

Serial measurement of *Wisteria floribunda* agglutinin positive Mac-2-binding protein is useful for predicting liver fibrosis and the development of hepatocellular carcinoma in chronic hepatitis C patients treated with IFN-based and IFN-free therapy

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Received: 23 February 2016 / Accepted: 24 June 2016 / Published online: 19 July 2016
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Abstract

Aim *Wisteria floribunda* agglutinin positive (WFA⁺) Mac-2-binding protein (M2BPGi) is a noninvasive glyco-marker for liver fibrosis. This study evaluated the utility of serial measurement of serum M2BPGi and total M2BP as a predictor of fibrosis and the development of hepatocellular carcinoma (HCC).

Methods This study included 119 patients with chronic hepatitis C (CHC). Of these patients, 97 were treated with IFN-based therapy and 22 were treated with daclatasvir and asunaprevir. Serum M2BPGi values were measured prior

to, at the end of, and at 24 weeks after the completion of treatment. As subanalysis, serum total M2BP levels were measured in patients treated with pegylated-interferon and ribavirin.

Results In patients treated with IFN-based therapy, M2BPGi levels were elevated at the end of treatment but decreased afterwards. In contrast, M2BPGi levels in patients treated with IFN-free therapy decreased immediately after starting the treatment without transient elevation. Though pre-treatment M2BPGi levels significantly correlated with fibrosis in both patients with a sustained virological response (SVR) and non-SVR, post-treatment M2BPGi levels decreased regardless of the degree of fibrosis in patients with SVR. In multivariate analysis, non-SVR and HCC development were independent factors associated with M2BPGi level ≥ 2.2 . In patients treated with pegylated-interferon and ribavirin, total M2BP levels were positively correlated with fibrosis and HCC development.

Conclusion Real-time monitoring of the serum M2BPGi level after antiviral therapy for CHC patients could be a helpful screening tool for assessing the risk of HCC. M2BP and its glycan structure could be associated together with hepatocarcinogenesis.

Keywords Chronic hepatitis C · Mac-2-binding protein glycosylation isomer · Liver fibrosis · Hepatocellular carcinoma

Electronic supplementary material The online version of this article (doi:10.1007/s12072-016-9754-1) contains supplementary material, which is available to authorized users.

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Abbreviations

HCV	Hepatitis C virus
WFA	<i>Wisteria floribunda agglutinin</i>
M2BP	Mac-2-binding protein
M2BPGi	Mac-2-binding protein glycosylation isomer
HCC	Hepatocellular carcinoma
CHC	Chronic hepatitis C
PEG-IFN	Pegylated-interferon
RBV	Ribavirin
SVR	Sustained virological response
COI	Cut off index
DAA	Direct-acting antiviral agent
ALT	Alanine aminotransferase
AST	Aspartate aminotransferase
AFP	α -Fetoprotein
US	Ultrasonography
CT	Computed tomography
MRI	Magnetic resonance imaging
PC	Positive control
NC	Negative control
PPV	Positive predictive value
NPV	Negative predictive value
SMV	Simeprevir
TVR	Telaprevir
ASV	Asunaprevir
DCV	Daclatasvir

Introduction

Hepatocellular carcinoma (HCC) is the third most frequent cause of cancer-related death in the world [1]. Hepatitis C virus (HCV) is a major cause of HCC, with 170 million people presently infected [2]. Although the efficacy of antiviral therapy has dramatically improved with direct-acting antiviral agents (DAAs), HCC continues to develop in patients, even in those with a sustained virological response (SVR), particularly in those with advanced liver fibrosis or severe steatosis or those from an older age group [3, 4]. Therefore, an assessment of liver fibrosis and/or the risk of HCC development are essential for the management of patients with chronic hepatitis C (CHC), not only before treatment but also after achieving SVR.

Recently, *Wisteria floribunda agglutinin* positive (WFA⁺) Mac-2-binding protein (M2BP), so called M2BP glycosylation isomer (M2BPGi) was originally discovered to be a novel serum glyco-marker for liver fibrosis in glycoproteomic biomarker screening studies [5, 6]. Recent cross-sectional studies have reported that the serum level of M2BPGi at one time point is associated not only with a progression in liver fibrosis during CHC infection, but also with hepatocarcinogenesis [7–10]. However, the glycan structure may change during the clinical course of CHC

including during anti-HCV therapy, and serial changes in M2BPGi levels during the course of antiviral treatment, especially after achieving SVR are unclear. Moreover it is also unknown which of the mechanisms are more important for liver fibrosis and HCC development, increase of M2BP itself or changes of its glycan structures.

In this report, we analyzed serial changes in serum M2BPGi and total M2BP levels during an anti-HCV treatment period in order to evaluate their clinical significance and usefulness for the assessment of liver fibrosis and HCC risk.

Patients and methods

Patients

One hundred nineteen patients with HCV who were treated at Tokyo Medical and Dental University Hospital or affiliated hospitals participating in the Ochanomizu-Liver Conference Study group were included in the present study. All patients had no history of HCC and other chronic liver diseases. Before starting antiviral treatment, patient characteristics, biochemical data, hematological data, virological data and treatment details were collected. A fibrosis 4 (Fib-4) index and aspartate aminotransferase (AST)-to-platelet ratio index (APRI) were also calculated as reported before [11, 12].

Histological evaluation

Liver biopsy was done before the initiation of therapy for 108 (90.8 %) of the 119 analyzed subjects. To obtain liver specimens, ultrasound-guided liver biopsies were performed using 18 gauge needles. The stage of fibrosis and the grade of inflammatory activity were scored by two pathologists according to the classification of Desmet et al. [13].

Anti-HCV therapy and definitions of responses to therapy

Fifty-five patients were treated by combination therapy with PEG-IFN- α 2b or PEG-IFN- α 2a and RBV. The standard duration of combination therapy was 24 weeks for genotype 2 and 48 weeks for genotype 1. Forty-two patients received 12 weeks of triple therapy that included SMV or TVR, PEG-IFN and RBV followed by 12 weeks of dual therapy that included PEG-IFN and RBV. Twenty-two patients received 24 weeks of IFN-free therapy with ASV and DCV. SVR was defined as serum HCV RNA undetectable 24 weeks after the cessation of treatment.

Measurement of M2BPGi

Serum M2BPGi levels were measured prior to, at the end of, and at 24 weeks after the completion of antiviral treatment. M2BPGi quantification was performed based on a lectin-antibody sandwich immunoassay using a fully automatic HISCL-2000i immunoanalyzer (Sysmex Co., Hyogo, Japan) [5]. Measured values of M2BP conjugated to WFA were indexed, with values calculated using the following equation:

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

where the $[\text{WFA}^+\text{M2BP}]_{\text{sample}}$ represents the $\text{WFA}^+\text{M2BP}$ count of the serum sample (PC is the positive control, NC is the negative control). The positive control was supplied as a calibration solution standardized to yield a COI value of 1.0 [6].

Measurement of serum total M2BP level

Serum total M2BP levels were measured prior to, at the end of, and at 24 weeks after the completion of treatment in 55 patients treated with PEG-IFN and RBV. M2BP concentration was determined by using an ELISA kit (Immuno-Biological Laboratory, Gunma, Japan, code# 27362) according to manufacturer's instruction.

Follow-up and diagnosis of HCC

All patients were followed up for between 3 and 12 months by the measurement of blood counts and liver biochemistry. Diagnostic imaging, either by US, CT, or MRI was performed at an interval of every 3–12 months. A diagnosis of HCC was made based on positive results of typical vascular patterns as revealed by either contrast-enhanced CT, contrast-enhanced MRI or angiography. The mean follow-up period was 7.1 years (range 0.83–11.4 years).

Statistical analysis

An SPSS software package (version 20; SPSS, Chicago, IL, USA) and graph pad prism (version 5.0; Graph Pad Software Inc., San Diego, CA, USA) were used for statistical analysis. Discrete variables were evaluated by Fisher's exact probability test, and distributions of continuous variables were analyzed by a Mann–Whitney *U* test.

All *p* values were calculated by two-tailed tests, and those less than 0.05 were considered statistically significant.

Results

Chronological changes in M2BPGi according to antiviral therapy

The clinical characteristics of the 119 patients prior to treatment are summarized in Table 1.

M2BPGi values were significantly elevated at the end of treatment and decreased after the completion of treatment in patients treated with IFN-based therapy (Fig. 1, $p < 0.0001$). On the other hand, M2BPGi levels uniformly decreased after starting IFN-free treatment (Fig. 1, $p = 0.003$). There were no differences between values of those infected with HCV genotype 1 and 2 in serum M2BPGi measured at pretreatment ($p = 0.366$), end of treatment ($p = 0.831$), and 24 weeks after treatment completion ($p = 0.093$), regardless of the standard duration of combination therapy.

Chronological changes in M2BPGi according to virological response

Among 64 patients with SVR, M2BPGi values were significantly decreased after the completion of treatment compared with those at start of treatment (Fig. 2, $p < 0.0001$), even with progression of liver fibrosis (Supplementary Fig. 1a, $p = 0.045$). In 12 patients without SVR, there were no significant differences of serum M2BPGi values between before and after treatment (Fig. 2, $p = 0.24$), regardless of the stage of fibrosis (Supplementary Fig. 1b).

Serum M2BPGi values according to HCC development

During follow-up, HCC developed in 8 of 119 patients [median period after completion of treatment 7.1 years (range 0.83–11.4 years)]. The clinical details of them are summarized in Supplementary Table 1. Serum M2BPGi levels before treatment in patients with HCC were significantly higher than those in patients without HCC (Fig. 3a, $p = 0.033$); this difference was more marked after treatment (Fig. 3a, $p = 0.0004$). And even in patients with SVR, serum M2BPGi levels after treatment in patients with HCC were also significantly higher than those in patients without HCC (Supplementary Fig. 2, $p = 0.0007$). In patients without HCC, M2BPGi values were significantly decreased after the completion of treatment (Supplementary Fig. 3a, $p < 0.0001$), whereas in patients with HCC there were no differences between before and after treatment (Supplementary Fig. 3a, $p = 0.70$). Though post-treatment AFP levels were also positively correlated with development of

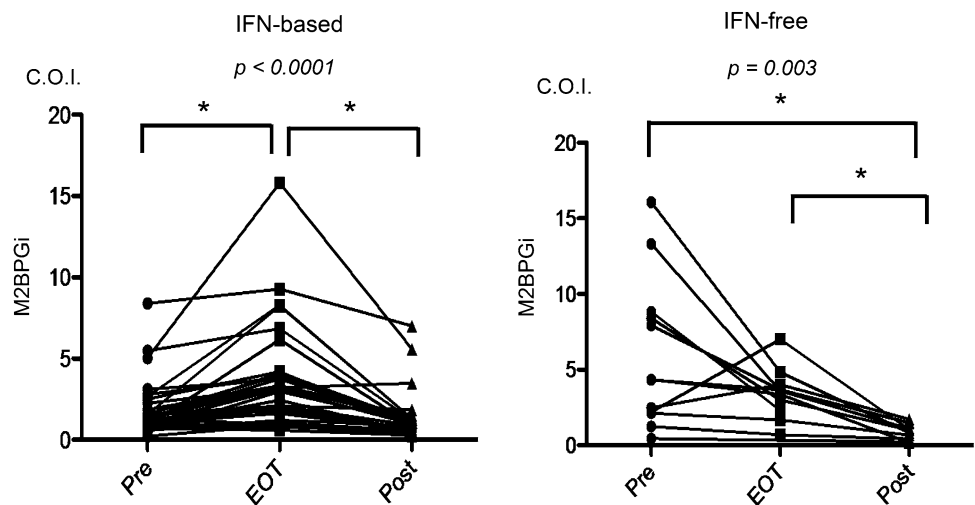
Table 1 Baseline characteristics of patients

Characteristic	IFN-based	IFN-free
Total number	97	22
Genotype (1b/2a/2b/unknown)	69/18/9/1	22/0/0/0
<i>IL28B</i> SNPs (rs8099917)		
TT/non TT/unknown	43/17/37	8/11/3
Age (years) ^a	60 (21–78)	71 (52–82)
<65/65–69/>70	65/16/16	8/4/10
Gender (male/female) (%male)	52/45 (54 %)	18/14 (56 %)
Body mass index (kg/m ²) ^a	22.6 (16.0–33.7)	22.8 (16.3–32.5)
Previous interferon therapy (no/yes/unknown)	51/34/12	8/14/0
Liver histology		
Grade of inflammation		
A0/1/2/3/unknown	0/41/46/3/7	0/3/11/1/7
Stage of fibrosis		
F0/1/2/3–4/unknown	9/43/21/19/5	4/6/4/2/6
Type of core 70 variants		
Wild/mutant/unknown	39/21/37	6/11/5
Type of ISDR (aa. 2209–2248) mutations		
0–1/≥2/unknown	31/16/50	10/6/7
SVR/non SVR (%SVR)	71/26 (74 %)	16/6 (73 %)

SNPs single nucleotide polymorphisms, ISDR interferon sensitivity determining region, SVR sustained virological response

^a Data are shown as median (range) values

Fig. 1 Chronological change in M2BPGi according to antiviral therapy. The solid line indicates serial change in M2BPGi level of each patient. “Pre” denotes pre-treatment, “EOT” denotes at the end of treatment, “Post” denotes 24 weeks after treatment, “COI” denotes cutoff index



HCC (supplementary Fig. 4, $p = 0.0023$), there were no correlations with HCC analyzed only in patients with SVR ($p = 0.27$).

Predictive potential of M2BPGi levels for advanced fibrosis and HCC development

To investigate the predictive potential of serum M2BPGi levels for advanced liver fibrosis and HCC development,

receiver operating characteristic (ROC) analyses were performed both before and after treatment. For predicting advanced fibrosis (F3–4), the area under the ROC curve (AUROC) for pre-treatment M2BPGi levels was greater compared with that in post-treatment M2BPGi levels (Fig. 4a, AUROC = 0.848 vs. 0.784). The predictive nature of pre-treatment levels of M2BPGi compared with post-treatment levels [positive predictive value (PPV) = 0.688 vs. 0.462] for advanced fibrosis was

Fig. 2 Chronological change in M2BPGi according to virological response. The *solid line* indicates serial change in M2BPGi level of each patient. “Pre” denotes pre-treatment, “Post” denotes 24 weeks after treatment, “COI” denotes cutoff index

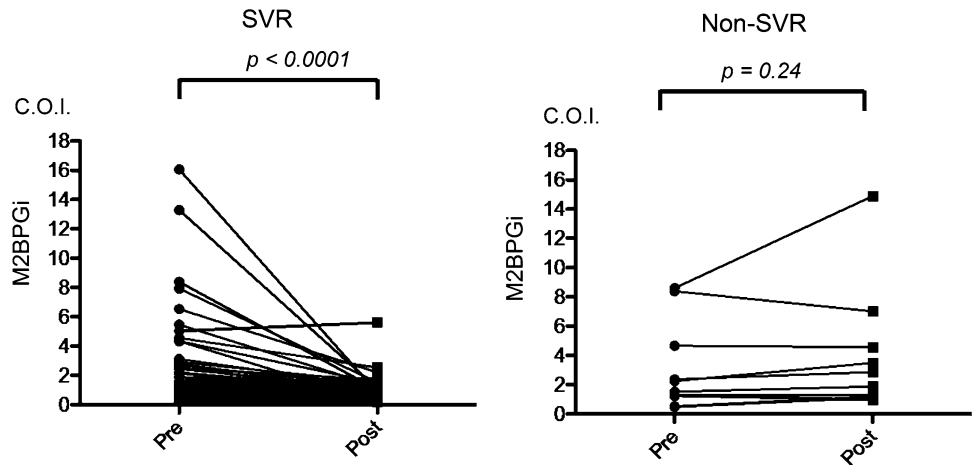
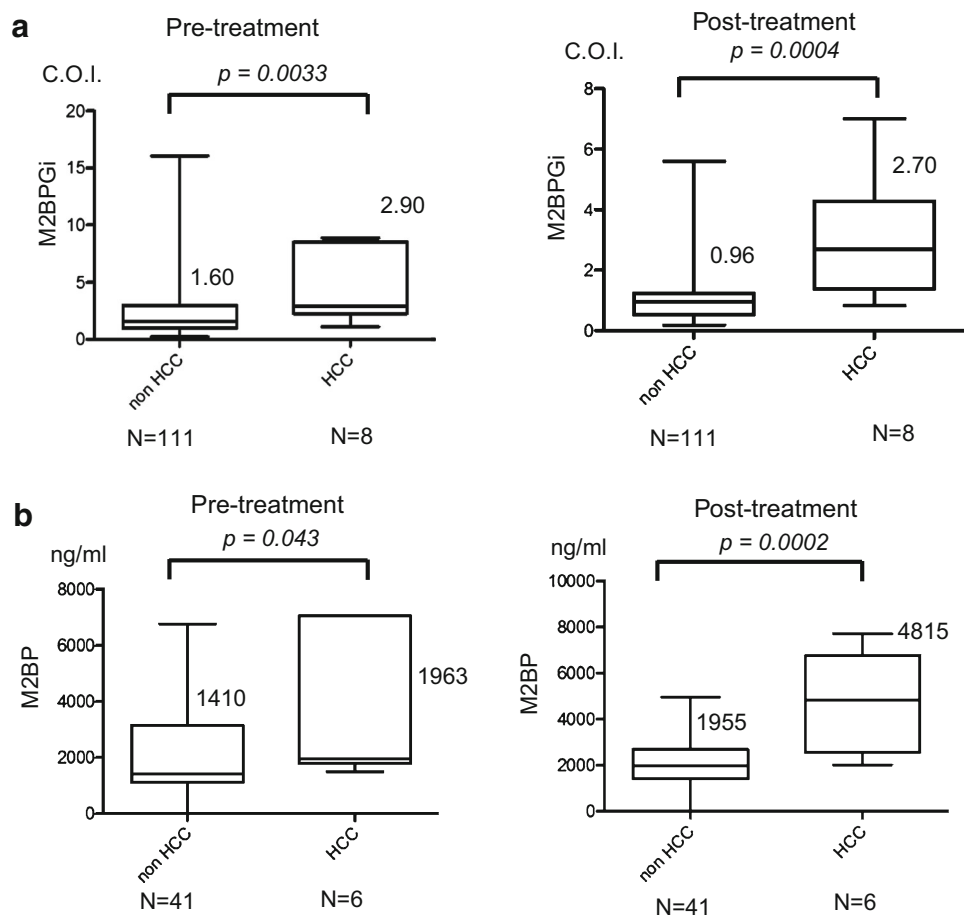


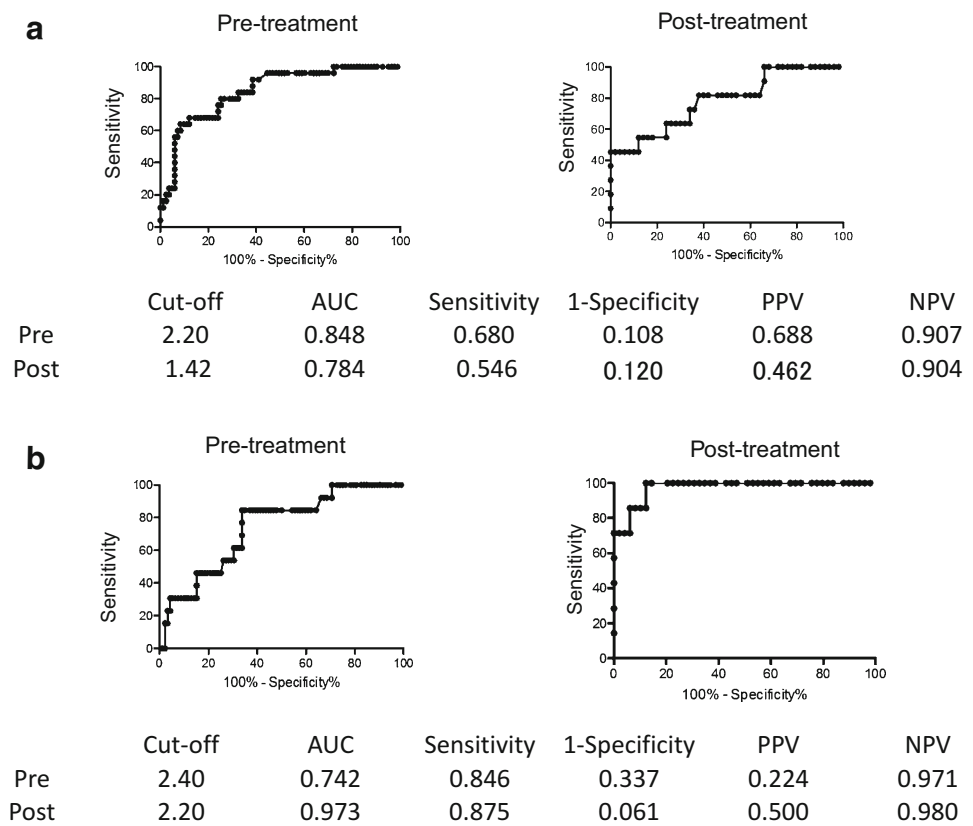
Fig. 3 M2BPGi and M2BP levels according to HCC development. M2BPGi (a) and M2BP (b) levels before and 24 weeks after treatment in patients without (non-HCC) and with HCC. The *top* and *bottom* of each *box* represent the first and third quartiles. The length of each *box* represents interquartile ranges within which are located 50 % of values. The *line through the middle* of each *box* represents the median. The *error bars* are 5–95 percentile. “COI” denotes cutoff index



superior. For predicting advanced fibrosis, pre-treatment M2BPGi levels yielded the highest AUROC with a level of 0.848 followed by hyaluronic acid (0.789), type 4 collagen (0.805), APRI (0.757), and Fib4 index (0.810) (Supplementary Fig. 5a). The predictive ability of M2BPGi levels for the future development of HCC was superior for post-treatment levels compared with pre-treatment levels

(Fig. 4b, AUROC = 0.973 vs. 0.742, PPV = 0.500 vs. 0.224). Both cut-off values for pre-treatment M2BPGi levels predicting advanced fibrosis and post-treatment M2BPGi levels predicting HCC development were 2.2 COI. Post-treatment M2BPGi value was also a useful predictor limited in patients with SVR (Supplementary Fig. 6). For predicting development of HCC, post-

Fig. 4 Predictive potential of M2BPGi for advanced fibrosis (F3 or F4) and HCC development (ROC analysis). Receiver operating characteristic (ROC) curves and areas under the ROC curves (AUROC) for serum M2BPGi levels in the diagnosis of F3 or F4 stage of liver fibrosis (a) and for the prediction of HCC development (b) before treatment and 24 weeks after treatment. “AUC” denotes area under the ROC curve. “PPV” denotes positive predictive value. “NPV” denotes negative predictive value



treatment M2BPGi levels yielded the highest AUROC with a level of 0.973 followed by APRI (0.743), Fib4 index (0.777) and AFP (0.752) (Supplementary Fig. 7). In multivariate analyses, the progression of liver fibrosis, pre-treatment APRI and HCC development were independent factors that were positively correlated with pre-treatment M2BPGi ≥ 2.2 COI (Table 2). On the other hand, failure of viral eradication and HCC development were identified as factors positively correlated with post-treatment M2BPGi ≥ 2.2 COI (Table 3).

Subgroup analysis of total M2BP level in patients treated with PEG-IFN and RBV

M2BP level was significantly positively correlated with M2BPGi values prior to ($r^2 = 0.229, p = 0.0004$), at the end of ($r^2 = 0.389, p < 0.0001$), and at 24 weeks after treatment completion ($r^2 = 0.361, p < 0.0001$). Serum M2BP level had significant correlation with fibrosis ($p = 0.01, r^2 = 0.20$), whereas they had no significant correlation with activity index ($p = 0.72, r^2 = 0.01$). Chronological changes in M2BP levels had no identical tendency after PEG-IFN and RBV therapy ($p = 0.09$). Moreover, there were no differences of M2BP levels between pre- and post-treatment regardless of the SVR status (Supplementary Fig. 8, $p = 0.41$ and $p = 0.080$).

Both pre- and post-treatment M2BP levels in patients with HCC were significantly higher than those in patients without HCC (Fig. 3b, $p = 0.043$ and $p = 0.0002$).

Discussion

Previous cross-sectional studies have shown M2BPGi to be a useful marker for liver fibrosis [5, 6, 14–16] and treatment outcome for triple therapy including SMV or TVR [17]. In the present study, we analyzed serial M2BPGi levels prior to, at the end of, and 24 weeks after treatment among patients treated with IFN-based and IFN-free therapy. M2BPGi levels were transiently elevated at the end of treatment, but decreased afterwards among patients treated with IFN-based therapy, while M2BPGi levels uniformly decreased after starting IFN-free treatment. In the subgroup analysis, M2BP levels in patients with HCC were significantly higher than those in patients without HCC, which suggested that M2BP and its glycan structure could be associated with hepatocarcinogenesis. On this basis, our study is the first to demonstrate a strict association between serial M2BPGi levels and HCC development after antiviral therapy both among IFN-based and IFN-free treatment.

Although pre-treatment M2BPGi values could distinguish the progression of fibrosis (Supplementary Fig. 9a),

Table 2 Factors associated with pre-treatment M2BPGi levels

Factor	M2BPGi levels		Univariate analysis <i>p</i> value	Multivariate analysis <i>p</i> value
	<2.2 (<i>n</i> = 69)	≥2.2 (<i>n</i> = 50)		
Age (years) ^a	60 (21–76)	65 (22–82)	0.599	
Gender (male/female)	36/33	24/26	0.653	
Stage of fibrosis (F0–2/3–4) (%F3–4)	64/5 (7 %)	22/28 (56 %)	<0.001	<0.001
Pre-treatment platelet count ($\times 10^{-3}/\mu\text{L}$) ^b	178 \pm 47	130 \pm 56	0.108	
Post-treatment platelet count ($\times 10^{-3}/\mu\text{L}$) ^b	183 \pm 53	143 \pm 49	0.347	
Pre-treatment ALT (U/mL) ^b	37 \pm 48	62 \pm 83	0.494	
Post-treatment ALT (U/mL) ^b	14 \pm 10	20 \pm 20	0.439	
Pre-treatment AFP (ng/mL) ^b	4.0 \pm 5.8	10.0 \pm 10.2	0.126	
Post-treatment AFP (ng/mL) ^b	2.8 \pm 5.2	3.9 \pm 10.7	0.212	
Pre-treatment Fib4 index ^b	2.1 \pm 9.6	4.5 \pm 3.3	0.101	
Post-treatment Fib4 index ^b	1.6 \pm 9.8	2.8 \pm 1.9	0.303	
Pre-treatment APRI ^b	0.6 \pm 5.4	1.4 \pm 13.7	0.005	<0.001
Post-treatment APRI ^b	0.3 \pm 16	0.5 \pm 8.0	0.599	
SVR/non SVR (%SVR)	54/15 (78 %)	35/15 (59 %)	0.062	
HCC yes/no (%HCC)	2/67 (3 %)	7/43 (14 %)	0.004	0.010

ALT alanine transaminase, AFP alphafetoprotein, APRI aspartate aminotransferase (AST)-to-platelet ratio index, SVR sustained virological response, HCC hepatocellular carcinoma

^a Data are show as median (range) values

^b Data are expressed as mean \pm SD

Table 3 Factors associated with post-treatment M2BPGi levels

Factor	<2.2 (<i>n</i> = 69)	≥2.2 (<i>n</i> = 50)	Univariate analysis <i>p</i> value	Multivariate analysis <i>p</i> value
	Age (years) ^a	61 (24–79)		
Gender (male/female)	30/30	1/6	0.144	
Stage of fibrosis (F0–2/3–4) (%F3–4)	6/54 (10 %)	2/5 (71 %)	0.001	0.075
Pre-treatment platelet count ($\times 10^{-3}/\mu\text{L}$) ^b	171 \pm 50	135 \pm 30	0.615	
Post-treatment platelet count ($\times 10^{-3}/\mu\text{L}$) ^b	14 \pm 7.3	13 \pm 43	0.287	
Pre-treatment ALT (U/mL) ^b	51 \pm 67	92 \pm 48	0.511	
Post-treatment ALT (U/mL) ^b	14 \pm 7.3	13 \pm 43	0.428	
Pre-treatment AFP (ng/mL) ^b	4.4 \pm 97.4	12.9 \pm 2.1	0.120	
Post-treatment AFP (ng/mL) ^b	3.1 \pm 2.0	7.3 \pm 2.3	0.125	
Pre-treatment Fib4 index ^b	2.3 \pm 2.6	3.5 \pm 3.0	0.727	
Post-treatment Fib4 index ^b	2.0 \pm 0.93	3.8 \pm 1.9	0.041	0.667
Pre-treatment APRI ^b	0.5 \pm 4.8	1.2 \pm 2.0	0.058	
Post-treatment APRI ^b	0.3 \pm 18	0.5 \pm 7.9	0.020	0.575
SVR/non SVR (%SVR)	54/6 (90 %)	3/4 (43 %)	0.002	0.019
HCC yes/no (%HCC)	3/57(5 %)	5/2 (71 %)	<0.001	0.038

ALT alanine transaminase, AFP alphafetoprotein, APRI aspartate aminotransferase (AST)-to-platelet ratio index, SVR sustained virological response, HCC hepatocellular carcinoma

^a Data are show as median (range) values

^b Data are expressed as mean \pm SD

post-treatment levels in SVR patients no longer differentiated fibrosis stages as shown in Fig. 2. Though APRI and Fib-4 index also significantly decreased in patients with

SVR (Supplementary Fig. 10a, b), such an improvement in liver fibrosis after 24-week-post-treatment is unlikely even in SVR patients. It would be reflected in the reduction of

inflammation (Supplementary Fig. 10c, d). Therefore the predictive potential of M2BPGi levels for fibrosis stage at a higher precision may require the presence of HCV. Previous cross-sectional studies for evaluation of M2BPGi levels in patients with chronic hepatitis B or non-alcoholic steatohepatitis, which demonstrated less predictive characteristics with lower cut-off levels for advanced liver fibrosis [18], support this hypothesis.

We found that M2BPGi levels at the end of treatment were uniformly elevated by IFN-based therapy, and decreased after completion of treatment (Fig. 1). These dynamic changes were similar to those of IFN-stimulated genes. On the other hand, M2BPGi levels uniformly decreased after starting IFN-free treatment with asunaprevir and daclatasvir (Fig. 1). Therefore, it is suggested that glycan structures of M2BP reflect not only the stage of liver fibrosis, but also the immune response induced by IFN or other cytokines in the presence of HCV. In contrast, M2BPGi levels after treatment more efficiently distinguished between patients showing HCC development and those without.

In multivariate analyses, pre-treatment M2BPGi levels were affected by APRI, stage of fibrosis, and HCC development, whereas post-treatment M2BPGi levels were affected by SVR and HCC development. Therefore, it is suggested that M2BPGi levels after treatment more directly reflect a potential for hepatocarcinogenesis, probably due to a decrease in the influence of factors, such as the immune response to HCV infection.

M2BP has been shown to be enhanced by IFN stimulation in an HCV replicon system [19] and to increase in breast, prostate, and lung cancers in humans [20–22] and HCC in a rat model [23]. More recently, it was reported that M2BP increased with the progression of liver fibrosis in CHC patients [24]. However, the biological mechanisms responsible for glycan alteration of M2BP in all points for immune response to HCV, liver fibrosis and hepatocarcinogenesis are still unknown. In our subgroup analysis treated with PEG-IFN and RBV, serum M2BP levels were significantly positively correlated with progression of fibrosis and HCC development. It was suggested that both the increase of M2BP production and the alteration of glycan structure of M2BP occurred by progression of fibrosis or development of HCC.

This study has a few limitations, the first being the retrospective nature and small number of patients with HCC studied. Because the incidence of HCC development after achieving SVR was low, longer-term longitudinal studies in larger cohorts are required to verify the generality of our results. A second limitation is the absence of post-treatment liver biopsies. However, a post-treatment liver biopsy from an ethical viewpoint is impossible, especially in patients showing SVR, while a remarkable

improvement in liver fibrosis immediately after achieving SVR is unlikely. Our results, which demonstrate the usefulness of post-treatment M2BPGi levels for predicting the future development of HCC in patients with SVR, were not affected by the absence of post-treatment liver biopsies.

In conclusion, post-treatment monitoring of serum M2BPGi has the potential to predict future HCC development in patients undergoing anti-HCV therapy; thus, constructing a post-treatment-surveillance system for HCC using this glyco-marker would be helpful in this regard.

Acknowledgements This study is based on the participation of six multicenter hospitals (Tokyo Medical and Dental University Hospital, Kashiwa City Hospital, Showa General Hospital, Toride Kyodo General Hospital, Mishima General Hospital).

Compliance with ethical standards

Conflict of interest Dr. Asahina and Dr. Kakinuma belong to donation-funded department funded by Chugai Pharmaceutical Co. Ltd., Toray Industries Inc., Bristol-Myers Squibb, Dainippon Sumitomo Pharma Co. Ltd., and Merck Sharp & Dohme.

Financial support This study was supported by grants for research received from Sysmex Corporation, grants from the Ministry of Education, Culture, Sports, Science and Technology-Japan, the Japan Society for the Promotion of Science, Japan Agency for Medical Research and Development, the Japan Health Sciences Foundation, the Miyakawa Memorial Research Foundation, and the National Institute of Biomedical Innovation.

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed consent Written informed consent was obtained from all patients. The Ethical Committee of Tokyo Medical and Dental University approved this study, which was conducted in accordance with the Declaration of Helsinki (Confirmation No. 1863).

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