In the Japanese population, both haplotypes *DPA1*01:03-DPB1*04:01* and *DPA1*01:03-DPB1*04:02* showed significant associations with protection against HBV infection ($P = 1.17 \times 10^{-5}$; OR = 0.32; 95% CI, 0.18–0.56 for *DPA1*01:03-DPB1*04:01* and $P = 1.95 \times 10^{-7}$; OR = 0.37; 95% CI, 0.24–0.55 for *DPA1*01:03-DPB1*04:02*). In the Korean subjects, a significant association of *DPA1*01:03-DPB1*04:02* was also demonstrated; however, no association was observed for *DPA1*01:03-DPB1*04:01*. Because the observed number of each haplotype was small, none of the other haplotypes showed a significant association with protection against HBV infection.

In order to identify trans-ethnic DPA1-DPB1 haplotypes associated with HBV infection, a meta-analysis was performed. A meta-analysis further revealed that the *DPA1*01:03-DPB1*02:01* haplotype was significantly associated with protection against HBV infection ($P = 1.45 \times 10^{-5}$; OR = 0.69; 95% CI, 0.58–0.82) (Fig. S1C).

Discussion

Among 2.2 billion individuals worldwide who are infected with HBV, 15% of these are chronic carriers. Of chronic carriers, 10–15% develops LC, liver failure and HCC, and the remaining individuals eventually achieve a state of nonreplicative infection, resulting in HBsAg negative and anti-HBc positive, i.e. HBV-resolved individuals. To identify host genetic factors associated with HBV-related disease progression may lead HBV patients to discriminate individuals who need treatment.

The *HLA-DPA1* and *HLA-DPB1* genes were identified as host genetic factors significantly associated with CHB infection, mainly in Asian populations [7–12], and not in European populations [13]. In the previous association analyses of *HLA-DPB1* alleles with HBV infection, one risk allele *HLA-DPB1*05:01* (OR = 1.52; 95% CI, 1.31–1.76), and two protective alleles, *HLA-DPB1*04:01* (OR = 0.53; 95% CI, 0.34–0.80) and *HLA-DPB1*04:02* (OR = 0.47; 95% CI, 0.34–.64), were identified in the Japanese population [7]. In this study, we further identified a new risk allele *HLA-DPB1*09:01* (OR = 1.94; 95% CI, 1.45–2.62) for HBV infection and a new protective allele *HLA-DPB1*02:01* (OR = 0.71; 95% CI, 0.56–0.89) in the Japanese population, in addition to the previously reported alleles (Table S2) [7]. The discrepancy in the association of *HLA-DPB1*09:01* allele with risk for HBV infection in a previous study [7] results from the elevated frequency of *HLA-DPB1*09:01* in the controls (12.2%), which is higher than our controls (8.7%). In this study, healthy subjects were recruited as controls. In contrast, individuals that were registered in BioBank Japan as subjects with diseases other than CHB were recruited as controls in the previous study [7], which may have included patients with diseases with which *HLA-DPB1*09:01* is associated. Although no significant association of *HLA-DPB1*09:01* with risk for HBV infection was observed in the Korean subjects, *HLA-DPB1*09:01* appears to have a susceptible effect on HBV infection, as it showed the same direction of association. When the association analyses in Japanese and Korean subjects were combined in meta-analysis, the association was statistically significant ($P = 1.36 \times 10^{-6}$; OR = 1.97; 95% CI, 1.50–2.59). Thus, *HLA-DPB1*09:01* may be a Northeast Asian-specific allele associated with risk for HBV infection.

Moreover, a significant association of *HLA-DPB1*13:01* with risk of HBV infection (OR = 2.17; 95% CI, 1.40–3.47) was identified in the Thai subjects. However, the frequency of *HLA-DPB1*13:01* in Thai healthy controls (11.5% in the present study) reportedly varies, ranging from 15.4% to 29.5%, due to the population diversity [15–17]. Therefore, a replication analysis is required to confirm the association of *HLA-DPB1*13:01* with HBV infection in the Thai subjects. There were four other marginally associated *HLA-DPB1* alleles with low allele frequencies below 5% in HBV patients and healthy controls, including *HLA-DPB1*28:01*, *-DPB1*31:01*, *-DPB1*100:01*, and *-DPB1*105:01*, in the Hong Kong and Thai subjects. Because these infrequent alleles may have resulted from false positive associations, the association needs to be validated in a large number of subjects.

*HLA-DPB1*02:01* showed a significant association with protection against HBV infection in both Japanese and Hong Kong populations (Table S2); however, the *HLA-DPB1*02:01* allele was not associated with HBV infection in the previous study [7]. Although *HLA-DPB1*02:01* showed no association in either Korean or Thai populations, a significant association of *HLA-DPB1*02:01* with protection against HBV infection among four Asian populations was detected in meta-analysis ($P = 5.22 \times 10^{-6}$; OR = 0.68; 95% CI, 0.58–0.81) (Fig. S1B). We therefore conclude that the present finding is not a false positive.

A recent report showed that *HLA-DPB1*02:01:02*, **02:02*, **03:01:01*, **04:01:01*, **05:01*, **09:01*, and **14:01* were significantly associated with response to booster HB vaccination in Taiwan neonatally vaccinated adolescents [18]. The *HLA-DPB1*02:01:02*, **02:02*, **03:01:01*, **04:01:01*, and **14:01* were significantly more frequent in recipients whose post-booster titers of antibodies against HBV surface antigen (anti-HBs) were detectable, on the other hand, *HLA-DPB1*05:01* and **09:01* were significantly more frequent in recipients who were undetectable. Moreover, the *HLA-DPB1*05:01* and **09:01* significantly increase the likelihoods of undetectable pre-booster anti-HBs titers. These results seem consistent with our findings, in which *HLA-DPB1*05:01* and **09:01* are associated with susceptibility to chronic hepatitis B infection.

We also identified a protective effect of *HLA-DPB1*02:01* allele on disease progression in Asian populations. Previous studies identified the association of HLA class II genes including *HLA-DQ* and *HLA-DR* with development of HBV related hepatocellular carcinoma in the Chinese population [6,19,20]. In this study using Japanese and Korean samples, we identified significant associations between *HLA-DPB1*02:01* and disease progression in CHB patients ($P = 4.26 \times 10^{-5}$; OR = 0.45; 95% CI, 0.30–0.67, for Japanese and $P = 8.74 \times 10^{-4}$; OR = 0.47; 95% CI, 0.29–0.75 for Korean) (Table S4). Although the association of *HLA-DPB1*02:01* with disease progression was weaker after adjustment for age and gender in Korean subjects ($P = 2.54 \times 10^{-2}$; OR = 0.55; 95% CI, 0.33–0.93), the same direction of association was observed (i.e. protective effect on disease progression) (Table 2). The protective effects of *HLA-DPB1*02:01* on disease progression showed a significant association after adjustment for age and gender in the Japanese population ($P = 1.77 \times 10^{-4}$; OR = 0.47; 95% CI, 0.32–0.70); moreover, a significant association between *HLA-DPB1*02:01* was observed among four Asian populations, under which population was adjusted by using dummy variables in a multivariate logistic regression analysis ($P = 1.55 \times 10^{-7}$; OR = 0.50; 95% CI, 0.39–0.65) (Table 2).

The *HLA-DPA1* and *HLA-DPB1* belong to the HLA class II alpha and beta chain paralogues, which make a heterodimer consisting of an alpha and a beta chain on the surface of antigen presenting cells. This HLA class II molecule plays a central role in the immune system by presenting peptides derived from extracellular proteins. We identified two susceptible haplotypes (*DPA1*02:02-DPB1*05:01* and *DPA1*02:01-DPB1*09:01*) and three protective haplotypes (*DPA1*01:03-DPB1*04:01*, *DPA1*01:03-DPB1*04:02*, and *HLA-DPA1*01:03-DPB1*02:01*) to chronic hepatitis B infection, which may result in different binding

affinities between HLA-DP subtypes and extracellular antigens. Although functional analyses of HLA-DP subtypes to identify HBV-related peptides are not fully completed, identification of susceptible and protective haplotypes as host genetic factors would lead us to understand the pathogenesis of HBV infection including viral factors.

In summary, we identified a new risk allele *HLA-DPB1*09:01*, which was specifically observed in Northeast Asian populations, Japanese and Korean. Moreover, a new protective allele *HLA-DPB1*02:01* was identified among four Asian populations: Japanese, Korean, Hong Kong and Thai. The protective allele *HLA-DPB1*02:01* was associated with both chronic HBV infection and disease progression in chronic HBV patients. Identification of a total of five alleles, including two risk alleles (*DPB1*09:01* and *DPB1*05:01*) and three protective alleles (*DPB1*04:01*, *DPB1*04:02* and *DPB1*02:01*), would enable HBV-infected individuals to be classified into groups according to the treatment requirements. Moreover, the risk and protective alleles for HBV infection and disease progression, identified in this study by means of trans-ethnic association analyses, would be key host factors to recognize HBV-derived antigen peptides. The present results may lead to subsequent functional studies into HLA-DP molecules and viral factors in order to understand the pathogenesis of HBV infection and development of hepatocellular carcinoma.

Materials and Methods

Ethics Statement

All study protocols conform to the relevant ethical guidelines, as reflected in the *a priori* approval by the ethics committee of National Center for Global Health and Medicine, and by the ethics committees of all participating universities and hospitals, including The University of Tokyo, Japanese Red Cross Kanto-Koshinetsu Block Blood Center, The University of Hong Kong, Chulalongkorn University, Yonsei University College of Medicine, Nagoya City University Graduate School of Medical Sciences, Musashino Red Cross Hospital, Tokyo Medical and Dental University, Teine Keijinkai Hospital, Hokkaido University Graduate School of Medicine, Kurume University School of Medicine, Okayama University Graduate School of Medicine, Yamaguchi University Graduate School of Medicine, Tottori University, Kyoto Prefectural University of Medicine, Osaka City University Graduate School of Medicine, Nagoya Daini Red Cross Hospital, Ehime University Graduate School of Medicine, Kanazawa University Graduate School of Medicine, National Hospital Organization Osaka National Hospital, Iwate Medical University, Kawasaki Medical College, Shinshu University School of Medicine, Saitama Medical University, Kitasato University School of Medicine, Saga Medical School, and University of Tsukuba.

Written informed consent was obtained from each patient who participated in this study and all samples were anonymized. For Japanese healthy controls, 419 individuals were de-identified with information about gender, and all were recruited after obtaining verbal informed consent in Tokyo prior to 1990. For the 419 Japanese healthy individuals, written informed consent was not obtained because the blood sampling was conducted before the "Ethical Guidelines for Human Genome and Genetic Sequencing Research" were established in Japan. Under the condition that DNA sample is permanently de-linked from the individual, this study was approved by the Research Ethics Committee of National Center for Global Health and Medicine.

Characteristics of studied subjects

All of the 3,167 genomic DNA samples were collected from individuals with HBV, HBV-resolved individuals (HBsAg-negative and anti-HBc-positive) and healthy controls at 26 multi-center hospitals throughout Japan, Korea, Hong Kong, and Thailand (Table 1). In a total of 1,291 Japanese and 586 Korean samples, 1,191 Japanese individuals and all 586 Korean individuals were included in our previous study [9]. With regard to additional Japanese individuals, we collected samples from 48 healthy controls at Kohnodai Hospital, and 52 HBV patients at Okayama University Hospital and Ehime University Hospital, including 26 individuals with LC and 26 individuals with HCC. A total of 661 Hong Kong samples and 629 Thai samples were collected at Queen Mary Hospital and Chulalongkorn University, respectively.

HBV status was measured based on serological results for HBsAg and anti-HBc with a fully automated chemiluminescent enzyme immunoassay system (Abbott ARCHITECT; Abbott Japan, Tokyo, Japan, or LUMIPULSE f or G1200; Fujirebio, Inc., Tokyo, Japan). For clinical staging, inactive carrier (IC) state was defined by the presence of HBsAg with normal ALT levels over 1 year (examined at least four times at 3-month intervals) and without evidence of liver cirrhosis. Chronic hepatitis (CH) was defined by elevated ALT levels (>1.5 times the upper limit of normal [35 IU/L]) persisting over 6 months (by at least 3 bimonthly tests). Acute exacerbation (AE) of chronic hepatitis B was defined as an elevation of ALT to more than 10 times the upper limit of normal (ULN, 58 IU/L) and bilirubin to at least three times ULN (15 μmol/L). LC was diagnosed principally by ultrasonography (coarse liver architecture, nodular liver surface, blunt liver edges and hypersplenism), platelet counts<100,000/cm^3, or a combination thereof. Histological confirmation by fine-needle biopsy of the liver was performed as required. HCC was diagnosed by ultrasonography, computerized tomography, magnetic resonance imaging, angiography, tumor biopsy or a combination thereof.

The Japanese control samples from HBV-resolved subjects (HBsAg-negative and anti-HBc-positive) at Nagoya City University-affiliated healthcare center were used by comprehensive agreement (anonymization in a de-identified manner) in this study. Some of the unrelated and anonymized Japanese healthy controls were purchased from the Japan Health Science Research Resources Bank (Osaka, Japan). One microgram of purified genomic DNA was dissolved in 100 μl of TE buffer (pH 8.0) (Wako, Osaka, Japan), followed by storage at −20°C until use.

Genotyping of *HLA-DPA1* and *HLA-DPB1* alleles

High resolution (4-digit) genotyping of *HLA-DPA1* and *-DPB1* alleles was performed for HBV patients, resolved individuals, and healthy controls in Japan, Korea, Hong Kong, and Thailand. LABType SSO HLA DPA1/DPB1 kit (One Lambda, CA) and a Luminex Multi-Analyte Profiling system (xMAP; Luminex, Austin, TX) were used for genotyping, in according with the manufacturer's protocol. Because of the small quantity of genomic DNA in some Korean samples, we performed whole genome amplification for a total of 486 samples using GenomiPhi v2 DNA Amplification kit (GE Healthcare Life Sciences, UK), in accordance with the manufacturer's instruction.

A total of 2,895 samples were successfully genotyped and characteristics of these samples are summarized in Table S1.

Statistical analysis

Fisher's exact test in two-by-two cross tables was used to examine the associations between *HLA-DP* allele and chronic HBV infection or disease progression in chronic HBV patients,

using statistical software R2.9. To avoid false-positive results due to multiple testing, significance levels were adjusted based on the number of observed alleles at each locus in each population. For *HLA-DPA1* alleles, the number of observed alleles was 3 in Japanese, 4 in Korean, 5 in Hong Kong, and 5 in Thai subjects. Therefore, the significant levels for α were set at $\alpha = 0.05/3$ in Japanese, $\alpha = 0.05/4$ in Korean, $\alpha = 0.05/5$ in Hong Kong, and $\alpha = 0.05/5$ in Thai subjects. In the same way, significant levels for *HLA-DPB1* alleles were $\alpha = 0.05/10$, $0.05/11$, $0.05/12$, and $0.05/16$, respectively. Multivariate logistic regression analysis adjusted for age and sex (used as independent variables) was applied to assess associations between the number of *DPB1*02:01* alleles (i.e., 0, 1, or 2) and disease progression in CHB patients. To examine the effect of *DPB1*02:01* allele on disease progression in all populations, population was further adjusted by using three dummy variables (i.e., (c1, c2, c3) = (0, 0, 0) for Japanese, (1, 0, 0) for Korean, (0, 1, 0) for Hong Kong, and (0, 0, 1) for Thai) in a multivariate logistic regression analysis. We obtained the following regression equation: logit(p) = −3.905+0.083*age+(−0.929)*sex+(−0.684)**DPB1*02:01*+1.814*c1+(−0.478)*c2+0.782*c3. Significance levels in the analysis of disease progression in CHB patients were set as $\alpha = 0.05/10$ in Japanese, $\alpha = 0.05/11$ in Korean, $\alpha = 0.05/15$ in Hong Kong, and $\alpha = 0.05/15$ in Thai subjects. The phase of each individual (i.e., a combination of two *DPA1-DPB1* haplotypes) was estimated using PHASE software [21], assuming samples are selected randomly from a general population. In comparison of the estimated *DPA1-DPB1* haplotype frequencies, significant levels were set as $\alpha = 0.05/14$ in Japanese, $\alpha = 0.05/17$ in Korean, $\alpha = 0.05/17$ in Hong Kong, and $\alpha = 0.05/18$ in Thai subjects. Meta-analysis was performed using the DerSimonian-Laird method (random-effects model) in order to calculate pooled OR and its 95% confidence interval (95% CI). We applied meta-analysis for alleles with frequency>1% in all four Asian populations. The significance levels in meta-analysis were adjusted by the total number of statistical tests; $\alpha = 0.05/20$ for *DPA1* alleles, $\alpha = 0.05/57$ for *DPB1* alleles, and $\alpha = 0.05/74$ for *DPA1-DPB1* haplotypes.

Supporting Information

Figure S1 Comparison of odds ratios in association analyses for *HLA-DP* with chronic HBV infection among four Asian populations: (A) *HLA-DPA1* alleles; (B) *HLA-DPB1* alleles; and (C) *HLA DPA1-DPB1* haplotypes. Meta-analysis was performed using the DerSimonian-Laird method (random-effects model) to calculate pooled OR and its 95% confidence interval (95% CI). Bold depicts a statistically significant association after correction of significance level.
(DOCX)

Table S1 Individuals with successfully genotyped for *HLA-DPA1* and *HLA-DPB1*.
(DOCX)

Table S2 Frequencies of HLA-DP alleles in HBV patients and healthy controls among Asian populations.
(XLSX)

Table S3 Frequencies of HLA-DP alleles in HBV patients and resolved individuals among Asian populations.
(XLSX)

Table S4 Associations of HLA-DPB1 alleles with disease progression in CHB patients among Asian populations.
(XLSX)

Table S5 Estimated frequencies of HLA DPA1-DPB1 haplotypes in HBV patients and healthy controls among Asian populations.
(XLSX)

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Author Contributions

Conceived and designed the experiments: NN HS MS KT M. Mizokami. Performed the experiments: NN HS KK Y. Mawatari M. Kawashima M. Minami. Analyzed the data: NN HS M. Kawashima JO. Contributed reagents/materials/analysis tools: W-KS M-FY NP YP SHA K-HH K. Matsuura YT M. Kurosaki YA NI J-HK SH TI KY IS Y. Murawaki YI AT EO YH MH SK EM KS KH ET SM MW YE NM K. Murata M. Korenaga KT M. Mizokami. Wrote the paper: NN HS JO KT M. Mizokami.

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ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

A novel serum marker, glycosylated *Wisteria floribunda* agglutinin-positive Mac-2 binding protein (WFA$^+$-M2BP), for assessing liver fibrosis

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Abstract

Background Recently, a novel marker, hyperglycosylated *Wisteria floribunda* agglutinin-positive Mac-2 binding protein (WFA$^+$-M2BP), was developed for liver fibrosis using the glycan "sugar chain"-based immunoassay; however, the feasibility of WFA$^+$-M2BP for assessing liver fibrosis has not been proven with clinical samples of hepatitis.

Methods Serum WFA$^+$-M2BP values were evaluated in 200 patients with chronic liver disease who underwent histological examination of liver fibrosis. The diagnostic accuracy of WFA$^+$-M2BP values was compared with various fibrosis markers, such as ultrasound based-virtual touch tissue quantification (VTTQ), magnetic resonance imaging based-liver-to-major psoas muscle intensity ratio (LMR), and serum markers, including hyaluronic acid, type 4 collagen, and aspartate transaminase to platelet ratio index (APRI).

Results Serum WFA$^+$-M2BP levels in patients with fibrosis grades F0, F1, F2, F3, and F4 had cutoff indices 1.62, 1.82, 3.02, 3.32, and 3.67, respectively, and there were significant differences between fibrosis stages F1 and F2, and between F2 and F3 ($P < 0.01$). The area under the receiver operating characteristic curves for the diagnosis of fibrosis ($F \geq 3$) using serum WFA$^+$-M2BP values (0.812) was almost comparable to that using VTTQ examination (0.814), but was superior to the other surrogate markers, including LMR index (0.766), APRI (0.694), hyaluronic acid (0.683), and type 4 collagen (0.625) ($P < 0.01$ each).

Conclusions Serum WFA$^+$-M2BP values based on a glycan-based immunoassay is an accurate, reliable, and reproducible method for the assessment of liver fibrosis. 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.

Keywords Liver fibrosis · Chronic hepatitis · M2BP · Mac-2 binding protein · VTTQ · Fibrosis marker

Abbreviations

ALT	Alanine aminotransferase
APRI	Aspartate transaminase-to-platelet ratio index
ARFI	Acoustic radiation force impulse
AST	Aspartate aminotransferase

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COI	Cutoff index
Gd-EOB-DTPA	Gadolinium ethoxybenzyl diethylenetriamine pentaacetic acid
HBsAg	Hepatitis B virus surface antigen
HBV	Hepatitis B virus
HCV	Hepatitis C virus
HCVAb	Hepatitis C virus antibody
LMR	Liver-to-major psoas muscle intensity ratio
m/s	Meters per second
MRI	Magnetic resonance imaging
NPV	Negative predictive value
nonBnonC	Negative for hepatitis B virus surface antigen and hepatitis C virus antibody
PBC	Primary biliary cirrhosis
PPV	Positive predictive value
ROC	Receiver operating characteristic
VTTQ	Virtual Touch™ Tissue Quantification
WFA$^+$-M2BP	*Wisteria floribunda* agglutinin-positive Mac-2 binding protein

Introduction

The management of chronic liver disease depends on the degree of liver fibrosis. Therefore, assessment of the degree of liver fibrosis is important for choosing a therapeutic strategy and for determining the prognosis [1, 2]. Liver biopsy is the gold standard method for evaluating the degree of liver fibrosis [3]. However, the invasiveness of liver biopsy, its potential for life-threatening complications, and sampling errors place a heavy burden on those patients with hepatitis who require follow-up [4–6]. Therefore, many reports have demonstrated non-invasive examination methods for assessing the degree of liver fibrosis, which might be alternatives to liver biopsy, such as new serum markers and transient elastography [7–9]. However, none of these studies developed a definitive method.

Recently, a novel marker for liver fibrosis was developed using the glycan "sugar chain"-based immunoassay, and the FastLec-Hepa system was used to determine serum values of "sweet-doughnut" hyperglycosylated *Wisteria floribunda* agglutinin-positive Mac-2 binding protein (WFA$^+$-M2BP) for the assessment of liver fibrosis [10, 11]. This unique technical approach supported by multiple lectin-assisted glycan profiling may be applicable to the development pipeline for a wide variety of glycodiagnostic tools. However, the feasibility of WFA$^+$-M2BP for assessing liver fibrosis has not been proven with clinical samples of hepatitis.

A method based on acoustic radiation force impulse (ARFI) imaging, with virtual touch tissue quantification (VTTQ), has been developed to evaluate liver fibrosis. VTTQ measurements can be performed during observation of a particular liver lesion with an ultrasonic probe, and measurements are reproducible compared with transient elastography [12, 13]. For assessment of liver imaging, we have developed magnetic resonance imaging (MRI) using gadolinium ethoxybenzyl diethylenetriamine pentaacetic acid (Gd-EOB-DTPA) enhancement to assess liver function, which may vary inversely for liver fibrosis with the passing grade [14]. Indeed, the increase in rate of liver-to-major psoas muscle intensity ratio (LMR) in the hepatobiliary phase compared with the precontrast image is best correlated with the degree of liver fibrosis, and significantly decreases as liver fibrosis progresses (F0–F4).

This study aimed to compare the diagnostic accuracy of serum WFA$^+$-M2BP values using the area under receiver operating characteristic (ROC) curves. The diagnostic performance of serum WFA$^+$-M2BP values was compared with validated surrogate fibrosis markers, including VTTQ, the MRI-LMR index, and serum markers, such as hyaluronic acid, type 4 collagen, and the aspartate aminotransferase-to-platelet ratio index (APRI).

Methods

Patients

The study cohort consisted of 200 adults, including 40 healthy volunteers and 160 patients with or without hepatitis, who underwent hepatectomy or living donor liver transplantation, and whose serum WFA$^+$-M2BP values and liver stiffness by VTTQ were measured at Kyushu University Hospital. Serum samples of inferior quality, which had the potential of measurement errors, such as those with hemolysis, milky fluid, or protein aggregation, were excluded [15]. Of the 160 patients, 106 were positive for antibody to hepatitis C virus (HCVAb), 21 were positive for hepatitis B virus (HBV) surface antigen (HBsAg), 12 had hepatitis due to alcohol, and 21 were negative for HBsAg and HCVAb. The study protocol conformed to the ethical guidelines of the 1975 Helsinki Declaration and was approved by our institutional review board.

Liver histology and quantification of liver fibrosis

All liver specimens were obtained by surgical resection and were fixed in formalin, embedded in paraffin wax, and stained with hematoxylin and eosin and Masson's trichrome. The fibrosis staging in all surgical specimens was determined independently by two pathologists who did not know the VTTQ values. In case of discrepancies, histological sections were simultaneously reviewed using a multi-pipe microscope to reach a consensus. Fibrosis was

staged on a scale of 0–4 according to the METAVIR classification [18], with F0 indicating no fibrosis; F1, enlarged, fibrotic portal tracts; F2, periportal or portal–portal septa but intact architecture; F3, fibrosis with architectural distortion but no obvious cirrhosis; and F4, probable or definite cirrhosis.

Direct measurement of serum Mac-2 binding protein

The method of assessment of WFA^+-M2BP was as follows [10, 11]. A glycan-based immunoassay, FastLec-Hepa, was developed as a simple and accurate system to automatically detect a unique fibrosis-related glyco-alteration in serum hyperglycosylated WFA^+-M2BP. Briefly, the *Wisteria floribunda* agglutinin (WFA)-antibody immunoassay using the HISCL-2000i bedside clinical chemistry analyzer was developed to measure WFA^+-M2BP values. These values were successfully adjusted every reaction condition during the automatic assay, and heat pretreatment of the serum was avoided to ensure binding avidity and a fast association rate within just 17 min. The measured values of WFA^+-M2BP conjugated to WFA were indexed with the obtained values using the following equation:

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

$[WFA^+\text{-}M2BP]_{sample}$, WFA^+-M2BP count of serum sample (PC, positive control; NC, negative control) [10, 11].

VTTQ and ARFI

The VTTQ system was installed on an ACUSON model S2000 ultrasound system (Siemens Medical Solutions, Inc., Ultrasound Division, Issaquah, WA, USA). The operators were surgeons trained by Siemens Medical Solutions, Inc. The VTTQ system uses an acoustic push pulse to generate shear waves, which pass through the liver parenchyma orthogonally to the acoustic push pulse, through a user-placed region of interest. When detection pulses interact with a passing shear wave, they reveal the wave's location at a specific time, allowing calculation of the shear wave speed. This absolute numerical value is related to the stiffness of the tissue within the region of interest [16, 17], and the results are expressed in meters per second (m/s). For each patient, seven successful measurements were performed several days before surgical operations during which histological specimens were obtained. The measurement of VTTQ in the right lobe of the liver was performed by placing the ultrasonic probe on the right intercostal space at a depth from 2 to 4 cm [9]. The median value of all measurements and the standard deviation of all right and left VTTQ measurements for each patient were considered for analysis.

Analysis of the MRI-LMR index

The analysis system of the LMR in MRI using Gd-EOB-DTPA enhancement was as follows [14]. Briefly, the signal intensities were measured by placing as large a region of interest as possible on the left and right lobes of the liver parenchyma and major psoas muscle, avoiding vessels, tumors and artifacts, and one slice without significant artifacts for the precontrast image and hepatobiliary phase was selected. The increase in rate of the LMR in the hepatobiliary phase compared with the precontrast image was calculated using the following equation:

(LMR in the hepatobiliary phase
−LMR on the precontrast image)/
LMR on the precontrast image

[14].

Surrogate serum markers

For all patients, blood samples were obtained on the same day that the VTTQ examination was performed, and they were examined in the same laboratory. The following parameters were determined: hyaluronic acid levels, type 4 collagen, platelet count, aspartate aminotransferase (AST) levels, alanine aminotransferase (ALT) levels, ICG-R15, and the APRI. The APRI was calculated as follows: AST level (per upper limit of normal; 33 U/L) × 100/platelet count (10^9/L) [18, 19].

Statistical analysis

Differences between quantitative variables for paired samples were analyzed using a nonparametric test (Wilcoxon rank sum test with Bonferroni's adjustment). The sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) of optimal cutoff values of liver surrogate fibrosis markers for the diagnosis of liver fibrosis were calculated as described [18, 19]. In addition, the diagnostic value of liver stiffness for predicting significant liver fibrosis (F1–F3) and cirrhosis (F4) was assessed by calculating the areas under the ROC curves. The ROC curve is a plot of sensitivity versus 1-specificity for all possible cutoff values. The most commonly used index of accuracy is the area under the ROC curve, where values close to 1.0 indicate high diagnostic accuracy, and 0.5 indicates a test of no diagnostic value. The optimal liver stiffness cutoff values used for the diagnosis of significant fibrosis and cirrhosis were selected based on the sensitivity, specificity, PPV, and NPV [18–20]. Statistical analysis of the differences between

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

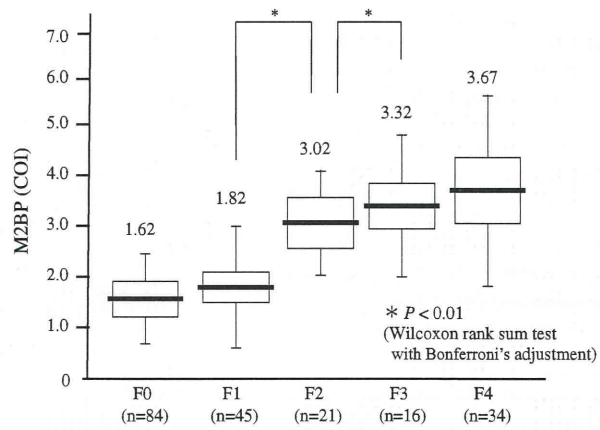

Fig. 1 Box-and-whisker plot of serum WFA$^+$-M2BP values for each fibrosis stage. The *tops* and *bottoms* of the boxes represent the first and third quartiles, respectively, with the *height* of the box represents the interquartile range, covering 50 % of the values. The *line* through the middle of 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

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.

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

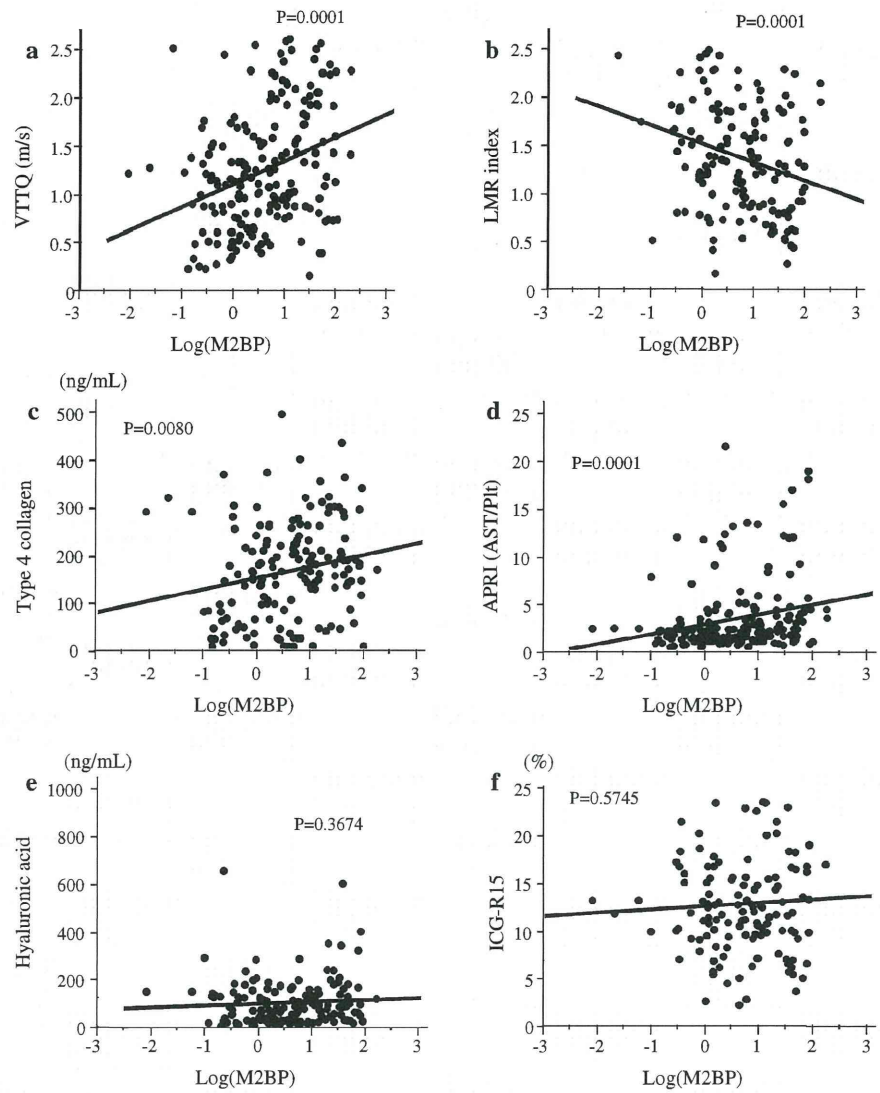

Fig. 2 Correlation of WFA^+-M2BP values with VTTQ, LMR index, and the other serum markers. The log of WFA^+-M2BP correlated with VTTQ results, a significant indicator of liver stiffness as liver fibrosis ($P = 0.0001$) (**a**). Log WFA^+-M2BP values were also inversely related with the LMR index, a significant 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

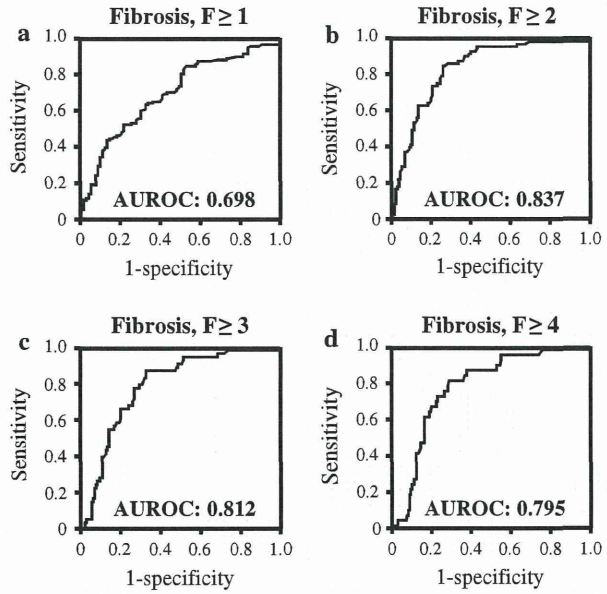

Fig. 3 Diagnostic ability of serum WFA$^+$-M2BP values to assess stages of liver fibrosis. The areas under the ROC curves of serum WFA$^+$-M2BP values for diagnosing liver fibrosis were **a** 0.698 for grade $F \geq 1$, **b** 0.837 for grade $F \geq 2$, **c** 0.812 for grade $F \geq 3$, and **d** 0.795 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™ 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

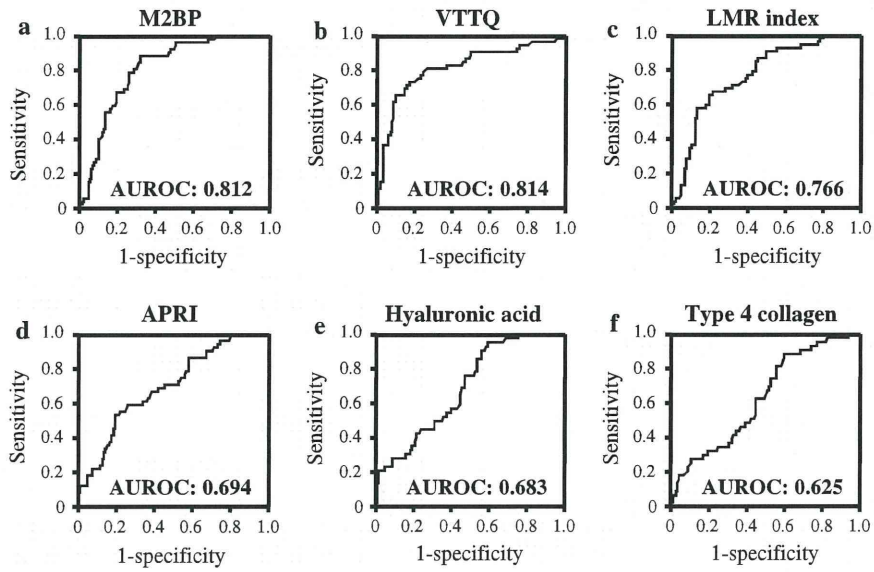

Fig. 4 Comparison of WFA$^+$-M2BP with other indicators for the diagnosis of fibrosis stage $F \geq 3$ by areas under the receiver operating curves (ROC). The areas under the ROC curves for the diagnosis of grade $F \geq 3$ fibrosis were **a** 0.812 for serum WFA$^+$-M2BP, **b** 0.814 for VTTQ, **c** 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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