an accurate, reliable, and reproducible method with which to assess liver fibrosis in patients with hepatitis. This approach could be clinically feasible for evaluation of beneficial therapy through the quantification of liver fibrosis in hepatitis patients if this measurement application is commercially realized.

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Conflict of interest The authors declare that they have no conflict of interest and have no financial interests linked to this work.

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Original Article

Wisteria floribunda agglutinin positive human Mac-2-binding protein as a predictor of hepatocellular carcinoma development in chronic hepatitis C patients

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Aims: *Wisteria floribunda* agglutinin (WFA)-positive human Mac-2-binding protein (WFA$^+$-M2BP) is a new glycol marker related to liver fibrosis. The aim of the present study was to evaluate WFA$^+$-M2BP as a predictor of hepatocellular carcinoma (HCC) development in patients with chronic hepatitis C.

Methods: This case–control study included 14 patients with chronic hepatitis C who developed HCC and 52controls, matched for age, gender, and fibrosis stage. WFA$^+$-M2BP was measured at biopsy and follow-up. Time zero was set at the date of liver biopsy.

Results: WFA$^+$-M2BP increased stepwise with progression of liver fibrosis ($p < 0.001$). Cumulative incidence of HCC development was significantly higher in patients with WFA$^+$-M2BP ≥4.2 ($p < 0.001$) or in those with time-course changes in WFA$^+$-M2BP (ΔWFA$^+$-M2BP/year) ≥0.3 ($p = 0.03$). Multivariate analyses demonstrated that WFA$^+$-M2BP ≥4.2 [hazard ratio (HR): 4.1, 95% confidence interval (CI): 1.1–15, $p = 0.04$], ΔWFA$^+$-M2BP/year ≥0.3 (HR: 5.5, 95% CI: 1.5–19, $p = 0.008$), and AFP ≥10 ng/ml (HR: 4.7, 95% CI: 1.1–19, $p = 0.03$) were independent predictive factors of HCC development. Based on these data, we developed a simple scoring system to predict HCC development using these three factors. Using these scores, patients were classified into four groups; cumulative incidence of HCC development significantly increased with increasing scores ($p < 0.001$).

Conclusions: WFA$^+$-M2BP measurements and time-course changes in WFA$^+$-M2BP can be used to identify patients at high risk of HCC development. Real-time monitoring of WFA$^+$-M2BP can be a novel predictor of HCC development.

Key words: chronic hepatitis C, hepatocellular carcinoma, liver fibrosis, WFA$^+$-M2BP

INTRODUCTION

HEPATITIS C VIRUS (HCV) INFECTION is the major cause of chronic hepatitis, which progresses to hepatocellular carcinoma (HCC) in many patients.[1] Advanced stage liver fibrosis is associated with HCC development;[2] therefore, accurate staging of liver fibrosis is extremely important in clinical practice. Although liver biopsy is the gold standard to diagnose liver fibrosis,[3,4] this method may be inaccurate because of sampling errors and interobserver variations.[5,6]

In recent years, several alternative non-invasive methods for evaluating liver fibrosis have emerged. It has been reported that liver fibrosis can be predicted by transient elastography,[7,8] acoustic radiation force impulse imaging[9] and real-time tissue elastography[10,11] using ultrasonography. In addition, blood tests, such as the aspartate aminotransferase (AST)/platelet ratio index (APRI)[12,13] and FIB-4 index,[14,15] have been reported to be useful in predicting liver fibrosis. Furthermore, these non-invasive markers have been reported to be associated with HCC development and liver-related mortality.[16–21]

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Glycans are referred to as the face of cells. Glycans mutate according to disease status, demonstrating their potential as biomarkers for chronic disease. In patients with chronic hepatitis, glycomic and glycoproteomic biomarker methods have also been reported to be useful in the diagnosis of liver fibrosis.[22,23]

Wisteria floribunda agglutinin (WFA) positive human Mac-2-binding protein (WFA^+-M2BP), a new glycol marker related to liver fibrosis, is obtained using sandwich immunoassay with WFA and anti-M2BP antibody. This marker glycoprotein has demonstrated fibrosis-related glyco-alteration potential.[24] The significance of WFA^+-M2BP as a predictor of liver fibrosis in chronic HCV infection has been previously reported;[24,25] however, the relationship between WFA^+-M2BP and HCC remains unclear. The aim of this study was to evaluate WFA^+-M2BP as a predictor of HCC development.

METHODS

Patients

WE CONDUCTED A matched case–control study to assess the relationship between WFA^+-M2BP and HCC development. Of patients with chronic HCV infection who underwent liver biopsy at Musashino Red Cross Hospital (Tokyo, Japan) between 2002 and 2010 and did not achieve sustained virological response to interferon therapy, 14 who developed HCC were enrolled in this study. Fifty-two patients who did not develop HCC served as matched controls on the basis of sex, age and histological fibrosis stage. WFA^+-M2BP was measured for all patients at biopsy and at more than 1-year follow up (mean interval, 2.6 ± 1.8 years). Exclusion criteria were as follows: (i) co-infection with hepatitis B virus or HIV; (ii) history of autoimmune hepatitis or primary biliary cirrhosis; or (iii) history of HCC at study entry. Time zero was set at the date of liver biopsy. Written informed consent was obtained from each patient. The study protocol conformed to the ethical guidelines of the Declaration of Helsinki and was approved by the institutional ethics review committees.

Histological evaluation

Liver biopsy specimens were laparoscopically obtained using 13-G needles. If laparoscopy was contraindicated because of a history of upper abdominal surgery, percutaneous ultrasound-guided liver biopsy was performed using 15-G needles. Specimens were fixed, paraffin-embedded and stained with hematoxylin–eosin and Masson-trichrome. All liver biopsy samples were independently evaluated by two senior pathologists who were blinded to the clinical data. Fibrosis staging was categorized according to the METAVIR score: F0, no fibrosis; F1, portal fibrosis without septa; F2, portal fibrosis with few septa; F3, numerous septa without cirrhosis; and F4, cirrhosis.[26] If staging was inconsistent between the two pathologists, an appropriate stage was determined by means of a discussion between them.

HCC surveillance and diagnosis

Ultrasonography and blood tests (including tumor marker testing) were performed on all patients every 3–6 months for HCC surveillance. In cases of an increase in tumor marker level or abdominal ultrasonography suggestive of lesions suspicious for HCC, contrast-enhanced computed tomography, magnetic resonance imaging or angiography was performed. HCC was diagnosed for tumors that showed vascular enhancement at an early phase with washout at a later phase. Tumor biopsy was used to diagnose tumors with non-typical imaging results.

WFA^+-M2BP quantification using sandwich immunoassay with WFA and anti-M2BP antibody

The fibrosis-specific glycosylated M2BP form was measured by sandwich immunoassay. Glycosylated M2BP was captured by WFA immobilized on magnetic beads. The bound product was assayed with an antihuman M2BP monoclonal antibody linked to alkaline phosphatase (ALP–αM2BP). Assay manipulation was fully automated using a chemiluminescence enzyme immunoassay machine (HISCL-2000i; Sysmex, Kobe, Japan) and acquired within 17 min.[24] All counts were standardized and converted to a cut-off index designated as WFA^+-M2BP.[27]

WFA^+-M2BP was measured two times for all patients: at biopsy and follow up. Time-course changes in WFA^+-M2BP (ΔWFA^+-M2BP/year) were calculated using the following formula:

$$\Delta WFA^+\text{-M2BP/year} = \frac{WFA^+ - \text{M2BP at follow up} - WFA^+\text{-M2BP at a liver biopsy}}{\text{interval between the two measurements (years)}}$$

Clinical and biological data

Patient age and sex were recorded. Serum samples were collected at liver biopsy and the following values were obtained through serum sample analyses: bilirubin, AST, alanine aminotransferase (ALT), platelet count and α-fetoprotein (AFP). APRI and FIB-4 were calculated at liver biopsy, as previously reported.[12,14]

Statistical analyses

Categorical data were compared using the χ^2-test and Fisher's exact test. Distributions of continuous variables were analyzed using Student's *t*-test or the Mann–Whitney *U*-test. Correlations between the WFA$^+$-M2BP and histological fibrosis stage were analyzed using Spearman's rank correlation coefficients. $P < 0.05$ was considered statistically significant. Receiver-operator curves (ROC) were constructed, and the area under the ROC (AUROC) was calculated. The cumulative incidence curve was determined by the Kaplan–Meier method, and differences among groups were assessed using a log-rank test. Factors associated with HCC risk were determined by the Cox proportional hazard model. Statistical analyses were performed using the Statistical Package for the Social Sciences software version 20.0 (SPSS, Chicago, IL, USA).

RESULTS

Patient characteristics

PATIENT CHARACTERISTICS AT biopsy are listed in Table 1. Age, sex, AST, ALT, bilirubin levels, platelet counts and histological fibrosis stage were not significantly different between patients with HCC development and patients without HCC development. The mean follow-up period for all patients was 4.1 years.

Table 1 Patient characteristics

	Patients with HCC development ($n = 14$)	Patients without HCC development ($n = 52$)	*P*
Age (years)	65.2 ± 6.2	60.8 ± 9.6	0.1
Sex (male/female)	9/5	22/30	0.1
AST (IU/L)	69.1 ± 43	50.4 ± 31	0.07
ALT (IU/L)	74.5 ± 61	52.5 ± 34	0.08
Bilirubin (mg/dL)	0.74 ± 0.3	0.72 ± 0.3	0.8
Platelet counts ($\times 10^9$/L)	125 ± 38	144 ± 51	0.2
Fibrosis stage (1/2/3/4)	3/3/5/3	15/18/15/4	0.4
AFP (ng/mL)	28.2 ± 36	11.3 ± 18	0.02
WFA$^+$-M2BP (COI)	4.70 ± 4.0	2.42 ± 2.2	0.007

AFP, α-fetoprotein; ALT, alanine aminotransferase; AST, aspartate aminotransferase; COI, cut-off index; HCC, hepatocellular carcinoma.

Relationship between histological findings and WFA$^+$-M2BP

The relationship between histological findings and WFA$^+$-M2BP was evaluated. Figure 1 shows median WFA$^+$-M2BP compared with the METAVIR fibrosis stage. Median WFA$^+$-M2BP increased stepwise with progression of liver fibrosis; levels for the F1, F2, F3 and F4 stages were 0.81, 1.82, 2.31 and 7.50, respectively ($P < 0.001$).

Prediction of HCC development by WFA$^+$-M2BP and time-course changes in WFA$^+$-M2BP

The AUROC of WFA$^+$-M2BP for prediction of HCC development within 5 years was 0.768, and a WFA$^+$-M2BP level of 4.2 was selected as the optimal cut-off value. The cumulative incidence of HCC development was significantly higher in patients with WFA$^+$-M2BP of 4.2 or more than those with WFA$^+$-M2BP less than 4.2 ($P < 0.001$, Fig. 2A). Similarly, AUROC of ΔWFA$^+$-M2BP/year for prediction of HCC development within 5 years was 0.607, and the optimal ΔWFA$^+$-M2BP/year cut-off value of 0.3 was selected. The cumulative incidence of HCC development was significantly higher in patients with ΔWFA$^+$-M2BP/year of 0.3 or more than those with ΔWFA$^+$-M2BP/year of less than 0.3 ($P = 0.03$, Fig. 2b). AUROC for APRI, FIB-4, platelet count and AFP was 0.708, 0.736, 0.674 and 0.822, respectively (Fig. 3). Besides AFP, WFA$^+$-M2BP was more accurate for predicting HCC development than fibrosis stage and other fibrosis markers.

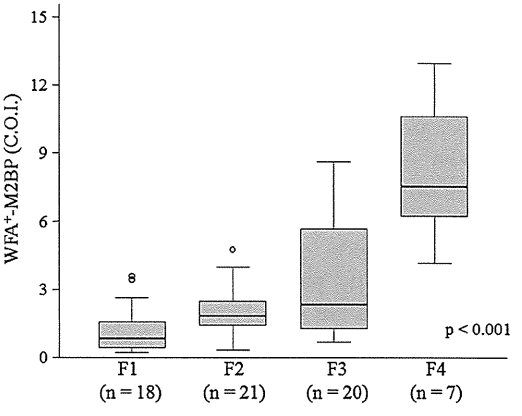

Figure 1 Correlation between WFA$^+$-M2BP and fibrosis stage. Box plot of WFA$^+$-M2BP is shown for each fibrosis stage. The box plot represents the 25th to 75th percentiles, giving the interquartile range. The line through the box indicates the median value, and an error bar indicates minimum and maximum non-extreme values.

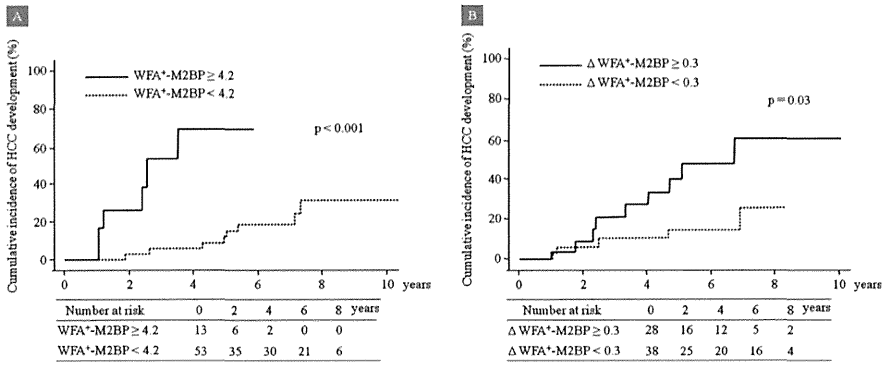

Figure 2 Cumulative incidence of hepatocellular carcinoma (HCC) development. Patients were categorized into two groups according to (a) WFA$^+$-M2BP and (b) time-course change in WFA$^+$-M2BP.

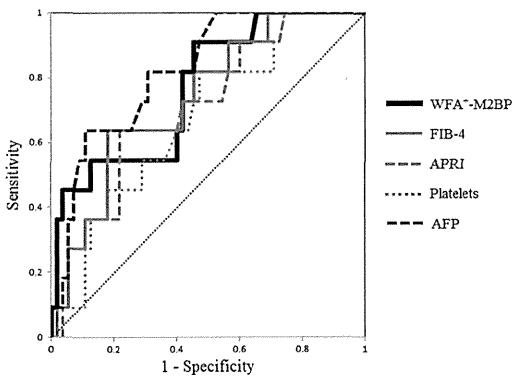

Figure 3 Receiver–operator curves of WFA+-M2BP, fibrosis markers and α-fetoprotein (AFP). APRI, aspartate aminotransferase-to-platelet ratio index.

Factors associated with HCC development

Univariate analysis revealed factors that increase the HR for HCC development (Table 2). High WFA$^+$-M2BP levels and high ΔWFA$^+$-M2BP/year levels were risk factors for HCC development. Compared with patients with WFA$^+$-M2BP of less than 4.2, HR for those with WFA$^+$-M2BP of 4.2 or more was 8.2 (95% confidence interval [CI], 2.6–26; $P < 0.001$). Similarly, patients with ΔWFA$^+$-M2BP/year of 0.3 or more had a HR of 3.1 compared with those with ΔWFA$^+$-M2BP/year of less than 0.3 (95% CI, 1.1–9.3; $P = 0.04$). Multivariate analyses demonstrated that WFA$^+$-M2BP, ΔWFA$^+$-M2BP/year and AFP levels were independent predictive factors for HCC development (Table 2). HR for HCC development with WFA$^+$-M2BP of 4.2 or more, ΔWFA$^+$-M2BP/year of 0.3 or more and AFP of 10 ng/mL or more were 4.1 (95% CI, 1.1–15; $P = 0.04$), 5.5 (95% CI, 1.5–19; $P = 0.008$) and 4.7 (95% CI, 1.1–19; $P = 0.03$), respectively. We developed a scoring system based on these three factors. WFA$^+$-M2BP of 4.2 or more, ΔWFA$^+$-M2BP/year of 0.3 or more and AFP of 10 ng/mL or more each contributed 1 point to the score. WFA$^+$-M2BP of less than 4.2, ΔWFA$^+$-M2BP/year of less than 0.3 and AFP of less than 10 ng/mL each contributed 0 points to the score. Using this scoring system, patients were classified into four groups according to the total score of 0, 1, 2 or 3. Cumulative incidence of HCC development significantly increased as the score increased ($P < 0.001$, Fig. 4).

DISCUSSION

RECENTLY, SEVERAL NON-INVASIVE methods to evaluate liver fibrosis have been developed. The WFA$^+$-M2BP glycol marker test using sandwich immunoassay with WFA and anti-M2BP antibody has demonstrated utility as a liver fibrosis marker.[24,25] However, the relationship between WFA$^+$-M2BP and HCC development remains unknown. The aim of this study was to determine whether WFA$^+$-M2BP could be used to predict HCC development.

The important findings in this study were that WFA$^+$-M2BP and time-course changes in WFA$^+$-M2BP independently predicted HCC development. It is widely known that advanced liver fibrosis is associated with HCC development.[2] Non-invasive markers of liver fibrosis are reported to be associated with HCC development and liver-related mortality.[16–21] The correlation of liver fibrosis and WFA$^+$-M2BP was demonstrated in the present study. Patients with a high level of WFA$^+$-M2BP have been suggested to have advanced liver fibrosis. Hence, we demonstrated that cumulative incidence of HCC development was higher in patients with high WFA$^+$-M2BP levels than those with low WFA$^+$-M2BP levels. WFA$^+$-M2BP proved