

Comparison of serum fibrosis biomarkers for diagnosing significant liver fibrosis in patients with chronic hepatitis B

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Abstract. Chronic hepatitis B (CHB) virus continues to be a leading cause of morbidity and mortality worldwide. The diagnosis of liver fibrosis has a key role in selecting patients with CHB for antiviral treatment. However, serum biomarkers demonstrate limited diagnostic utility. The present study aimed to compare the performances of fibrosis biomarkers for diagnosing significant liver fibrosis that indicates the need for antiviral therapy in patients with CHB and to identify the most appropriate biomarker for these patients. The current study included 96 antiviral-naïve patients with CHB who underwent liver biopsy. METAVIR scoring system was used to assess liver fibrosis and necroinflammation. The diagnostic performances were evaluated of the platelet (PLT) count; the levels of hyaluronan, serum 7S domain of type 4 collagen, procollagen type III N-terminal peptide, tissue inhibitor of metalloproteinases 1, Mac-2 binding protein glycosylation isomer (M2BPGi) and N-terminal type III collagen propeptide (Pro-C3); the fibrosis index based on four factors; the aspartate aminotransferase-to-platelet ratio index; and enhanced liver fibrosis score for identifying significant liver fibrosis [\geq fibrosis stage 2 (F2)]. All fibrosis biomarkers, except the Pro-C3 level,

correlated with the fibrosis stage. M2BPGi was better than other biomarkers for diagnosing \geq F2, with the highest area under the curve of 0.902. M2BPGi demonstrated a higher diagnostic accuracy for significant fibrosis than mild/severe fibrosis or cirrhosis. However, no significant correlation was observed between the M2BPGi level and fibrosis stage in patients with CHB having significant liver necroinflammation defined as \geq necroinflammatory activity 2. The M2BPGi level and PLT count were exclusively correlated with the fibrosis stage in 73 patients without significant liver necroinflammation. M2BPGi demonstrated the highest diagnostic performance for significant fibrosis in patients having significant liver fibrosis with no significant liver necroinflammation. In conclusion, the M2BPGi level can accurately diagnose significant liver fibrosis that indicates the need for antiviral therapy in patients with CHB.

Introduction

Liver fibrosis is a common pathological manifestation of various chronic liver diseases, which can advance to cirrhosis and hepatocellular carcinoma (1) Liver biopsy is the gold standard approach to assess fibrosis progression in chronic liver diseases, such as chronic hepatitis B (CHB), chronic hepatitis C (CHC), autoimmune liver diseases and non-alcoholic fatty liver disease, even though it is an invasive procedure (2). As the progression of liver fibrosis can be reversed with treatment, serial hepatic biopsy analysis is important for chronic liver diseases (3). However, repetitive procedures are difficult to perform because of various limitations regarding the principle, cost and sampling error (3). Therefore, non-invasive methods for assessing liver fibrosis are required to overcome these limitations. Non-invasive techniques, such as magnetic resonance imaging (MRI) (4) and ultrasound-based transient elastography, are considered helpful for estimating the advanced stages of fibrosis (5). Serum surrogate biomarkers can also be employed and they are broadly classified as direct markers, which reflect alterations in the contents of extracellular matrix proteins, and indirect markers, which reflect changes in liver function. Direct markers include hyaluronan (HA), 7S domain of type 4 collagen (7S collagen) and procollagen type III N-terminal peptide (PIIINP), and with all these markers, there are

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Abbreviations: 7S collagen, 7S domain of type 4 collagen; ALT, alanine aminotransferase; APRI, aspartate aminotransferase-to-platelet ratio index; AST, aspartate aminotransferase; CHB, chronic hepatitis B; ELF, enhanced liver fibrosis score; FIB-4, fibrosis index based on four factors; HA, hyaluronan; M2BPGi, mac-2-binding protein glycosylation isomer; PIIINP, type 3 procollagen-N-peptide; Pro-C3, N-terminal type III collagen propeptide; TIMP-1, tissue inhibitor of metalloproteinase 1

Key words: chronic hepatitis B, liver fibrosis, liver necroinflammation, Mac-2 binding protein glycosylation isomer, N-terminal type III collagen propeptide

difficulties in discriminating accurately between the early and adjacent stages of fibrosis (6,7). Conversely, indirect markers involve routine laboratory parameters and are calculated from these laboratory parameters [for example, fibrosis index based on four factors (FIB-4 index) and aspartate aminotransferase (AST)-to-platelet ratio index (APRI)] (8). These are inefficient in detecting early stage fibrosis in patients with CHB (9).

The early diagnosis of liver fibrosis is important to control disease progression. The clinical practice guidelines for the management of hepatitis B proposed by the American Association for the Study of Liver Diseases (10) and the Japan Society of Hepatology (11) state that the decision to initiate antiviral therapy should be taken if a patient has F1 fibrosis without necroinflammation. The underlying mechanisms of histological progression vary among patients with chronic liver diseases (12,13). Recently, plasma N-terminal type III collagen propeptide (Pro-C3) has been introduced as a novel non-invasive marker for the assessment of liver fibrosis in patients with CHC (14) and non-alcoholic steatohepatitis (15). The authors of the present study have previously demonstrated that the serum angiotensin-converting enzyme level is a beneficial non-invasive marker for evaluating significant fibrosis in patients with CHB without fatty liver or habitual alcoholic consumption (16). To date, no surrogate marker that accurately quantifies hepatic fibrosis has been identified in patients with CHB. The present study aimed to compare the performances of fibrosis biomarkers for diagnosing significant liver fibrosis that indicate the need for antiviral therapy in patients with CHB to identify the most appropriate biomarker in these patients.

Materials and methods

Patients. The present study enrolled 96 treatment-naïve patients who were diagnosed with CHB serologically and histologically between September 2005 and May 2017 (Table I). The typical characteristics of CHB infection were as follows: i) Hepatitis B surface antigen (HBsAg) positivity for at least 6 months; and ii) serum hepatitis B virus (HBV) DNA level ≥ 1.3 log IU/ml. Detections of HB envelope antigen, anti-HBe IgG and anti-HB core IgG were not considered as inclusion criteria in the present assessment. The exclusion criteria were clinical findings suggestive of concomitant liver diseases (including CHC), autoimmune hepatitis, primary biliary cholangitis, alcoholic liver disease, non-alcoholic fatty liver disease and hepatocellular carcinoma. The present study was performed in accordance with the standards of the Declaration of Helsinki and written informed consent was obtained from all the study participants. The Ethics Committee of Nara Medical University affiliated Hospital approved this study (approval no. 1077).

Laboratory analysis and measurement of clinical laboratory parameters. Variables, including age, platelet (PLT) count and levels of AST, alanine aminotransferase (ALT), albumin (ALB), total bilirubin, HBV DNA, and HBsAg, were assessed and recorded on admission (Table I). Additionally, the HA level, 7S collagen level, PIIINP level, tissue inhibitor of metalloproteinases 1 (TIMP-1) level, Mac-2 binding protein glycosylation isomer (M2BPGi) level, Pro-C3 level, FIB-4 index, APRI and

enhanced liver fibrosis (ELF) score were used as non-invasive biomarkers for the assessment of liver fibrosis. The following formulas were used: FIB-4 index = $(\text{age} \times \text{AST}) / [(\text{PLT count}) \times (\text{ALT})^{1/2}]$; APRI = $[(\text{sample AST}/\text{reference AST}) \times 100] / \text{PLT count}$; ELF score = $2.278 + [0.851 \ln(\text{HA}) + 0.751 \ln(\text{PIINP}) + 0.394 \ln(\text{TIMP-1})]$. The levels of HA, TIMP-1 and PIIINP were measured using chemiluminometric immunoassays performed on the ADVIA Centaur XP Immunoassay System (Siemens Healthineers) (17,18). Serum 7S collagen was determined using radioimmunoassay kits (7S-RIA; Nippon DPC Corporation) (19). The Wisteria floribunda agglutinin-positive Mac-2 binding protein assay was performed using an automated chemiluminescence enzyme immunoassay analyzer (HISCL-5000; Sysmex Corporation) (20). Pro-C3 level was measured using UniQ PIIINP RIA assay (Orion Diagnostica Ltd.) (21). HBs antigen and HBV DNA levels were measured as previously described (22).

Liver biopsy. Percutaneous liver biopsy was performed before the initiation of therapy, using ultrasound localization. Liver samples were fixed in formalin at a room temperature of 20–22°C, embedded in paraffin and sectioned to 5 μm . Each section was stained with hematoxylin-eosin and reticular fiber stain for 30 sec at a room temperature of 20 to 22°C or Masson's stain for 60 min at 54–64°C. Professor Chiho Obayashi and Dr Kohei Morita (Department of Diagnostic Pathology, Nara Medical University) independently reviewed all cases for validation of the histological features of CHB. The METAVIR scoring system (23) was used to evaluate fibrosis and necroinflammation. The degree of hepatic fibrosis was scored from F0 to F4 (F0, no fibrosis; F1, portal fibrosis without septa; F2, portal fibrosis with few septa; F3, numerous septa without cirrhosis; and F4, cirrhosis) (11,24). The degree of necroinflammatory activity was scored from A0 to A3 (A0, no histological necroinflammatory activity; A1, minimal necroinflammatory activity; A2, moderate necroinflammatory activity; and A3, severe necroinflammatory activity) (25). F0-F1 and A0-A1 were considered to indicate no to mild fibrosis and no to mild necroinflammation, whereas F2-F4 and A2-A3 were considered to indicate moderate to severe fibrosis and cirrhosis and moderate to severe necroinflammation, respectively. Significant liver fibrosis and necroinflammation were defined as the fibrosis stage \geq F2 and necroinflammation grade \geq A2, respectively.

Statistical analysis. Patient characteristics are presented as the mean \pm standard error of the mean. Differences in continuous variables were assessed using Student's t-tests or one-way ANOVAs followed by Tukey's post-hoc tests. The Mann-Whitney U test was used to compare two groups of nonparametric data. Chi-square test was used to analyze categorical variables. Correlations were evaluated using Spearman's correlation coefficient for continuous variables. All statistical tests were two-tailed and $P < 0.05$ was considered to indicate a statistically significant difference. The areas under the receiver operating characteristic (ROC) curves (AUCs) were used to evaluate the diagnostic values of the fibrosis biomarkers with regard to the correct identification of significant liver fibrosis. The sensitivities, specificities, positive-predictive values (PPVs), negative-predictive values

(NPVs), diagnostic accuracies and cut-off values of the fibrosis biomarkers were calculated from the ROC curves. All analyses were performed using SPSS software version 24 (IBM Corp.).

Results

Baseline clinical characteristics of patients with different fibrosis stages. The demographic and baseline characteristics of the patients are summarized in Table I. The fibrosis stages were F0, F1, F2, F3 and F4 in 25 (26.0%), 44 (45.8%), 14 (14.6%), 10 (10.4%) and 3 (3.2%) patients, respectively (Table II). Spearman's rank correlation coefficients between the fibrosis stage and PLT count, HA level, 7S collagen level, PIIINP level, TIMP-1 level, M2BPGi level, Pro-C3 level, FIB-4 index, APRI and ELF score were -0.43, 0.38, 0.52, 0.54, 0.38, 0.61, 0.08, 0.42, 0.58 and 0.55, respectively (Fig. 1A-J). All fibrosis biomarkers, except the Pro-C3 level, were significantly associated with the liver fibrosis stage in patients with CHB (Table II).

Levels of serum fibrosis biomarkers according to the degree of liver fibrosis in patients with CHB. It was identified that the M2BPGi level was markedly higher in patients with F2 fibrosis compared with those with F1 fibrosis [F1, 0.75±0.45 vs. F2, 1.29±0.46 Cutoff index (COI) P<0.01; Fig. 2F]. Unlike the M2BPGi findings, there were significant differences between patients with F2 fibrosis and those with F3 fibrosis with regard to the HA level (F2, 53.4±23.6 vs. F3, 245.9±278.9 ng/ml; P<0.01; Fig. 2B), 7S collagen level (F2, 4.0±0.9 vs. F3, 6.9±2.7 ng/ml; P<0.01; Fig. 2C), PIIINP level (F2, 11.4±4.2 vs. F3, 17.3±7.2 ng/ml; P<0.05; Fig. 2D), APRI (F2, 0.77±0.34 vs. F3, 1.52±1.03; P<0.01; Fig. 2I) and ELF score (F2, 9.51±0.68 vs. F3, 10.79±1.15; P<0.01; Fig. 2J) and between patients with F3 fibrosis and those with F4 fibrosis with regard to the FIB-4 index (F3, 2.71±1.62 vs. F4, 4.97±3.02; P<0.05; Fig. 2H). However, no differences were found among the fibrosis groups with regard to the PLT count and serum TIMP-1 and Pro-C3 levels (Fig. 2A, E and G).

Diagnostic performances of serum fibrosis biomarkers for identifying significant liver fibrosis in patients with CHB. The diagnostic sensitivity, specificity, PPV, NPV and accuracy of the PLT count, HA level, 7S collagen level, PIIINP level, TIMP-1 level, M2BPGi level, Pro-C3 level, FIB-4 index, APRI and ELF score for the differentiation of ≥F2 fibrosis in patients with CHB are shown in Table III. The AUCs of these markers for the accurate diagnosis of significant liver fibrosis (≥F2) were 0.757, 0.776, 0.739, 0.778, 0.713, 0.902, 0.452, 0.676, 0.812 and 0.816, respectively (Table III). These findings indicated that the serum M2BPGi level was most accurately identifying significant liver fibrosis when compared with other non-invasive fibrosis biomarkers in patients with CHB.

Diagnostic performance of M2BPGi for identifying the different stages of liver fibrosis in patients with CHB. The AUCs for identifying mild fibrosis (F1-4), significant fibrosis (F2-4), severe (F3-4) and advanced fibrosis/cirrhosis (F4) were 0.773 (sensitivity, 52.1%; specificity, 100%; Fig. 3A) 0.902 (sensitivity, 92.6%; specificity, 82.4%; Fig. 3B), 0.865 (sensitivity, 100%; specificity, 63.4%; Fig. 3C) and 0.774 (sensitivity,

Table I. Baseline characteristics of patients with CHB.

Variable	CHB patients (n=96)
Sex	
Male	49
Female	47
Age, years	51.1±13.7
Fibrosis stage	
F0	25
F1	44
F2	14
F3	10
F4	3
Inflammatory activity	
A0	36
A1	37
A2	20
A3	3
Platelet (10 ⁴ /μl)	19.7±5.4
AST (IU/l)	37.7±27.2
ALT (IU/l)	46.8±57.3
Serum Albumin (g/dl)	4.2±0.3
Total Bilirubin (mg/dl)	0.9±0.3
HBV DNA (Log copies/ml)	4.8±2.6
HBs Antigen (IU/ml)	13,782±30,650
Hyaluronic acid (ng/ml)	83.7±15.0
Type 4 collagen 7S (ng/ml)	4.3±2.2
PIIINP (ng/ml)	10.4±6.4
TIMP-1 (ng/ml)	224.9±73.8
M2BPGi (cutoff index)	0.87±0.51
Pro C3 (ng/ml)	16.6±5.2
FIB-4	1.77±1.31
APRI	0.71±0.73
ELF score	9.34±1.10

CHB, chronic hepatitis type B; AST, aspartate aminotransferase; ALT, alanine aminotransferase; PIIINP, type 3 procollagen-N-peptide; TIMP-1, tissue inhibitor of metalloproteinase 1; M2BPGi, Mac-2-binding protein glycosylation isomer; Pro-C3, N-terminal type III collagen propeptide; FIB-4, fibrosis index based on four factors; APRI, the aspartate aminotransferase-to-platelet ratio index; ELF, enhanced liver fibrosis.

100%; specificity, 56.5%; Fig. 3D), respectively, indicating that M2BPGi has higher diagnostic accuracy for significant fibrosis than mild/severe fibrosis or cirrhosis. Together, these results suggested that serum M2BPGi had the best performance for identifying significant liver fibrosis in patients with CHB. The diagnostic accuracy of serum M2BPGi level was compared to that of other fibrosis markers including the PLT count; HA, 7S collagen, PIIINP, TIMP-1 and Pro-C3 levels; FIB-4 index; APRI; and ELF score. Significant differences were observed in the AUCs between M2BPGi level and PLT count, HA level, 7S collagen level, PIIINP level, TIMP-1 level, Pro-C3 level and FIB-4 index (P<0.05, P<0.05, P<0.01,

Table II. Baseline characteristics of patients with chronic hepatitis B stratified according to liver fibrosis stages.

Variable (reference range)	F0 (n=25)	F1 (n=44)	F2 (n=14)	F3 (n=10)	F4 (n=3)	Overall P-value
Male/female	11/14	23/21	7/7	6/4	2/1	NS
Age (years)	53.7±2.4	50.0±2.1	49.1±3.4	51.9±5.4	51.6±6.7	NS
Platelet ($10^4/\mu\text{l}$) (14.0-37.9)	21.6±1.1	20.6±0.7	18.0±1.0	15.4±4.8	12.0±2.9	<0.01
AST (IU/l) (10-40)	26.1±2.5	34.1±3.4	42.9±6.0	66.3±46.3	67.3±15.9	<0.01
ALT (IU/l) (5-45)	22.0±2.3	42.4±5.4	65.9±19.9	97.4±118.3	60.7±3.9	<0.01
Serum albumin (g/dl) (3.7-5.5)	4.2±0.0	4.2±0.0	4.1±0.0	4.0±0.4	3.9±0.0	NS
Total Bilirubin (mg/dl) (0.3-1.2)	0.8±0.0	0.8±0.0	0.9±0.0	1.0±0.3	1.8±0.0	NS
HBV DNA (Log copies/ml)	3.5±0.4	4.8±0.4	5.8±0.5	6.3±2.9	3.0±1.4	0.01
HBsAg (IU/ml) (<0.05)	12,128±5,088	42,946±24,337	11,051±5,832	11,801±24,431	754±340	NS
Hyaluronic acid (ng/ml) (<50.0)	61.7±24.9	52.5±10.2	53.4±6.3	245.9±88.1 ^b	325.1±165.6	<0.01
Type 4 collagen 7S (ng/ml) (<6.0)	3.6±0.2	3.8±0.2	4.0±0.2	6.9±0.9 ^b	9.1±3.5	<0.01
PIIINP (ng/ml) (3.62-9.52)	7.9±0.4	8.9±0.6	11.4±1.1	17.3±2.3 ^b	24.7±10.5	<0.01
TIMP-1 (ng/ml)	198.6±13.7	213.7±5.9	247.9±22.1	291.2±38.3	277.7±27.1	<0.01
M2BPGi (cutoff index) (<1.00)	0.53±0.03	0.75±0.07	1.29±0.12 ^a	1.44±0.11	1.42±0.38	<0.01
ProC3 (ng/ml)	18.5±1.3	15.5±0.5	16.5±1.6	18.0±1.9	14.3±0.5	NS
FIB-4	1.52±0.14	1.46±0.13	1.85±0.30	2.71±0.51 ^c	4.97±1.74	0.05
APRI	0.43±0.03	0.56±0.06	0.77±0.09	1.52±0.33 ^b	2.56±1.07	<0.01
ELF score	8.91±0.18	9.04±0.13	9.51±0.18	10.79±0.36 ^b	11.28±0.77	<0.01

^aP<0.01 vs. F1; ^bP<0.01 vs. F2; ^cP<0.01 vs. F4. CHB, chronic hepatitis B; AST, aspartate aminotransferase; ALT, alanine aminotransferase; PIIINP, type 3 procollagen-N-peptide; TIMP-1, tissue inhibitor of metalloproteinase 1; M2BPGi, Mac-2-binding protein glycosylation isomer; Pro-C3, N-terminal type III collagen propeptide; FIB-4, fibrosis index based on four factors; APRI, aspartate aminotransferase-to-platelet ratio index; ELF score, enhanced liver fibrosis score; F, fibrosis stage.

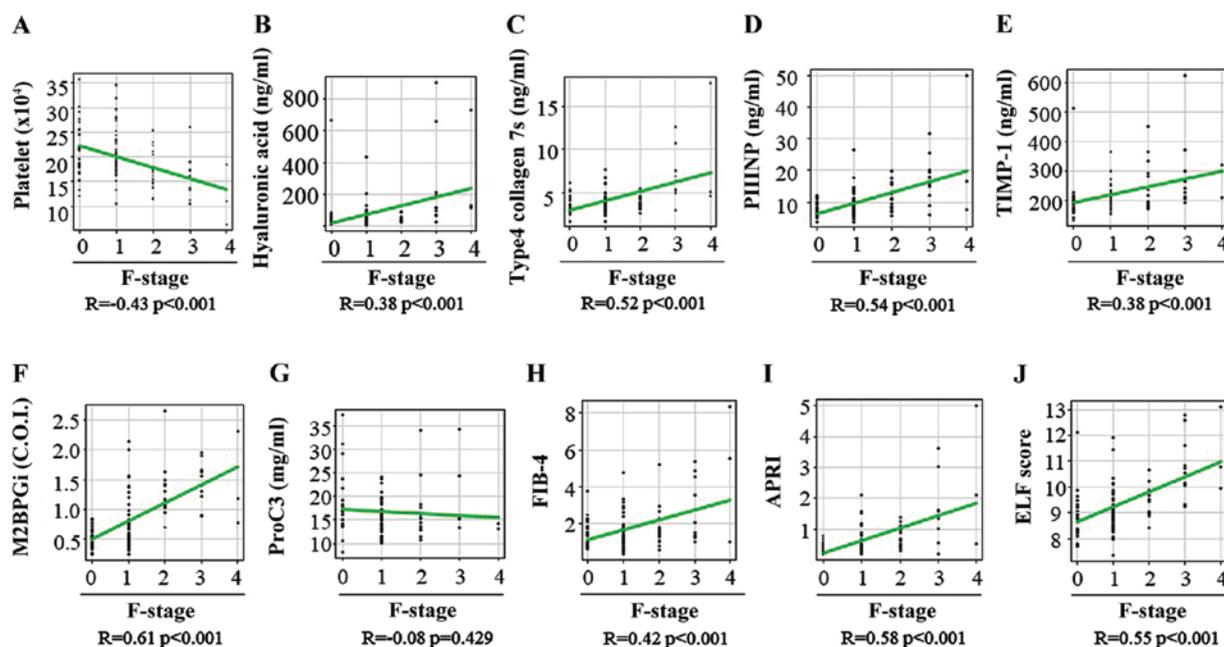


Figure 1. Correlation between 10 liver fibrosis biomarkers and fibrosis stage in patients with chronic hepatitis B. (A) Platelet count, (B) hyaluronan acid, (C) 7S domain of type 4, (D) PIIINP, (E) TIMP-1, (F) M2BPGi, (G) Pro-C3, (H) FIB-4 index, (I) APRI and (J) ELF score. PIIINP, procollagen type III N-terminal peptide; TIMP-1, tissue inhibitor of metalloproteinases 1; M2BPGi, Mac-2 binding protein glycosylation isomer; Pro-C3, N-terminal type III collagen propeptide; FIB-4 index, fibrosis index based on four factors; APRI, aspartate aminotransferase-to-platelet ratio index; ELF, enhanced liver fibrosis; F, fibrosis.

P<0.05, P<0.01, P<0.001 and P<0.001, respectively) and no significant difference was found between M2BPGi level and

APRI and ELF score (P=0.093 and P=0.072, respectively) in patients with CHB (Table SI).

Table III. Diagnostic accuracy of serum fibrosis markers for significant fibrosis in patients with chronic hepatitis B.

Biomarker	AUC	95% CI	Cut-off	Sensitivity (95% CI)	Specificity (95% CI)	PPV (95% CI)	NPV (95% CI)	Accuracy (95% CI)
Platelet count	0.757	0.641-0.872	17.5	70.4 (49.8-86.2)	76.8 (65.1-86.1)	54.3 (36.6-71.2)	86.9 (75.8-94.2)	75.0 (65.1-83.3)
Hyaluronic acid	0.776	0.675-0.877	61.08	63.0 (42.4-80.6)	84.1 (73.3-91.8)	60.7 (40.6-78.5)	85.3 (74.6-92.7)	78.1 (68.5-85.9)
Type 4 collagen 7S	0.739	0.621-0.856	4.6	59.3 (38.8-77.6)	83.6 (72.5-91.5)	59.3 (38.8-77.6)	83.6 (72.5-91.5)	76.6 (66.7-84.7)
PIIINP	0.778	0.668-0.888	15.0	48.1 (28.7-68.1)	97.1 (89.9-99.6)	86.7 (59.5-98.3)	82.7 (72.7-90.2)	83.3 (74.4-90.2)
TIMP-1	0.713	0.591-0.833	264.3	44.4 (25.5-64.7)	94.2 (85.8-98.4)	75.0 (47.6-92.7)	81.2 (71.0-89.1)	80.2 (70.8-87.6)
M2BPGi	0.902	0.841-0.962	0.890	92.6 (75.7-99.1)	82.4 (71.2-90.5)	67.6 (50.2-82.0)	96.6 (88.1-99.6)	85.3 (76.5-91.7)
Pro-C3	0.452	0.325-0.579	10.781	100 (81.7-100)	11.6 (5.1-21.6)	30.7 (21.3-41.4)	100 (51.8-100)	36.5 (26.9-46.9)
FIB-4	0.676	0.555-0.798	1.264	77.8 (57.7-91.4)	50.7 (38.4-63.0)	38.2 (25.4-52.3)	85.4 (70.8-94.4)	58.3 (47.8-68.3)
APRI	0.812	0.709-0.914	0.534	81.5 (61.9-93.7)	75.4 (63.5-84.9)	56.4 (39.6-72.2)	91.2 (80.7-97.1)	77.1 (67.4-85.0)
ELF score	0.816	0.723-0.909	9.250	77.8 (57.7-91.4)	73.9 (61.9-83.7)	53.8 (37.2-69.9)	89.5 (78.5-96.0)	75.0 (65.1-83.3)

PIIINP, type 3 procollagen-N-peptide; TIMP-1, tissue inhibitor of metalloproteinase 1; M2BPGi, Mac-2-binding protein glycosylation isomer; Pro-C3, N-terminal type III collagen propeptide; FIB-4, the fibrosis index based on four factors; APRI, the aspartate aminotransferase-to-platelet ratio index; ELF score, enhanced liver fibrosis score.

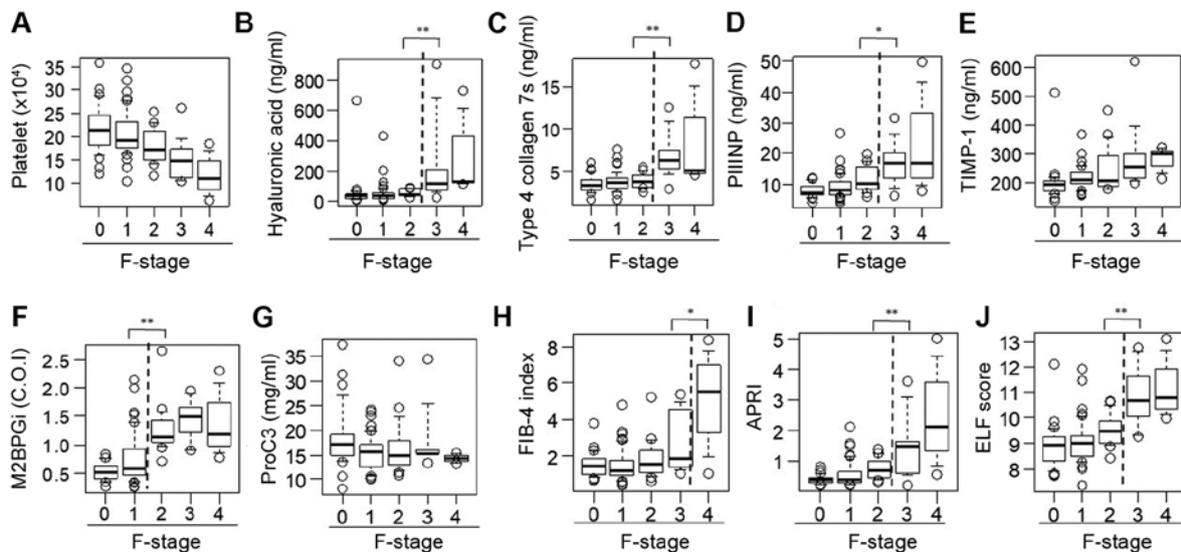


Figure 2. Serum levels of fibrosis biomarkers for staging liver fibrosis in patients with chronic hepatitis B. A box plot of each fibrosis marker according to the fibrosis stage is presented. (A) Platelet count, (B) hyaluronan acid, (C) 7S domain of type 4, (D) PIIINP, (E) TIMP-1, (F) M2BPGi, (G) Pro-C3, (H) FIB-4 index, (I) APRI and (J) ELF score. Data are presented as mean \pm standard deviation. *P<0.05 and **P<0.01 as indicated. M2BPGi, Mac-2 binding protein glycosylation isomer; PIIINP, procollagen type III N-terminal peptide; APRI, aspartate aminotransferase-to-platelet ratio index; ELF, enhanced liver fibrosis; FIB-4 index, fibrosis index based on four factors; TIMP-1, tissue inhibitor of metalloproteinases 1; Pro-C3, N-terminal type III collagen propeptide; F, fibrosis.

Association of the M2BPGi level with the fibrosis stage in terms of histological necroinflammatory activity. The ALT level was significantly higher in patients with significant

liver necroinflammation (n=23) compared with those without significant necroinflammation (n=73; P<0.01; Table SII). A significant correlation was observed between the M2BPGi

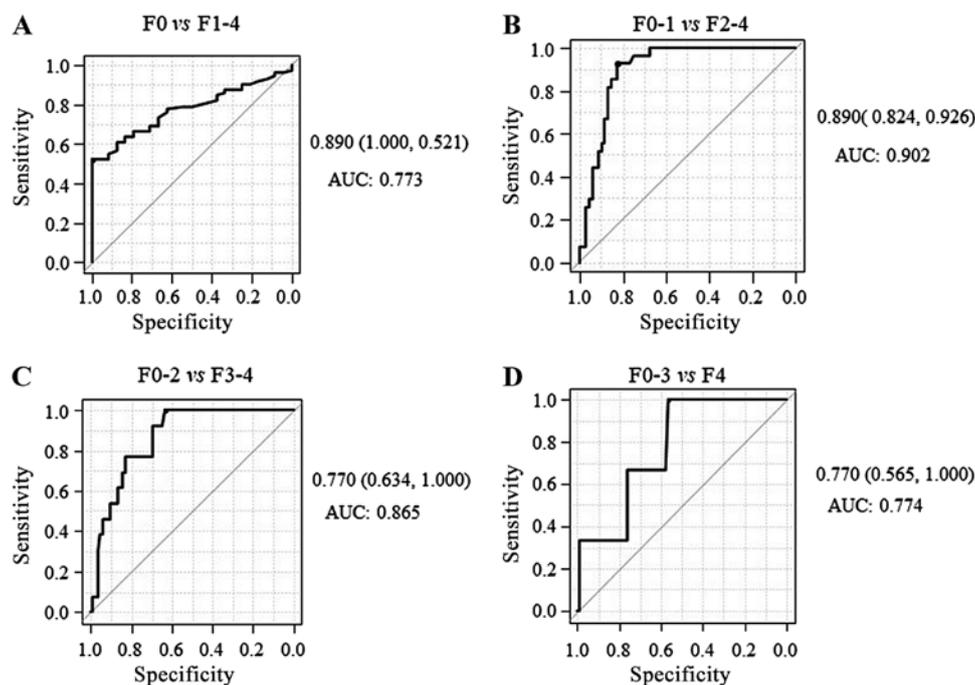


Figure 3. Diagnostic performance of serum M2BPGi for identifying the different stages of liver fibrosis in patients with chronic hepatitis B. Receiver operating characteristic curve analysis and AUC for the diagnostic performance of serum M2BPGi for identifying different stages of liver fibrosis. (A) F0 vs. F1-4, (B) F0-1 vs. F2-4 (C) F0-2 vs. F3-4 and (D) F0-3 vs. F4. M2BPGi, Mac-2 binding protein glycosylation isomer; AUC, area under the curve; F, fibrosis stage.

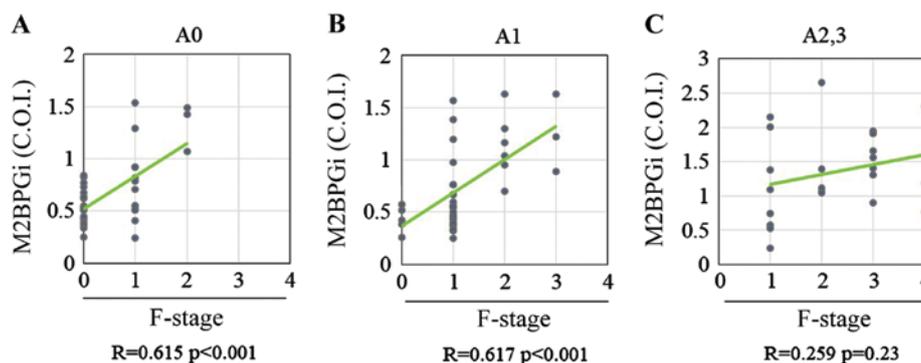


Figure 4. Correlation of the M2BPGi level with the fibrosis stage in terms of liver necroinflammation activity. (A and B) Significant correlation between the M2BPGi level and fibrosis stage is noted in patients with chronic hepatitis B without significant liver necroinflammation, A0-A1. (C) No correlation was observed in patients with CHB with significant liver necroinflammation, A2 or A3. COI, cutoff index; M2BPGi, Mac-2 binding protein glycosylation isomer; F, fibrosis.

level and fibrosis stage in patients with CHB without significant liver necroinflammation (A0-1; Fig. 4A and B), whereas no significant correlation was observed between these two variables in those with significant liver necroinflammation (\geq A2; Fig. 4C). These findings indicated that significant liver necroinflammation might affect the M2BPGi level in patients with CHB. Among patients with CHB without significant liver necroinflammation, Spearman's rank correlation coefficients between the fibrosis stage and PLT count, HA level, 7S collagen level, PIIINP level, TIMP-1 level, M2BPGi level, Pro-C3 level, FIB-4 index, APRI and ELF score were -0.31, 0.04, 0.21, 0.20, 0.15, 0.59, -0.10, 0.19, 0.28 and 0.26, respectively (Fig. 5A-J). The PLT count and M2BPGi level were the only variables associated with the liver fibrosis stage in patients with CHB without significant liver necroinflammation (Table IV).

Levels of serum fibrosis biomarkers according to the degree of liver fibrosis in patients with CHB without significant liver necroinflammation. Significant differences were observed between patients with F1 fibrosis and those with F2 fibrosis with regard to the PLT count (F1, 21.0 ± 5.0 vs. F2, $16.4 \pm 3.2 \times 10^4/\mu\text{l}$; $P < 0.01$; Fig. 6A) and M2BPGi level (F1, 0.68 ± 0.34 vs. F2, 1.20 ± 0.28 COI; $P < 0.01$; Fig. 6B). There were significant differences between patients with F2 fibrosis and those with F3 fibrosis with regard to the TIMP-1 level (F2, 193.5 ± 19.5 vs. F3, 264.4 ± 77.7 ng/ml; $P < 0.01$; Fig. 6C), ELF score (F2, 9.21 ± 0.52 vs. F3, 10.20 ± 0.69 ; $P < 0.05$; Fig. 6D) and HA level (F2, 47.4 ± 23.1 vs. F3, 123.9 ± 51.9 ng/ml; $P < 0.01$; Fig. 6E). However, no differences were observed among the fibrosis groups with regard to the 7S collagen level, PIIINP level, Pro-C3 level, FIB-4 index and APRI (Fig. 6F-J).

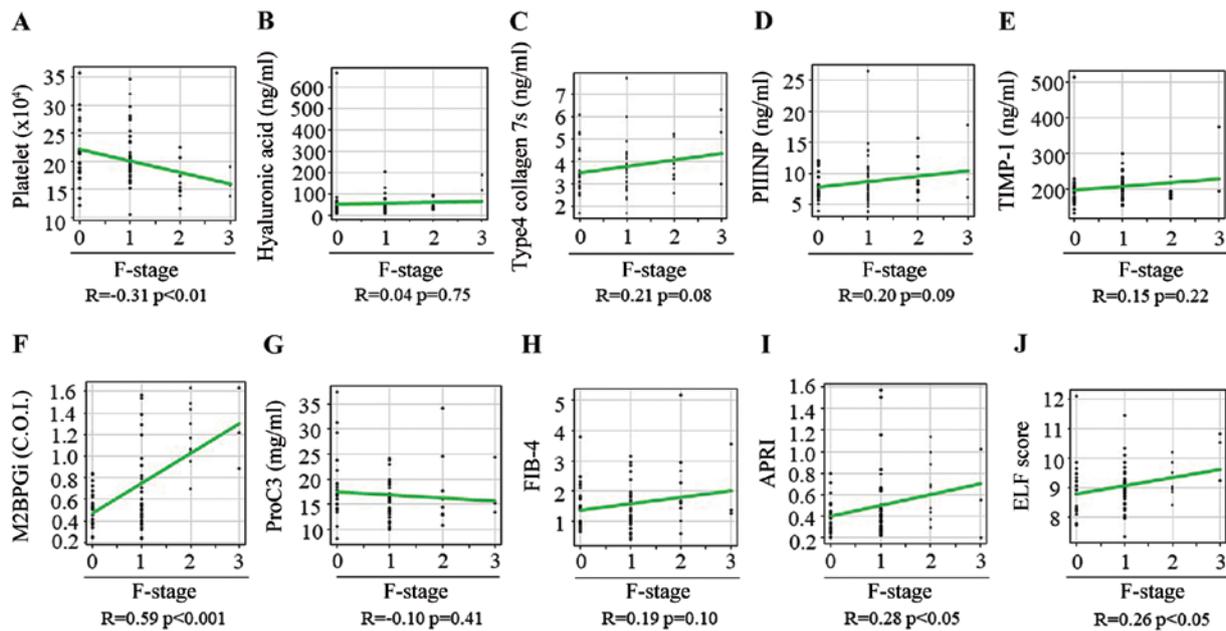


Figure 5. Correlation between 10 liver fibrosis biomarkers and fibrosis stage in patients with chronic hepatitis B without significant liver necroinflammation. (A) Platelet count, (B) hyaluronan acid, (C) 7S domain of type 4, (D) PIIINP; (E) TIMP-1, (F) M2BPGi, (G) Pro-C3, (H) FIB-4 index, (I) APRI and (J) ELF score. PIIINP, procollagen type III N-terminal peptide; TIMP-1, tissue inhibitor of metalloproteinases 1; M2BPGi, Mac-2 binding protein glycosylation isomer; Pro-C3, N-terminal type III collagen propeptide; FIB-4 index, fibrosis index based on four factors; APRI, aspartate aminotransferase-to-platelet ratio index; ELF, enhanced liver fibrosis; F, fibrosis.

Diagnostic performances of serum fibrosis biomarkers for identifying significant liver fibrosis in patients with CHB without significant liver necroinflammation. The optimal cut-off value and diagnostic performance of each serum fibrosis biomarker for identifying significant liver fibrosis in patients with CHB without significant liver necroinflammation are presented in Table V. The AUCs of the PLT count, HA level, 7S collagen level, PIIINP level, TIMP-1 level, M2BPGi level, Pro-C3 level, FIB-4 index, APRI and ELF score for the correct diagnosis of significant liver fibrosis were 0.807, 0.678, 0.603, 0.632, 0.501, 0.921, 0.458, 0.678, 0.708 and 0.688, respectively. These findings indicated that the serum M2BPGi level more accurately identified significant liver fibrosis when compared with other non-invasive fibrosis biomarkers in patients with CHB without significant liver necroinflammation.

Diagnostic performance of serum M2BPGi for identifying the different stages of liver fibrosis in patients with CHB without significant liver necroinflammation. The AUCs of the M2BPGi level for identifying F1-3, F2-3 and F3 were 0.704, 0.921 and 0.882, respectively (Fig. 7), indicating that M2BPGi exhibited a higher diagnostic accuracy for significant fibrosis than mild or severe fibrosis. These results suggested that serum M2BPGi had the best performance for identifying significant liver fibrosis in patients with CHB who had significant liver fibrosis but did not have significant liver necroinflammation. In addition, significant differences were identified in the AUCs between M2BPGi level and HA level, 7S collagen level, PIIINP level, TIMP-1 level, Pro-C3 level, FIB-4 index, APRI and ELF score ($P < 0.01$, $P < 0.05$, $P < 0.01$, $P < 0.001$, $P < 0.001$, $P < 0.05$, $P < 0.05$ and $P < 0.05$, respectively) but not between M2BPGi level and PLT count in patients with CHB without significant liver necroinflammation (Table SI).

Discussion

Liver fibrosis staging plays an important role in the selection of patients with CHB for antiviral therapy and liver biopsy is a reference tool for the decision to start therapy. However, biopsy has limitations, such as high cost, invasiveness, bleeding complications and sampling variability. The serum biomarkers of liver fibrosis are considered to have limited diagnostic utility (22). There is no established biomarker for patients with CHB who require antiviral therapy. To the best of the authors' knowledge, the present study is the first to report that the serum M2BPGi level is a useful marker for identifying liver histological findings in patients with CHB without significant necroinflammation and in need of antiviral therapy.

M2BPGi is a glycosylated secretory protein synthesized by activated hepatic stellate cells (Ac-HSCs) and is emerging as a serum marker for liver fibrosis (26). M2BPGi serves as a juxtacrine messenger between Ac-HSCs and Kupffer cells during progression of liver fibrosis (27). Bekki *et al* (28) demonstrated that M2BPGi is exclusively produced in Ac-HSCs and plays an important role in the progression of liver fibrosis. M2BPGi has been recently developed as a novel serum biomarker that is strongly correlated with liver fibrosis in patients with CHC (29). Several studies have identified that M2BPGi can serve as a serum fibrosis marker in patients with CHB (30-33), although M2BPGi levels vary for the same fibrosis stage between patients with CHB and CHC (31). This may be partly explained by the fact that the generative nodule size and fibrous septum thickness (composed of collagen fibrils) substantially differ between patients with CHB and CHC (34). These findings support the potential role of M2BPGi as a surrogate biomarker that reflects hepatic stellate cell function (28).

Table IV. Baseline characteristics of patients with chronic hepatitis B without significant liver inflammation stratified according to liver fibrosis stages.

Variable (reference range)	F0 (n=25)	F1 (n=36)	F2 (n=9)	F3 (n=3)	Overall P-value
Male/female	11/14	19/17	3/6	2/1	NS
Age (years)	53.7±2.4	50.0±2.3	51.2±4.6	55.9±8.0	NS
Platelet count (10 ⁴ /μl) (10.4-37.9)	21.6±1.1	21.0±0.8 ^b	16.4±1.1	16.4±1.2	0.03
AST (IU/l) (10-40)	26.1±2.6	31.3±3.4	31.9±3.4	27.2±7.6	NS
ALT (IU/l) (5-45)	22.0±2.4	38.3±6.0	30.0±5.0	22.7±6.2	NS
Serum albumin (g/dl) (3.7-5.5)	4.2±0.1	4.2±0.1	4.0±0.1	4.4±0.2	NS
Total Bilirubin (mg/dl) (0.3-1.2)	0.8±0.0	0.8±0.1	0.9±0.1	1.0±0.2	NS
HBV DNA (Log copies/ml)	3.5±0.4	4.5±0.4	5.5±0.6	3.4±1.4	NS
HBsAg (IU/ml) (<0.05)	12,128±5,088	41,852±2,863	10,580±8,840	1,602±520	NS
Hyaluronic acid (ng/ml) (<50.0)	61.7±25.8	45.0±6.1	47.4±7.7	123.9±30.0 ^c	NS
Type 4 collagen 7S (ng/ml) (<6.0)	3.6±0.2	3.8±0.1	3.8±0.3	4.9±0.8 ^c	NS
PIIINP (ng/ml) (3.6-9.52)	7.9±0.5	8.7±7.3	9.4±1.0	11.0±2.9	NS
TIMP-1 (ng/ml)	198.6±13.8	211.1±5.7	193.5±6.5 ^c	264.4±45.0	NS
M2BPGi (COI) (<1.00)	0.53±0.32	0.68±0.6 ^b	1.20±0.1	1.25±0.17	<0.01
ProC3 (ng/ml)	18.5±1.3	15.8±0.6	17.4±2.3	17.7±2.8	NS
FIB-4	1.52±0.14	1.38±0.12	2.15±0.43	2.07±0.61	NS
APRI	0.43±0.05	0.50±0.05	0.66±0.09	0.59±0.20	NS
ELF score	8.91±0.18	8.99±0.12 ^c	9.21±0.17	10.20±0.40 ^d	NS

^bP<0.01 vs. F2; ^cP<0.01 vs. F3; ^dP<0.01 vs. F4; AST, aspartate aminotransferase; ALT, alanine aminotransferase; PIIINP, type 3 procollagen-N-peptide; TIMP-1, tissue inhibitor of metalloproteinase 1; M2BPGi, Mac-2-binding protein glycosylation isomer; Pro-C3, N-terminal type III collagen propeptide; FIB-4, fibrosis index based on four factors; APRI, aspartate aminotransferase-to-platelet ratio index; ELF score, enhanced liver fibrosis score; F, fibrosis stage.

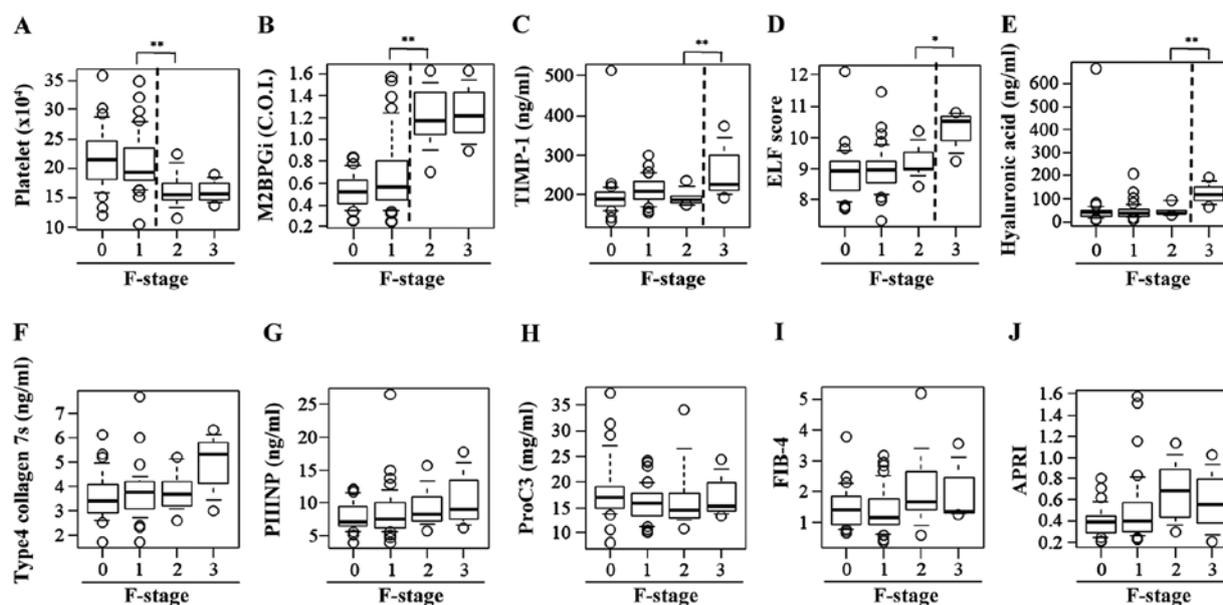


Figure 6. Serum levels of fibrosis biomarkers for staging liver fibrosis in patients with chronic hepatitis B without significant liver necroinflammation. A box plot of each fibrosis marker according to the fibrosis stage is presented. (A) Platelet count, (B) M2BPGi, (C) TIMP-1, (D) ELF score, (E) hyaluronan acid, (F) 7S domain of type 4 collagen, (G) PIIINP, (H) Pro-C3, (I) FIB-4 index and (J) APRI. Data are presented as mean ± standard deviation. *P<0.05 and **P<0.01 as indicated. M2BPGi, Mac-2 binding protein glycosylation isomer; TIMP-1, tissue inhibitor of metalloproteinases 1; ELF, enhanced liver fibrosis; PIIINP, procollagen type III N-terminal peptide; Pro-C3, N-terminal type III collagen propeptide; FIB-4 index, fibrosis index based on four factors; APRI, aspartate aminotransferase-to-platelet ratio index; F, fibrosis.

M2BPGi levels have been found to rapidly decrease with reduced hepatic inflammation during direct-acting antiviral

therapy for HCV infection in patients with CHC (27,35). In agreement with the current findings, serum M2BPGi

Table V. Diagnostic accuracy of serum fibrosis markers for significant fibrosis in patients with chronic hepatitis B without significant liver inflammation.

Biomarker	AUC	95% CI	Cut-off	Sensitivity (95% CI)	Specificity (95% CI)	PPV (95% CI)	NPV (95% CI)	Accuracy (95% CI)
Platelet	0.807	0.670-0.947	16.2	66.7 (34.9-90.1)	88.5 (77.8-95.3)	53.3 (16.6-78.7)	93.1 (83.3-98.1)	84.9 (74.6-92.2)
Hyaluronic acid	0.678	0.522-0.833	26.6	100 (64.0-100)	37.7 (25.6-51.0)	24.0 (13.1-38.2)	100 (78.9-100)	47.9 (36.1-60.0)
Type 4 collagen 7S	0.603	0.416-0.791	5.1	33.3 (9.9-65.1)	91.8 (81.9-97.3)	44.4 (13.7-78.8)	87.5 (76.8-94.4)	82.2 (71.5-90.2)
PIIINP	0.632	0.454-0.810	8.27	66.7 (34.9-90.1)	65.6 (52.3-77.3)	27.6 (12.7-47.2)	90.9 (78.3-97.5)	65.8 (53.7-76.5)
TIMP-1	0.501	0.329-0.674	174.7	100 (64.0-100)	24.6 (14.5-37.3)	20.7 (11.2-33.4)	100 (69.8-100)	37.0 (26.0-49.1)
M2BPGi	0.921	0.856-0.986	0.890	91.7 (61.5-99.8)	86.7 (75.4-94.1)	57.9 (33.5-79.7)	98.1 (89.9-100)	87.5 (77.6-94.1)
Pro-C3	0.458	0.251-0.664	24.35	25.0 (5.5-57.2)	95.1 (86.3-99.0)	50.0 (11.8-88.2)	86.6 (76.0-93.7)	83.6 (73.0-91.2)
FIB-4	0.678	0.500-0.855	1.264	83.3 (51.6-97.9)	50.8 (37.7-63.9)	25.0 (12.7-41.2)	93.9 (79.8-93.3)	56.2 (44.1-67.8)
APRI	0.708	0.524-0.892	0.438	75.0 (42.8-94.5)	65.6 (52.3-77.3)	30.0 (14.7-49.4)	93.0 (80.9-98.5)	67.1 (55.1-77.7)
ELF score	0.688	0.530-0.846	8.87	91.7 (61.5-99.8)	44.3 (31.5-57.6)	24.4 (12.9-39.5)	96.4 (81.7-99.9)	52.1 (40.0-63.9)

PIIINP, type 3 procollagen-N-peptide; TIMP-1, tissue inhibitor of metalloproteinase 1; M2BPGi, Mac-2-binding protein glycosylation isomer; Pro-C3, N-terminal type III collagen propeptide; FIB-4, fibrosis index based on four factors; APRI, aspartate aminotransferase-to-platelet ratio index; ELF score, enhanced liver fibrosis score.

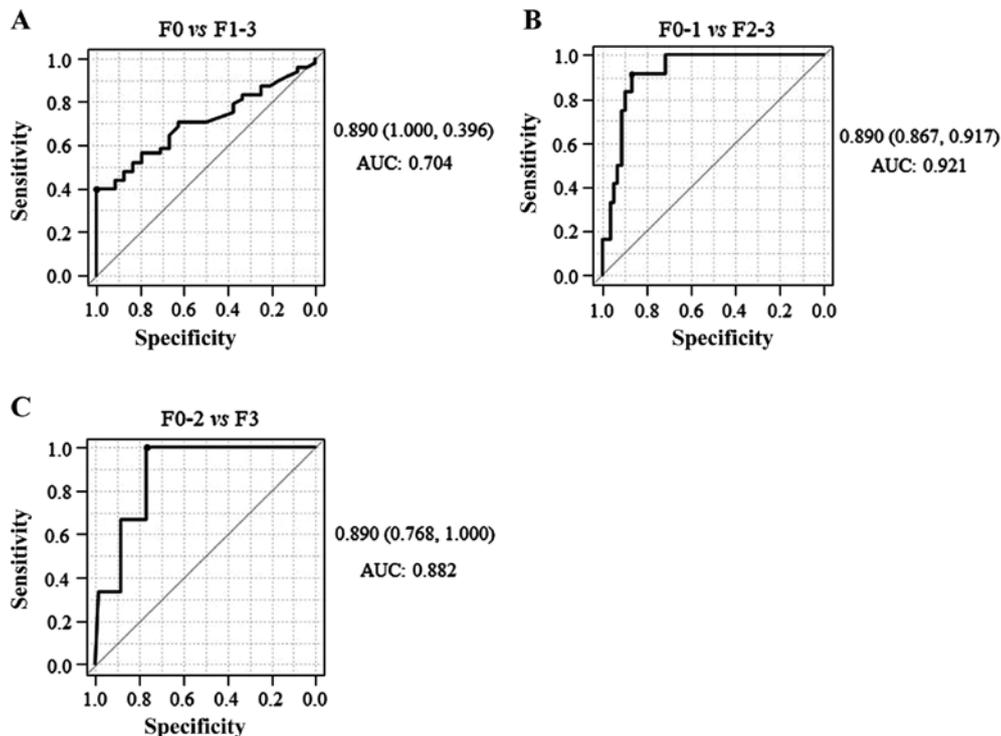


Figure 7. Diagnostic performance of serum M2BPGi for identifying the different stages of liver fibrosis in patients with chronic hepatitis B without significant liver necroinflammation. Receiver operating characteristic curve analysis and AUC for the diagnostic performance of serum M2BPGi for identifying the different stages of liver fibrosis. (A) F0 vs. F1-3, (B) F0-1 vs. F2-3 and (C) F0-2 vs. F3. M2BPGi, Mac-2 binding protein glycosylation isomer; AUC, area under the curve; F, fibrosis.

levels were significantly higher in patients with CHB with significant liver necroinflammation than in those without significant liver necroinflammation (36). All serum fibrosis biomarkers, except the Pro-C3 level, were correlated with the fibrosis stage in all patients with CHB, whereas the PLT count and M2BPGi level were exclusively associated with the fibrosis stage in patients with CHB without significant liver necroinflammation. In addition, in a previous study, the M2BPGi level was found to be correlated with the serum C-X-C motif chemokine 10 level, which is closely related to the migration of inflammatory cells to the local focus in the liver (30). These results further supported the hypothesis that fibrosis markers are substantially affected by liver inflammation. However, Miyaki *et al* (32) and Liu *et al* (33) reported that M2BPGi levels reflect fibrosis progression and are not affected by inflammation or ALT fluctuations in treatment-naïve patients with CHB. The reasons for the different results between the studies remain unclear. However, one possible explanation is the difference in the percentage of patients with cirrhosis between the studies, which warrants further investigation.

The present study found that the M2BPGi level had the highest diagnostic performance for identifying significant liver fibrosis in patients with CHB without significant necroinflammation. Recently, the ELF score and M2BPGi level demonstrated comparable diagnostic performances for identifying significant liver fibrosis in patients with CHB (32). A recent report by Jekarl *et al* (20) demonstrated that the PLT count, ELF score and M2BPGi level accurately identified significant liver fibrosis in treatment-naïve patients with CHB. Serum M2BPGi has been shown to have a good performance for diagnosing severe fibrosis in patients with CHB treated with nucleoside analogs (33). Mak *et al* (37) demonstrated that M2BPGi was significantly correlated with severe fibrosis and cirrhosis in patients with CHB treated with nucleoside analogs. The difference in the ability of serum M2BPGi to identify the liver fibrosis stage between the studies might be attributed to the patient distribution according to the fibrosis stage at inclusion and the administration of nucleoside analogs that might reduce hepatic fibrosis, in addition to the influence of hepatic inflammation. The current results confirm that M2BPGi is a novel non-invasive diagnostic biomarker that can identify treatment-naïve patients with CHB in need of treatment.

The present study has several limitations. First, this was a retrospective study. Second, liver biopsy has the drawback of being prone to sampling errors in fibrosis staging and inflammation grading, potentially leading to bias. Third, the sample size (especially the number of patients with cirrhosis) was small for analysis. It is difficult to obtain a representative liver biopsy specimen from patients with cirrhosis whose platelet counts are lower than $5 \times 10^4/\mu\text{l}$. Thus, further research with a large number of patients is required to validate the use of serum M2BPGi level in the detection of significant fibrosis in patients with CHB.

In conclusion, serum M2BPGi level is a useful marker for identifying liver histological findings in patients with CHB without significant necroinflammation in need of antiviral therapy, although M2BPGi level not only identifies the status of liver fibrosis but also reflects liver necroinflammation (27).

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

YT, KKa, HT, KN, SSat, SSai, YS, KKi, NS, HK, KM, RN, TA and AM performed data analysis. AM supervised all statistical analyses performed in this study. HY and TN made substantial contributions to the conception and design of the study and analysis and interpretation of the data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Written informed consent for the use of resected tissue was obtained from all patients and the study protocol was approved by the Ethics Committee of Nara Medical University (approval no. 1077).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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