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Serum M2BPGi level and risk of hepatocellular carcinoma after oral anti-viral therapy in patients with chronic hepatitis B

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Summary

Background: Mac-2 binding protein glycosylation isomer (M2BPGi) is an emerging biomarker for risk prediction of liver disease, but data remain sparse for patients with chronic hepatitis B (CHB) who are treated with nucleos(t)ide analogues (NA).

Aim: To clarify serial changes in M2BPGi and its association with subsequent hepatocellular carcinoma (HCC) development in NA-treated CHB patients.

Methods: We enrolled 384 previously untreated CHB patients who received NAs. Among them, 195 had baseline cirrhosis (n = 142:48:5 for Child A:B:C). Sera were collected at NA initiation, and after 1 and 2 years. Serum M2BPGi levels were measured and expressed as cut-off index (COI) at different time points. The association between M2BPGi and HCC was evaluated by the Cox proportional hazard model.

Results: The median M2BPGi levels significantly decreased from 1.68 COI at baseline, to 1.0 at year 1, and 0.88 at year 2. During median follow-up of 72.7 months, HCC occurred in 37 patients, 36 of whom had cirrhosis. In patients with cirrhosis, baseline M2BPGi level was associated with HCC risk (adjusted hazard ratio, 1.07 per COI; 95% CI, 1.01-1.14) on the multivariable Cox analysis, whereas levels at year 1 or 2 were not independently predictive. A risk score for HCC was developed using baseline M2BPGi, age and body mass index with *c* statistics of 0.77, 0.79 and 0.87 at 3, 5 and 10 years, respectively.

Conclusions: Serum M2BPGi level significantly decreases after NA treatment in CHB patients. Baseline level can be factored into the risk prediction of HCC in NA-treated patients with cirrhosis.

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1 | INTRODUCTION

Mac-2 binding protein (M2BP) is a glycoprotein involved in intercellular adhesion and interactions with the extracellular matrix.[1] M2BP is widely expressed in various human tissues, but a liver-specific glycosylation isomer (M2BPGi) can be determined and quantified using the *Wisteria floribunda* agglutinin immunoassay that is already commercially available in Japan.[2] In the recent years, M2BPGi has emerged as a novel biomarker that correlates well with hepatic fibrosis in patients with chronic liver diseases.[3] Recent literature also suggests that M2BPGi may correlate with hepatocellular carcinoma (HCC) development in patients with chronic viral hepatitis, but most have been conducted in patients with chronic hepatitis C (CHC) or untreated chronic hepatitis B (CHB) without serial measurements.[4-8]

Indeed, the serum concentration of M2BPGi is not static and may change in a relatively short period of time, especially in the setting of anti-viral therapies. This was well described by Nagata et al in their recent study of CHC patients treated with either interferon-based or interferon-free direct acting anti-viral therapies.[9] However, data on the longitudinal effect of anti-viral therapy on M2BPGi levels in treated CHB patients are still limited. While CHB patients treated with nucleos(t)ide analogue (NA) can expect significantly reduced risk of HCC, this risk remains, especially in patients with cirrhosis.[10] Therefore, it is particularly important to evaluate the dynamic changes and predictive potential of novel biomarkers such as M2BPGi longitudinally and in relation to anti-viral therapy because levels can change with treatment,[8] treated patients may still develop HCC,[11] and long-term suppressive treatment with NAs remains the primary therapeutic strategy in the management of active CHB.[12-14] Moreover, a serum-based marker is more convenient and accessible for clinical practice and warrants investigation.

To clarify the serial changes of M2BPGi in NA-treated CHB patients and to evaluate the association of M2BPGi levels with subsequent HCC development, we conducted this multicenter cohort study of treatment-naïve patients who had pre-treatment and serial blood collection after the initiation of NA therapies. A group of untreated patients were also enrolled to serve as a reference frame to help interpret the data from the treated patients. To accurately estimate this risk, we used time-dependent analytic methods and performed stratified analyses by cirrhosis status, given that M2BPGi is well-known to strongly correlate with liver fibrosis/cirrhosis.

2 | METHODS AND MATERIALS

2.1 | Study design and setting

This cohort study included a treated cohort of CHB patients with serum samples prospectively collected prior to NA therapy (baseline sample) and serially at year 1 and 2 after treatment initiation and an untreated cohort of CHB patients who did not receive NA therapy during study period. Patients were enrolled and observed at two teaching hospitals in Kaohsiung, Taiwan (Kaohsiung Medical University Hospital and E-Da Hospital) between end of January 2000 and end of September 2017. All patients gave written informed consents. The laboratory analysis for the serum samples was conducted at a single laboratory at the Nagoya City University, Nagoya, Japan. Clinical and laboratory data were submitted to the data centre at Stanford University Medical Center, Palo Alto, California, USA for data management and analysis. This study was approved by the institutional review board at each participating institution.

2.2 | Patient population

Patients were eligible if they were 18 years or older, had CHB (positive hepatitis B virus [HBV] surface antigen [HBsAg] or HBV DNA and a documented history of chronic infection for 6 months or longer), were treatment-naïve at the time of the first serum collection (baseline level) and were subsequently treated with a commercially approved NA and had serum collection again at 1 year and/or 2 years after NA treatment. Patients were ineligible if they had a positive serological test of HCV, excessive alcohol consumption (generally defined as more than 14 drinks per week in men and seven drinks per week in women), or other causes of liver diseases such as autoimmune or drug-induced liver injury according to the clinical judgement of the treating physicians. However, we did not exclude patients with a report of fatty liver on routine sonography. Patients with any malignancy at the time of NA initiation and those who developed HCC within 1 year of therapy were also excluded. In view of the doubling time of HCC,[15] 1-year duration should be sufficient to exclude the vast majority of prevalent cases.

The indications of anti-viral therapy principally followed the practice guidelines endorsed by the Asian Pacific Association for the Study of the Liver.[16] In general, the manifestation of hepatic decompensation, severity of liver fibrosis, status of hepatitis B e antigen (HBeAg), serum levels of HBV DNA and alanine aminotransferase (ALT) were all taken into consideration.

The selection of untreated control patients followed the same eligibility criteria as described above, except for treatment status.

2.3 | Data collection and M2BPGi measurement

Data including demographics, comorbid diseases, laboratory tests and other pertinent radiological, pathological and clinical information at baseline and follow-up were extracted from each clinical centres and were recorded using the same data frame and variable definitions. One investigator reviewed the datasets and audited the accuracy. Cirrhosis was determined via histology or by clinical criteria mainly composed of radiological features (nodular hepatic surface, coarse echotexture, irregular vasculature and splenomegaly).[17]

Noninvasive scores based on routinely available laboratory tests were also used to estimate levels of liver fibrosis and hepatic dysfunction. The scores of the aspartate aminotransferase (AST) to

platelet ratio index (APRI), Fibrosis 4 (FIB-4), and MELD (model for end-stage liver disease) were calculated according to the following equations: [AST/38 (U/L)/platelet count (10^3/μL)] × 100, [AST (U/L) × age (years)]/[ALT (U/L)$^{1/2}$ × platelet count (10^3/μL)], and 3.78 × ln [serum bilirubin (mg/dL)] + 11.2 × ln[INR] + 9.57 × ln[serum creatinine (mg/dL)] + 6.43, respectively.[18-20]

Serum M2BPGi levels were measured using sera collected prior to NA initiation, at 1 and at 2 years after the therapy. Archived sera from the two hospitals were sent to a single laboratory (Nagoya City University, Nagoya, Japan) for the measurement of M2BPGi. The protocol has been reported previously.[21] In brief, it was measured by an automated analyser applying the lectin-antibody sandwich immunoassay (HISCL-2000i; Sysmex Corporation, Hyogo, Japan). M2BPGi that was conjugated to *W. floribunda* agglutinin was quantified in cut-off index (COI) and calculated by the formula: ($M2BPGi_{sample}$ − $M2BPGi_{negative\ control}$)/($M2BPGi_{positive\ control}$ − $M2BPGi_{negative\ control}$). For standardised calibration, the supplied solution of positive control would yield a COI of 1.0.

2.4 | Longitudinal follow-up and outcome measure

The primary outcome was the development of incident HCC. Surveillance for HCC was carried out by abdominal sonography in principle, whereas computed tomography or magnetic resonance image served as the second-line modality for diagnostic confirmation.[22] Generally, the frequency of surveillance was every 6 months in patients without cirrhosis and 3 months in those with cirrhosis. Patients were censored at loss to follow-up, death, or end of the observation in the end of September 2017. HCC was diagnosed via histology, cytology, or non-invasive criteria using dynamic images as recommended by the American Association for the Study of Liver Diseases.[22]

2.5 | Statistical analysis

Continuous variables were expressed as medians and interquartile ranges (IQR) and categorical variables as percentages. All patients with at least one measurement of M2BPGi were retained and the serial levels were first explored by the Skillings-Mack test, which can account for missing data in analyses with repeated measurements.[23] The differences between the respective serum M2BPGi levels were further examined by the Wilcoxon signed-rank test in patients with available sera at all three time points. Thus, no data were imputed.

The cumulative incidence of HCC was estimated by the Kaplan-Meier method. We performed the Cox proportional hazard regression to evaluate the association between M2BPGi levels and subsequent development of HCC. The measurements of M2BPGi at different time points were examined individually and compared against each other for the association with HCC. M2BPGi levels were also analysed as a time-varying variable in the model.

Next, we developed a risk score based on the most predictive M2BPGi for HCC as well as other risk factors significantly associated with HCC in the multivariable Cox model. The model examined all potential predictors regardless of the results in the univariable analyses and was determined by stepwise regression to remove insignificant factors. The scoring factors were weighted according to their regression coefficients in Cox models. We constructed time-dependent receiver operating characteristic (ROC) curves for censored survival data to appraise the risk score that included M2BPGi as an explanatory variable.[24] The new M2BPGi-based score was also compared against the PAGE-B and CAMD scores in area under the ROC curve, statistical significance of which was evaluated by two-sided bootstrap *P*-value. Both PAGE-B and CAMD scores were developed in NA-treated CHB patients and have been validated.[25,26]

All data analysis was performed using either the STATA software (13.0 version, College Station, TX) or the R program (version 3.4.0). All statistical tests were two-tailed and a *P*-value less 0.05 defined statistical significance.

3 | RESULTS

3.1 | Characteristics of the study cohorts

We enrolled a total of 384 eligible patients who received NA therapy and 122 untreated controls. (Table 1 and Table S1). For the treated cohort (Table 1), approximately three-fourths were male (n = 282), one half (n = 195) had liver cirrhosis, and slightly over half (n = 224) were treated with entecavir. One third (n = 139) used older generations of NAs and the M2BPGi levels did no differ according to the regimens (Table S2). The median follow-up for this cohort was 72.73 (IQR, 44.33, 103.75) months. During this period, 36 (9.38%) patients developed HCC and all but one occurred in patients with cirrhosis at baseline.

Median baseline serum M2BPGi (prior to NA therapy, available in all 384 patients) was 1.68 (IQR, 0.78, 4.40) COI. At year 1 and 2 after the NA treatment, the measurements were available in 314 and 282 patients, respectively, with the medians of 1.0 (IQR, 0.61, 1.83) and 0.88 (95% CI, 0.58-1.75) respectively. The distributions of M2BPGi were significantly skewed to the right (Figure S1). The correlation between baseline M2BPGi level and FIB-4 index was significant but modest (Figure S2A; Spearman's ρ = 0.6, $P < 0.0001$), and the M2BPGi levels varied considerably within the same FIB-4 category (Figure 2B).

3.2 | Serial changes of M2BPGi during the anti-viral therapy

The three measurements of M2BPGi significantly differed. In view of the difference between patients with and those without liver cirrhosis, the analysis was stratified by cirrhosis to confirm that M2BPGi levels decreased after NA treatment regardless of baseline cirrhosis status (Table 2).

In the paired comparison among 274 patients with all three measurements, the baseline M2BPGi measurement (median, 1.83; IQR,

0.97, 4.66) was significantly higher than those measured 1 year (median, 0.99; IQR, 0.61, 1.77) and 2 years (median, 0.87; IQR, 0.55, 1.71) afterwards ($P < 0.0001$ for both comparisons, Wilcoxon signed-rank test). The trend of decline was significant over time ($P_{trend} < 0.0001$) among both the cirrhotic and noncirrhotic subgroups but the difference was much more pronounced in the first year (pre-treatment to year 1 post-treatment level: median, 0.57; IQR, 0.07, 2.07) while the difference during the second year (between year 1 to 2 post-treatment) was only modest (median, 0.08; IQR, −0.18, 0.40) (Table 3).

TABLE 1 Baseline characteristics of the treated study cohort

Characteristics	No HCC (n = 348)	HCC (n = 36)	P[a]
Age (y)	46 (36-55)	56 (52-61)	<0.001
Male gender, n (%)	256 (73.56)	26 (72.22)	0.85
BMI (kg/m^2)	24.5 (22.4-27.0)	25.7 (22.6-29.8)	0.14
DM, n (%)	45 (12.93)	10 (27.78)	0.02
Cirrhosis, n (%)	160 (45.98)	35 (97.22)	<0.001
Child A	120 (75.0)	22 (62.86)	0.04
Child B	38 (23.75)	10 (28.57)	
Child C	2 (1.25)	3 (8.57)	
Anti-viral regimen, n (%)			
Lamivudine	86 (24.71)	12 (33.33)	0.2
Adefovir	2 (0.57)	1 (2.78)	
Telbivudine	35 (10.06)	3 (8.33)	
Entecavir	204 (58.62)	20 (55.56)	
Tenofovir	21 (6.03)	0	
HBeAg-positive, n (%)	150/333 (45.05)	8/33 (24.24)	0.03
HBV DNA, log IU/mL	6.45 (4.96-7.64)	5.98 (4.85-7.25)	0.18
AST (U/L)	82 (53-172)	87 (50-150)	0.99
ALT (U/L)	116 (65-274)	83 (46.5-193.5)	0.06
Alfa-fetoprotein (ng/mL)	6.87 (3.9-18.1)	11.8 (6.3-22.3)	0.02
Albumin (g/dL)	4.0 (3.5-4.4)	3.5 (2.96-3.96)	<0.001
Creatinine (mg/dL)	0.98 (0.81-1.11)	0.86 (0.72-1.07)	0.04
Platelet count, $10^3/\mu L$	162 (107-210)	80 (70-122)	<0.001
Bilirubin (mg/dL)	1.3 (0.87-2.1)	1.3 (0.95-3.2)	0.3
INR	1.08 (1.01-1.2)	1.09 (1.18-1.35)	<0.001
FIB-4	2.22 (1.27-5.11)	5.77 (3.77-11.15)	<0.001
APRI	1.47 (0.81-2.94)	2.48 (1.38-5.20)	0.009
MELD	8.35 (5.88-11.06)	8.26 (6.87-14.41)	0.24
Follow-up until HCC	74.2 (45.5-104.7)	59.2 (37.0-92.0)	0.06
Baseline M2BPGi, COI	1.57 (0.76-3.8)	3.88 (1.78-10.66)	<0.001
Year 1 M2BPGi, COI[b]	0.96 (0.6-1.63)	2.57 (1.35-4.0)	0.002
Year 2 M2BPGi, COI[b]	0.84 (0.53-1.52)	2.23 (1.28-5.20)	<0.001

ALT, alanine aminotransferase; APRI, aspartate aminotransferase-to-platelet ratio index; AST, aspartate aminotransferase; BMI, body mass index; DM, diabetes mellitus; FIB-4, fibrosis-4; HCC, hepatocellular carcinoma; INR, international normalised ratio; M2BPGi, mac-2 binding protein glycan isomer; MELD, model for end-stage liver disease.
[a]Compared between patients with and without HCC events.
[b]M2BPGi was measured in 314 and 282 patients after 1 and 2 years of therapy respectively.

3.3 | Serial M2BPGi levels in the untreated patients

Baseline M2BPGi were available in all 122 untreated patients. Of these, 91 and 60 patients remained untreated and had serum collection at one and 2 years later, respectively; 59 patients had all three serum collections (Table S3). There was no difference in serial measurements of M2BPGi in the untreated controls overall ($P = 0.17$, Skillings-Mack test). Similarly, no difference was noted among the 59 untreated patients with all three paired sera ($P = 0.25$, Friedman's test). Because only one untreated patient had cirrhosis, the analysis was not further stratified.

3.4 | Association between pre-treatment or on-therapy M2BPGi levels and risk of HCC

Hepatocellular carcinoma occurred in 36 patients (35 in patients with cirrhosis) with a cumulative incidence of 19.8% (95% CI, 13.7%-28.2%) at 15 years (Figure 1A). As HCC almost exclusively occurred in the 195 patients with cirrhosis (Figure 1B), the

TABLE 2 Serum levels of M2BPGi from baseline to 2 y after treatment in all 384 patients with baseline measurements[a]

	Baseline[b]	First year[b]	Second year[b]
Cirrhosis	3.02 (1.18, 7.25)[1] N=195	1.52 (0.85, 4.13)[2] N = 168	1.47 (0.81, 3.58)[3] N = 156
No cirrhosis	1.11 (0.65, 2.08)[1] N = 189	0.71 (0.51, 1.05)[2] N = 146	0.71 (0.46, 0.88)[3] N = 126

[a]Analysis adjusted for missing samples at year 1 and 2 by Skillings-Mack test; M2BPGi levels presented as median and interquartile ranges.
[b]$P < 0.0001$ for the comparisons among the three measurements at different time points in all patients, the subgroup with cirrhosis, as well as the subgroup without cirrhosis (Skillings-Mack test).
[1,2,3]$P < 0.0001$ for all comparisons between cases with cirrhosis and without cirrhosis at the three time points (Wilcox rank sum test).

TABLE 3 Decline of M2BPGi after initiation of anti-viral therapy in 274 patients with all three measurements

	Decline during the 1st year	Decline during the 2nd year	P[a]
All patients	0.57 (0.07-2.07)	0.08 (−0.18 to 0.4)	<0.0001
Cirrhosis (n = 148)	0.55 (−0.14 to 2.74)	0.16 (−0.32 to 0.74)	<0.0001
No cirrhosis (n = 126)	0.57 (0.17-1.5)	0.05 (−0.15 to 0.2)	<0.0001

Data presented as median and interquartile range.
[a]Examined by the Wilcoxon signed-rank test.

analysis to identify risk factors of HCC was performed only in the cirrhosis subgroup.

In univariable Cox proportional hazard model (Table 4), the baseline M2BPGi level was significantly associated with the development of HCC (crude HR, 1.10 per COI; 95% CI, 1.05-1.16). With regard to M2BPGi levels at year 1 and 2 after treatment, the associations with subsequent occurrence of HCC did not reach the predefined level of statistical significance. The multivariable Cox model revealed that only the baseline M2BPGi level was significantly associated with HCC (adjusted HR, 1.07, 95% CI, 1.01-1.14) after adjustment for age and body mass index, which were the other two independent risk factors. The serial change in M2BPGi was further analysed as a single variable that varied with time in the time-dependent Cox model. Time-varying M2BPGi level was associated with subsequent HCC development in the univariable Cox proportional hazard model (crude HR, 1.09 per COI, 95% CI, 1.01-1.18), but it was not an independent risk factor in the adjusted multivariable analysis (adjusted HR, 1.08 per COI, 95% CI, 0.98-1.196; $P = 0.10$) (Table 4).

3.5 | Development of a risk score using baseline M2BPGi to predict HCC risks in patients with cirrhosis

The regression coefficients for the three variables included in the final multivariable Cox model (Model I) were 0.08 per year for age, 0.07 per COI for baseline M2BPGi, and 0.10 per kg/m^2 for body mass index (Table S4), and were used to generate a risk score with the following formula: 8 × age (year) + 7 × baseline M2BPGi (COI) + 10 × body mass index (kg/m^2). The score was calculable in 171 patients with cirrhosis with a median of 652.5 (IQR, 581.3, 709.4) points. Twenty nine patients among them developed HCC. The performance of the score to predict HCC was illustrated by the time-dependent ROC curves in Figure 2. The areas under the curves were 0.77, 0.79 and 0.87 at year 3, 5 and 10 respectively (Figure 2A). The new M2BPGi-based risk score outperformed both the PAGE-B and CAMD scores in this cohort of patients with cirrhosis at 5 and 10 years, though not at 3 years (Figures 2B-D). To illustrate the potential for clinical application, the median of the scores (652.5) was used to categorise patients into higher or lower risks.[27] The two risk subgroups significantly differed in the 15-year cumulative incidence of HCC (67.2% with 95% CI of 34.9%-94.5% vs 15.8% with 95% CI of 6.2%-36.8%; Figure 3). The sensitivity, specificity, positive and negative predictive values for HCC were also appended (Table S5).

4 | DISCUSSION

Our study demonstrates that (a) serum M2BPGi levels were significantly higher in CHB patients with cirrhosis compared to those without cirrhosis regardless of treatment status; (b) serum level of M2BPGi significantly decreased after NA treatment while serial changes were not observed in untreated patients; (c) the decline in NA-treated patients occurred mostly during the first year of therapy, when more than half of the treated patients experienced a decrease of at least 0.5 COI, a finding that was consistent in cirrhotic as well as non-cirrhotic patients, while the changes between year 1 and 2 were less appreciable; (d) baseline M2BPGi level was independently associated with the risk of HCC in patients with cirrhosis but not post-treatment or time-varying levels and (e) a risk score including baseline M2BPGi and physiologic variables such as age and body mass index can predict long-term HCC risk in NA-treated CHB patients with cirrhosis. Collectively, these findings illustrated how serum M2BPGi level would change serially after NA therapy in CHB patients and identified baseline M2BPGi as the most significant level for the risk prediction of HCC in NA-treated patients.

M2BPGi is secreted by hepatic stellate cells[28] and have been shown to correlate with liver fibrosis in a variety of chronic liver diseases including viral hepatitis,[29,30] non-alcoholic fatty liver disease,[31]

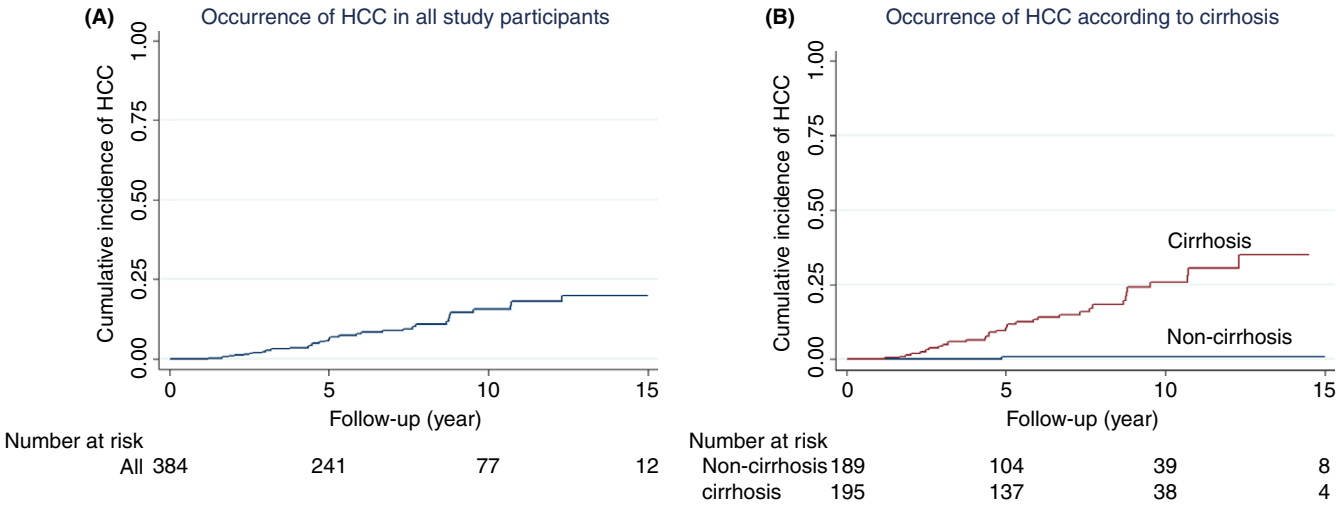

FIGURE 1 The cumulative incidence of hepatocellular carcinoma up to 15 y after the initiation of NA therapy in the overall treated study cohort (A) and the subgroups stratified by liver cirrhosis (B)

TABLE 4 The Cox proportional hazard model for the development of hepatocellular carcinoma in patients with liver cirrhosis

	Univariable analysis			Multivariable analysis I			Multivariable analysis II		
	HR	95% CI	P	Adjusted HR	95% CI	P	Adjusted HR	95% CI	P
Age (y)	1.08	1.03-1.13	0.001	1.08	1.03-1.14	0.003	1.09	1.03-1.14	0.002
Male gender	1.01	0.48-2.12	0.97						
Body mass index (kg/m^2)	1.14	1.03-1.27	0.01	1.11	1.00-1.22	0.05	1.10	0.99-1.22	0.09
Diabetes mellitus	2.17	1.04-4.54	0.04						
Child class B or C	1.69	0.85-3.36	0.13						
Less preferred NA regimen[a]	0.58	0.28-1.18	0.13						
HBV DNA, log IU/mL	1.03	0.82-1.29	0.81						
HBeAg-positive[b]	0.32	0.14-0.72	0.01						
AST (U/L)	1.0	0.999-1.001	0.71						
ALT (U/L)	1.0	0.999-1.001	0.90						
Alpha-fetoprotein (ng/mL)	0.998	0.991-1.004	0.49						
Albumin (g/dL)	0.53	0.30-0.93	0.03						
Creatinine (mg/dL)	1.49	0.83-2.69	0.18						
Platelet count, $10^3/\mu L$	0.99	0.98-1.0	0.04						
Bilirubin (mg/dL)	1.04	0.97-1.11	0.27						
INR	1.91	0.78-4.64	0.16						
FIB4	1.02	0.99-1.06	0.13						
APRI	1.01	0.96-1.06	0.70						
MELD score	1.08	1.01-1.15	0.02						
M2BPGi at baseline, COI	1.10	1.05-1.16	<0.001	1.07	1.01-1.14	0.02			
M2BPGi at year 1, COI	1.09	1.0-1.18	0.05						
M2BPGi at year 2, COI	1.10	1.0-1.22	0.06						
Time-varying M2BPGi, COI	1.09	1.01-1.18	0.03				1.08	0.98-1.20	0.10

All the listed factors were examined with stepwise regression in the multivariable model. The M2BPGi levels at baseline, at year 1, and 2 were tested one by one in the model I. The serial measurement of M2BPGi was managed as a single time-varying variable in the model II.

ALT, Alanine aminotransferase; APRI, aspartate aminotransferase-to-platelet ratio index; AST, aspartate aminotransferase; CI, confidence interval; FIB-4, fibrosis-4; HBV; hepatitis B virus; HR, hazard ratio; INR, international normalised ratio; M2BPGi, mac-2 binding protein glycan isomer; MELD, model for end-stage liver disease.

[a]Less preferred NA regimen included lamivudine, adefovir, or telbivudine.

[b]Data of HBeAg status were available in 366 patients.

biliary cirrhosis,[32] and autoimmune hepatitis.[33] Consistent with prior studies, our study also showed that M2BPGi levels were higher in CHB patients with cirrhosis than those without. However, our findings further suggested that serum M2BPGi level represented something more than just liver fibrosis, because the rapid drop of M2BPGi within 1 year could not be entirely attributable to changes in the fibrosis status. Presumably, it was accounted for by the amelioration of hepatocellular injury that preceded the regression of fibrosis which would likely take a few years or longer. This result was consistent with recent studies that reported rapid decrease in serum M2BPGi levels among HCV-infected patients who cleared the virus and those who recovered from acute liver injury.[33,34] These lines of evidence suggested that M2BPGi levels might also reflect liver inflammation, hepatocellular necrosis or regeneration. Therefore, the ability of M2BPGi measurement to predict long-term outcomes such as HCC should be interpreted in the clinical context which the levels were derived from, including the consideration of liver aetiology, disease activity, fibrosis stages and anti-viral treatment status.

Previous studies of treated CHB patients have reported decreased in M2BPGi levels after NA therapy but few have evaluated serial levels between the first and second years after NA initiation in patients with CHB. One recent study observed that the median levels of M2BPGi at baseline and at 48 weeks were 1.22 and 0.77 COI in patients who did not develop HCC, and higher at 1.48 and 1.34 COI in those who later developed HCC, respectively; but this study did not investigate post-treatment M2BPGi levels beyond week 48.[8] In another recent study, serial M2BPGi levels after therapy were examined. The mean M2BPGi also decreased from 3.1 COI at baseline to 1.9 COI at 48 weeks and 1.5 COI at 96 weeks[29]; however, this study only included 89 patients with serial serum samples, analysed patients with and those without cirrhosis together, and did not evaluate correlation between M2BPGi with HCC development.

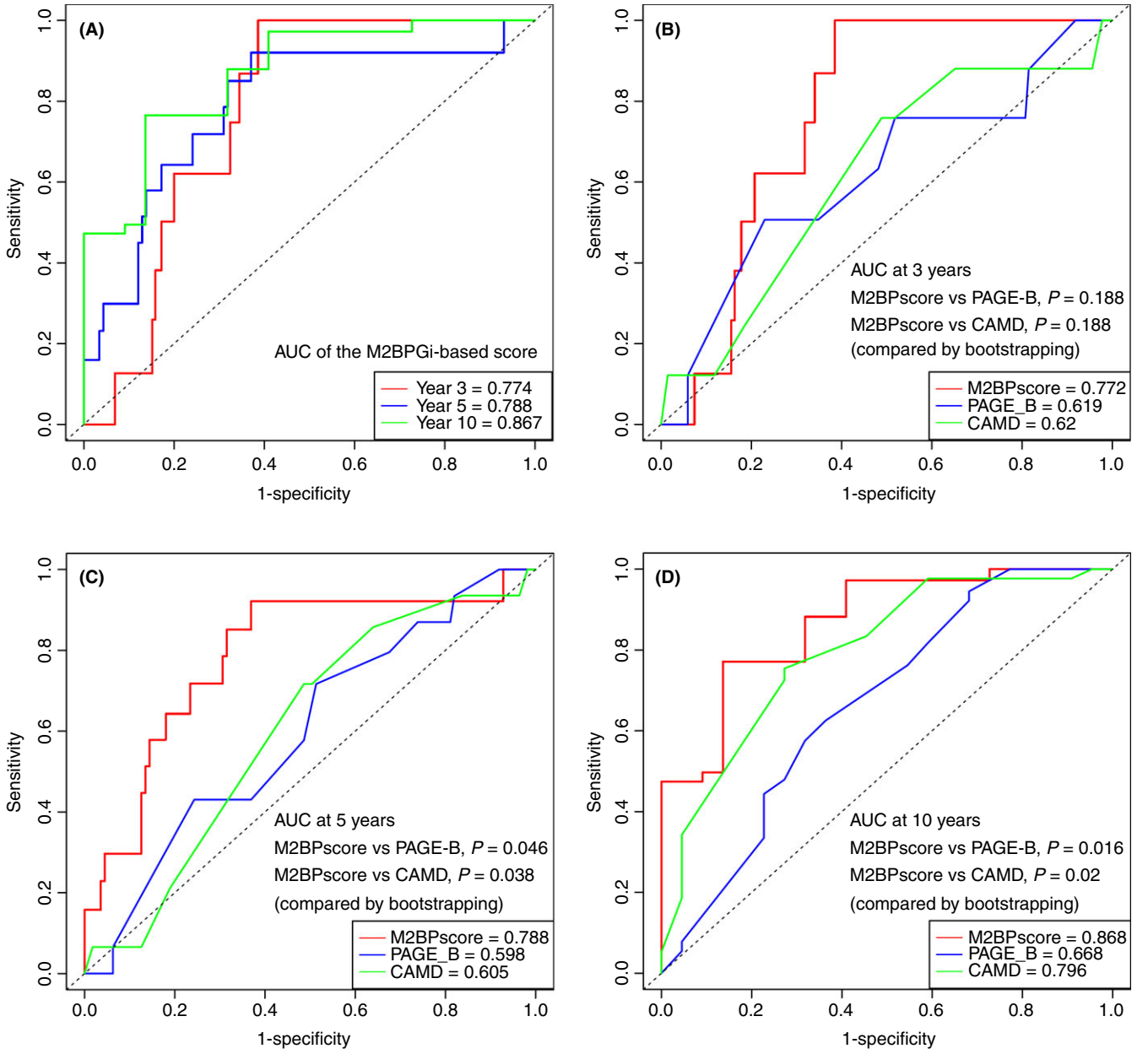

FIGURE 2 The receiver operating characteristic curves of the risk score based on age, body mass index, and baseline M2BPGi level to predict hepatocellular carcinoma in patients with cirrhosis. Areas under the curves at 3, 5, and 10 years were 0.77, 0.79 and 0.87 respectively (A). The newly developed M2BPGi-based score, PAGE-B, and CAMD score were similar in the area under the receiver operating characteristic curves at 3 y (B), but the M2BPGi-based score outperformed the other two scores at 5 and 10 y of follow-up (C, D); AUC, area under the curve (comparison was carried out by bootstrapping)

Besides its association with fibrosis, M2BPGi has also been studied as a marker of future HCC development in untreated CHB patients.[35] Data on M2BPGi as a predictor for HCC for NA-treated patients are much more limited by small sample size, lack of stratified analysis by cirrhosis which is a major confounder, and insufficient account for the time-dependent nature of the association of M2BPGi levels and HCC development. Shinkai and colleagues observed 234 CHB NA-treated patients (37 with cirrhosis) and reported that M2BPGi level measured at week 48 after NA initiation was predictive of subsequent occurrence of HCC rather than baseline levels as seen in our study; however, this result was not drawn from stratified analysis by cirrhosis and multivariate analysis did not control for fibrosis or cirrhosis limiting its conclusions.[8] On the other hand, another recent study of NA-treated CHB patients (57 HCC cases and 57 non-HCC controls) found significant differences in pre-treatment M2BPGi levels between cases and controls, but not post-treatment levels, a finding similar to ours.[7] This study also provided data for stratified analysis by cirrhosis, but its case-control design did not allow for time-to-event analysis and evaluation of M2BPGi as a predictor of future HCC.

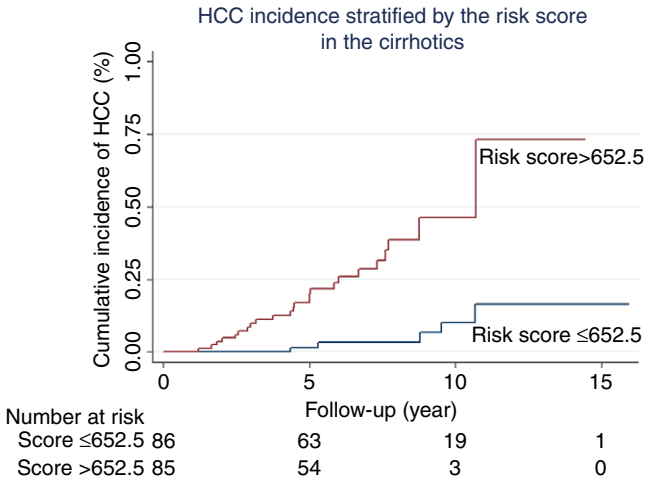

FIGURE 3 Using the median of the risk score to stratify patients at different risks for hepatocellular carcinoma, the cumulative incidences at 15 y were 67.2% (95% CI, 34.9%-94.5%) for the higher risk and 15.8% (95% CI of 6.2%-36.8%) for the lower risk patients

Patients with CHB remained at risk of HCC while on NA therapy.[11] Previous studies have found age, cirrhosis, platelet count, liver stiffness, severity of hepatic dysfunction, alfa-fetoprotein, male sex and diabetes mellitus to be predictive of HCC in NA-treated patients.[25,26,36-38] Although we explicitly set out to explore the serial changes of M2BPGi and how it might be associated with subsequent HCC, it was beyond our scope to compare this biomarker with other predictive tools such as liver elastography,[39] which has been shown to be predictive of HCC in both treated and untreated patients.[37,40] Neither did this study aim to conclude an exact cut-off of M2BPGi nor establish an "optimal model" ready for clinical application. Accordingly, we chose the stepwise regression instead of criterion-based methods for variable selection in the multivariable Cox model, and applied the median split to illustrate how M2BPGi might be incorporated into a predictive score while deliberately avoiding the "optimal cutoff" approach that could raise the concern of multiple comparisons.[27]

In addition to a larger sample size of 384 NA-treated patients (195 with cirrhosis) drawn from two clinical centres and a long duration of observation, our study also has the following strengths. First, an untreated cohort with serial serum collections served as a reference to contrast the rapid decrease seen in treated patients. This finding addressed the paucity of data in serial M2BPGi measurements in untreated CHB patients, although the study was not specifically designed to directly compare treated and untreated patients, as these two populations were expected to differ with the untreated one less likely to have advanced disease. Moreover, we performed stratified analysis by cirrhosis which is well-known to be one of the strongest factors associated with both M2BPGi level and HCC risk, thus removing cirrhosis from affecting our results as a potential confounder. Last, our conclusions were based on the consistent results from different angles of analyses using nonparametric methods, paired comparison, and time-dependent approaches.

There are some limitations that require attention. First, only one patient without baseline cirrhosis developed HCC in our study; therefore, we could not investigate the association between M2BPGi and HCC in the noncirrhotic population. This issue required further research. Second, older generations of anti-viral agents were still included to reflect the heterogeneous composition in the real-world practice. However, changes in M2BPGi levels appeared similar in patients receiving first-line agents versus older generations in the analysis (Table S2). We did not find the type of NA was a significant HCC risk determinant, either (Table 4), in accordance with recent studies.[37] Third, as a result of incomplete or missing data, our analysis could not be fully adjusted for some potential confounders such as viral genotype, HBeAg status, and family history; however, previous studies have shown that these factors are not significant predictors for HCC in NA-treated CHB patients.[36,41] Fourth, although we found body mass index to be associated with HCC, this study could not examine if fatty liver was an independent risk factor of HCC in NA-treated CHB patients because there was no standardised measurement of fatty liver in this cohort, but this important issue should be further studied. Finally, limitations in generalisability are recognised: all participants are Taiwanese and our results may not be generalisable for other Asian and non-Asian ethnicities. In addition, the untreated cohort inevitably included mostly patients with inactive or less severe diseases. Otherwise, treatment would have been indicated. Therefore, our untreated patient data could not be extrapolated to all untreated patients, particularly those with liver cirrhosis. As most of our treated patients with cirrhosis also had Child-A disease, caution is advised before generalising our data to treated patients with decompensated cirrhosis.

In summary, our findings demonstrate a significant drop of serum M2BPGi after NA treatment in patients with CHB during the first year of therapy and less pronounced afterwards; it is the pre-treatment baseline level that is most significantly associated with future HCC occurrence on long-term follow-up. Our study also demonstrates a risk score using the baseline M2BPGi level to predict HCC occurrence up to 10 years later in CHB patients with cirrhosis. External validation of this risk score is needed.

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SUPPORTING INFORMATION

Additional supporting information will be found online in the Supporting Information section at the end of the article.

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