pathology of NASH, such as the severities of steatosis, inflammation, hepatocellular disorders, and fibrosis. To the best of our knowledge, this is the first report on the association of the liver tissue fatty acid composition ratio with the severities of liver tissue inflammation and hepatocellular disorders in NASH. The fatty acid content of liver tissue was expected to increase in patients with advanced hepatic steatosis; however, significant changes in the fatty acid composition ratios suggested that not all fatty acids homogenously increase. Of the changes in fatty acid composition ratios observed in the SS and NASH groups, a decrease in the C18:0/C16:0 ratio and an increase in the C18:1n9/18:0 ratio (i.e. relative increases in C16:0 and C18:1n9) were associated with steatosis and insulin resistance, and an increase in the C16:1n7/16:0 ratio (i.e. a relative increase in C16:1n7) was associated with liver tissue inflammation and hepatocellular disorders. These results revealed that fatty acid components change depending on pathological differences in liver tissue in NAFLD patients.

There are two main pathways of fatty acid accumulation in the liver. The close involvement of insulin resistance in both pathways has been clarified (20, 21). The hydrolysis of fat tissue occurs in the presence of insulin resistance and increases free fatty acid inflow into the liver in one pathway. In the other, related genes, such as the SREBP-1c gene and downstream SCD1 and FAS genes, are activated in the liver in the presence of high

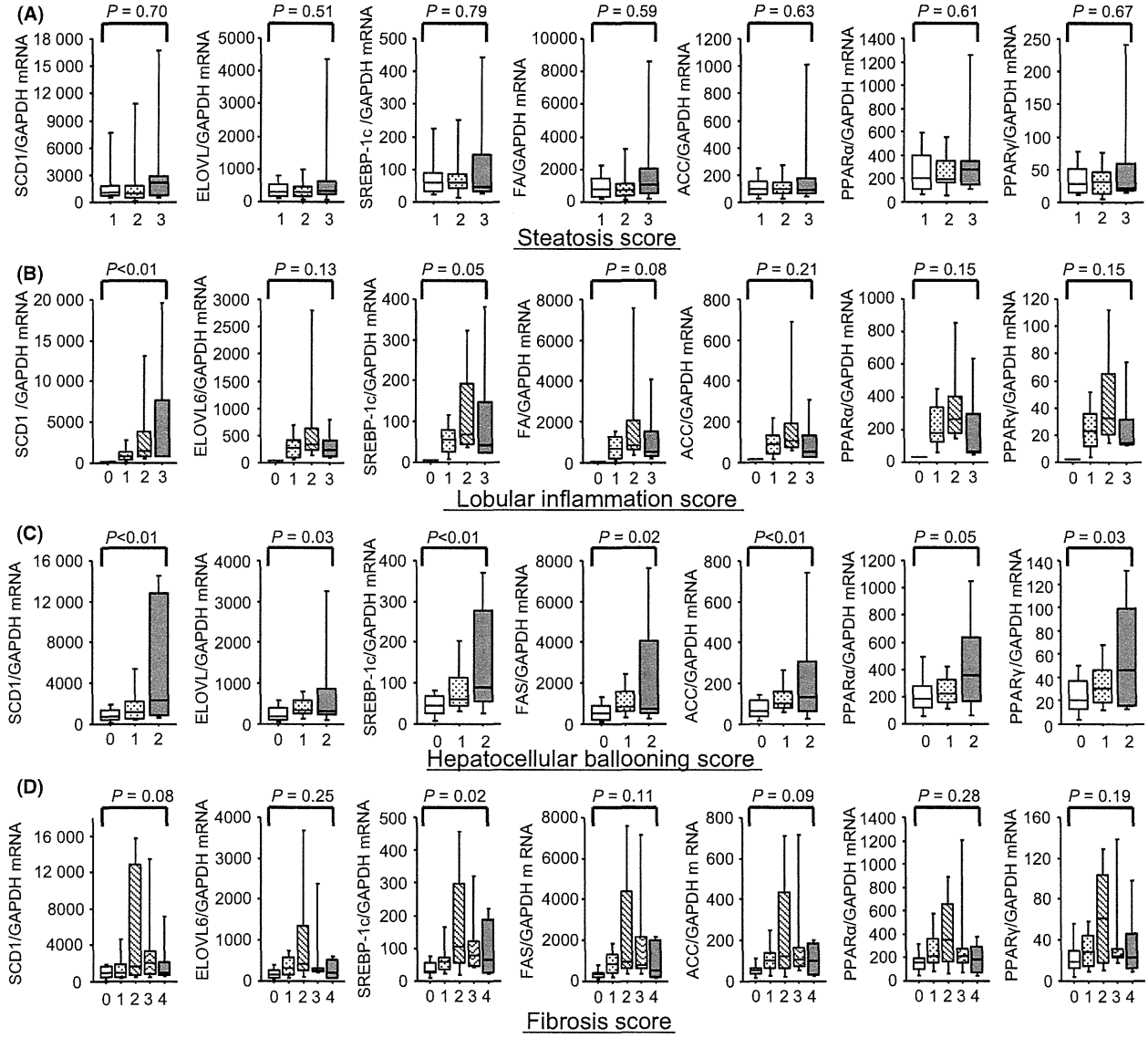

Fig. 4. Relationship between the histopathological findings of the liver and the expression levels of fatty acid metabolism-related genes. The association of the progression of the following items and expression of the fatty acid metabolism-related genes measured in liver tissue using RT-PCR was investigated: (A) steatosis score, (B) lobular inflammation score, (C) hepatocellular ballooning score and (D) fibrosis.

Table 4. Multivariate correlation between histological scores, insulin resistance and genes adjusted for age, gender and BMI

	Steatosis score			Inflammation score			Ballooning score			Fibrosis score		
	Coefficient	UA *P*-value	MA *P*-value	Coefficient	UA *P*-value	MA *P*-value	Coefficient	UA *P*-value	MA *P*-value	Coefficient	UA *P*-value	MA *P*-value
C18:0/C16:0	−0.610	<0.0001	0.0092	−0.315	0.0022	0.4248	−0.310	0.0016	0.3965	−0.289	0.0040	–
C16:1n7/C16:0	0.084	0.4071	–	0.339	0.0010	0.0191	0.255	0.0106	0.2753	0.141	0.1695	–
C18:1n9/C18:0	0.575	<0.0001	0.2387	0.224	0.0302	0.5603	0.243	0.0140	0.5163	0.184	0.0689	–
HOMA-IR	0.070	0.5211	–	0.137	0.2268	–	0.112	0.3083	–	−0.007	0.9485	–
QUICK I	−0.282	0.0108	0.1421	−0.183	0.1180	–	−0.271	0.0163	0.0200	−0.123	0.2901	–
SCD1	0.093	0.4725	–	0.266	0.0386	0.1785	0.321	0.0077	0.2904	0.067	0.5904	–
ELOVL6	0.16	0.1941	–	0.161	0.2177	–	0.283	0.0201	0.8737	0.037	0.7673	–
SREBP-1c	0.104	0.4349	–	0.249	0.0591	–	0.336	0.0064	0.0559	0.118	0.3534	–
FAS	0.148	0.2543	–	0.195	0.1340	–	0.320	0.0083	0.3309	0.085	0.4949	–
ACC	0.142	0.2902	–	0.159	0.2380	–	0.254	0.0441	0.1917	0.040	0.7539	–
PPARα	0.131	0.3222	–	0.170	0.2005	–	0.232	0.0637	–	0.028	0.8227	–
PPARγ	0.136	0.3243	–	0.155	0.2631	–	0.215	0.1003	–	0.030	0.8195	–

MA, multivariate analysis; PPARα, peroxisome proliferator-activated receptor-α; UA, univariate analysis.

blood insulin and glucose levels (22) and promote glucose uptake in the liver, enhancing the *de novo* synthesis of C16:0 through acetyl-CoA.

C16:0 is considered to be a toxic fatty acid for liver tissue. TGs in the liver and microsomal saturated fatty acids increased in mice fed a saturated fatty acid-enriched diet, and elevations in the activity of liver caspase-3 and transaminase levels were confirmed (23). Saturated fatty acids, such as C16:0, are not readily esterified and exhibit strong cytotoxicity in the liver (24). It is assumed that toxicity is avoided by the conversion of these saturated fatty acids to unsaturated fatty acids, such as C16:1n7 and C18:1n9, through elongation by ELOVL6 and desaturation by SCD1. As both ELOVL6 and SCD1 were controlled by SREBP-1c, their expressions are related to each other.

It has been previously reported that the expression of these genes was associated with the pathology of NASH in an animal model (25). Matsuzaka et al. have also shown that the expression level of ELOVL6 in the liver was correlated with the inflammation of liver tissue in a mouse model with NASH and was also increased in NASH patients (26). These results are consistent with our results. In this study, we evaluated the relationship between fatty acid metabolism and NASH pathology by the simultaneous examination of the fatty acid composition ratio around C16:0, fatty acid metabolic gene expression and histopathology of the liver in the same liver samples of many patients. The analysis of age-, sex- and BMI-adjusted associations between the histological scores of the liver and experimental parameters showed that a decrease in the C18:0/C16:0 ratio, an increase in the C16:1n7/16:0 ratio, and an increase in the expression of fatty acid metabolism-related genes including SCD1 and ELOVL6 correlated with inflammation or ballooning of liver tissue. Taking our results together with previous reports, fatty acid metabolism in the liver according to the development of NASH can be explained as follows.

First, a decrease in the C18:0/C16:0 ratio is because of an increase in C16:0 without an increase in the fatty acid metabolism-related genes. Next, an increase in the expression of the fatty acid metabolism-related genes including SCD1 and ELOVL6 occurs and correlates with inflammation and the ballooning of hepatocytes in liver tissue. Finally, it becomes difficult to sufficiently convert C16:0 to C18:0 by ELOVL6, and a compensatory increase in the conversion of C16:0 to C16:1n7 controlled by SCD1 occurs. Consequently, the increase in C16:1n7/C16:0 correlates with inflammation in liver tissue with the highest correlation coefficient. Therefore, our results suggest that the acceleration of overall hepatic fatty acid metabolism is more important for the pathogenesis of NASH than the expression levels of ELOVL6 in patients with NASH.

In conclusion, analysis of the liver tissue fatty acid composition and gene expression showed that an enhancement of the fatty acid metabolic pathway centring on C16:0 contributed to the progression of SS to NASH. Elucidating these changes in the metabolic pathway may lead to the development of a drug that could prevent the progression to NASH.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Table S1. Differences of fatty acid composition rates in liver tissue among male, premenopausal female, postmenopausal female.

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ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

Non-invasive prediction of hepatocellular carcinoma development using serum fibrosis marker in chronic hepatitis C patients

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Abstract
Background The FIB-4 index is a simple formula to predict liver fibrosis. This study aimed to evaluate the utility of the FIB-4 index and associated time-course changes as a predictor of hepatocellular carcinoma (HCC) development.
Methods A total of 171 chronic hepatitis C patients who underwent paired liver biopsies and 875 patients who underwent a single liver biopsy (validation group) were investigated during mean follow-up periods of 6.4 and 5.9 years, respectively. All patients had received interferon therapy and had not achieved a sustained virological response. Factors associated with HCC development were analyzed in these patients.
Results HCC developed in 30 patients in the paired biopsy group and 89 patients in the validation group. Univariate analysis demonstrated that the FIB-4 index >3.25 and change in the FIB-4 index per year (ΔFIB-4/year) ≥0.3 were predictive factors for HCC development in both groups. Multivariate analysis in the combined population revealed that these two factors were independent. The hazard ratio (HR) for the FIB-4 index >3.25 was 2.7 ($p < 0.001$) and ΔFIB-4/year ≥0.3 was 1.8 ($p = 0.003$). Patients with a FIB-4 index >3.25 and a ΔFIB-4/year ≥0.3 were defined as high risk, and those with a FIB-4 index ≤3.25 and a ΔFIB-4/year <0.3 were defined as low risk. The HR of HCC development in patients at high risk was 7.3 (95 % confidence interval 4.3–12.5, $p < 0.001$).
Conclusions It was possible to define a group at high risk of developing HCC by intermittently measuring the FIB-4 index and considering time-course changes in this index.

Keywords FIB-4 index · Hepatocellular carcinoma · Chronic hepatitis C · Liver fibrosis · Non-invasive

Introduction

Persistent hepatitis C virus infection induces chronic hepatitis and eventually develops into liver cirrhosis and hepatocellular carcinoma (HCC) [1]. An advanced stage of liver fibrosis in chronic hepatitis C is associated with HCC development and complications such as esophageal variceal bleeding and liver failure [2, 3]. Therefore, accurate evaluation of the stage of liver fibrosis is necessary to predict its progression to liver cirrhosis and HCC development for optimal clinical disease management.

Although the gold standard for evaluating liver fibrosis is liver biopsy [4, 5], it has been reported that this method may be inaccurate because of sampling errors and inter-observer variations [6, 7]. Moreover, because the invasiveness of liver biopsy precludes repeated examinations [8], evaluation of liver fibrosis time-course changes is difficult.

Recently, various non-invasive methods for evaluating liver fibrosis have rapidly improved as alternatives to liver biopsy. Liver fibrosis was reportedly predicted by transient elastography [9, 10], acoustic radiation force impulse imaging [11], and real-time tissue elastography [12]. In

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addition, methods using blood test data, including the aspartate aminotransferase (AST)/alanine aminotransferase (ALT) ratio [13], AST/platelet ratio index [14], the Forns test [15], and the Fibro test [16] have been reported to be useful. These tests have exhibited high accuracy in predicting severe liver fibrosis.

The FIB-4 index is a simple formula used for predicting liver fibrosis based on the standard biochemical values (AST, ALT and platelet count) and age, and is reported to be significantly useful for predicting advanced liver fibrosis [17–19]. Because the FIB-4 index can be repeatedly calculated using age and general biochemistry results, it offers the advantage of easy follow-up of time-course changes with repeated measurements. We have reported that time-course changes in the FIB-4 index correlate with changes in liver fibrosis, and advancement of liver fibrosis can be predicted by changes in the FIB-4 index [20].

Progression of liver fibrosis in chronic hepatitis C is closely associated with the high risk of developing HCC [2, 3]. In addition, we have reported that the risk of developing HCC increases with aging [21]. Because the FIB-4 index correlates with liver fibrosis and considers age, it is possible that it can also be used to predict the risk of developing HCC. In this study we investigated the significance of the FIB-4 index and time-course changes in the FIB-4 index as predictors of HCC development.

Methods

Paired biopsy group

Study subjects comprised 314 chronic hepatitis C patients who underwent liver biopsies twice between 1991 and 2010 at Musashino Red Cross Hospital. The average interval between two biopsies was 4.9 ± 2.9 years. The subject characteristics were detailed previously [20]. All patients were treated by interferon after the first liver biopsy and had non-sustained virological response. They underwent the second biopsy and were treated again by interferon. After excluding 110 patients who achieved a sustained virological response with the second interferon therapy, 171 patients were followed-up ≥1 year and were included in this analysis. Exclusion criteria comprised the follows: (1) co-infection with hepatitis B virus or human immunodeficiency virus, (2) alcohol abuse, (3) the presence of nonalcoholic steatohepatitis, (4) the presence of HCC at entry, (5) interval between paired biopsies <1.5 years, and (6) length of biopsy sample <15 mm. The relationship between HCC development and the FIB-4 index at liver biopsy or change in the FIB-4 index between the two liver biopsies was investigated. To determine the optimal cut-off values of change in the FIB-4 index for prediction of HCC development, patients with HCC development within 10 years were considered. Time zero was set at the date of the second biopsy.

Single liver biopsy group (validation group)

A total of 1,377 patients received interferon therapy after liver biopsy at Musashino Red Cross Hospital between 1991 and 2010 and were followed-up for ≥1 year after treatment. Of those in follow-up, 875 patients who exhibited non-sustained virological response were included in the validation group. Exclusion criteria were the same as those for the paired biopsy group. Because these patients did not undergo a second liver biopsy, change in the FIB-4 index was calculated between the liver biopsy and 1, 2 and 3 years after the end of interferon therapy. The relationship between HCC development and the FIB-4 index at liver biopsy or change in the FIB-4 index was investigated. Time zero was set at the date of liver biopsy.

Ethical approval

Written informed consent was obtained from each patient in the paired biopsy group and in the validation group, and the study protocol conformed to the ethical guidelines of the Declaration of Helsinki and was approved by the institutional ethics review committees.

Histological evaluation

Liver biopsy specimens were obtained using 13G needles laparoscopically, or by percutaneous ultrasound-guided liver biopsy using 15G needles. Specimens were fixed, paraffin-embedded, and stained with hematoxylin–eosin and Masson's trichrome. A minimum 15 mm biopsy sample was required for diagnosis. All liver biopsy samples were independently evaluated by two senior pathologists who were blinded to the clinical data. Fibrosis staging was categorized according to the METAVIR score: F0, no fibrosis; F1, portal fibrosis without septa; F2, portal fibrosis with few septa; F3, numerous septa without cirrhosis; and F4, cirrhosis [22]. When staging was inconsistent between the two pathologists, an appropriate stage was determined by discussion between the two. Fibrosis progression was defined as a 1 point or more increase in the METAVIR score, and fibrosis non-progression was defined as no change or a 1 point or more decrease in the METAVIR score.

HCC surveillance and diagnosis

Ultrasonography and a blood test including tumor markers were performed every 3–6 months for HCC surveillance. When tumor marker levels showed an abnormal rise and/or

abdominal ultrasonography suggesting a lesion suspicious for HCC, contrast-enhanced computed tomography, magnetic resonance imaging or angiography were performed. HCC was diagnosed for tumors showing vascular enhancement at an early phase with washout at a later phase. Tumor biopsy was used to diagnose tumors with non-typical imaging findings.

Clinical and biological data

The age and gender of the patients were recorded. Serum samples were collected within 1 month prior to the liver biopsy. The following variables were obtained by analyzing the serum samples: AST, ALT, and platelet count. The FIB-4 index was calculated according to the following formula: FIB-4 index = age [years] × AST [IU/L]/(platelets [10^9/L] × ALT [IU/L]$^{1/2}$). Cutoff value of the FIB-4 index was set at 3.25 according to the previously established value for the prediction of advanced fibrosis [17]. Change in the FIB-4 index per year (ΔFIB-4 index/year) in the paired biopsy group was calculated by the following formula: ΔFIB-4 index/year = (the FIB-4 index at the second liver biopsy − the FIB-4 index at the first liver biopsy)/the interval between paired biopsies (years). ΔFIB-4 index/year in the validation group was calculated similarly between the liver biopsy and 1, 2, and 3 years after the end of interferon therapy. Change in AST, ALT, platelets per year (ΔAST/year, ΔALT/year, ΔPlatelets/year) were calculated similarly.

Statistical analysis

Categorical data were compared using the Chi-square and Fisher's exact test. Distributions of continuous variables were analyzed using the Student's t test or the Mann–Whitney U test. A p value of <0.05 was considered statistically significant. The cumulative incidence curve was determined by the Kaplan–Meier method and differences among groups were assessed using a log-rank test. Receiver operating characteristic (ROC) curves were constructed, and the area under the ROC curve (AUROC) was calculated. Optimal cut-off values were selected using Youden's index. Factors associated with HCC risk were determined by the Cox proportional hazard model. Statistical analyses were performed using the Statistical Package for the Social Sciences software version 15.0 (SPSS Inc., Chicago, IL, USA)

Results

Patient characteristics

Table 1 shows the characteristics of patients in the paired biopsy group and validation group. There were no significant differences in the FIB-4 index between the two groups. Mean follow-up periods were 6.4 years in the paired biopsy group and 5.9 years in the validation group, respectively. HCC developed in 30 patients (14 %) in the

Table 1 Patient characteristics

	Paired biopsy group		Validation group	p value*	p value**
	First biopsy	Second biopsy			
Patients (n)	171		875		
Age (SD) (years)	56.1 (8.5)	60.8 (8.1)	58.0 (10.3)	0.02	<0.001
Gender [n (%)]					
Female	95 (56)		493 (56)		
Male	76 (44)		382 (44)	0.85	
Fibrosis stage [n (%)]					
F0–1	67 (39)	57 (33)	388 (44)		
F2	57 (34)	60 (35)	269 (31)		
F3	43 (25)	43 (25)	186 (21)		
F4	4 (2)	11 (7)	32 (4)	0.41	0.04
AST (SD) (IU/L)	68.3 (38.2)	60.3 (38.7)	62.9 (35.4)	0.08	0.51
ALT (SD) (IU/L)	90.8 (63.2)	68.3 (54.0)	77.9 (52.7)	0.008	0.06
Platelets (SD) (10^9/L)	159 (48)	153 (51)	157 (50)	0.71	0.28
FIB-4 index	2.90 (1.6)	3.38 (1.9)	3.20 (2.1)	0.08	0.28
Interferon response (relapse/no response/ND)		71/100	335/366/174		0.15
HCC development [n (%)]		30 (14)	89 (10)		0.01
Follow-up period (SD) (years)		6.4 (2.7)	5.9 (2.8)		0.04

AST aspartate aminotransferase, *ALT* alanine aminotransferase, *ND* not determined

* Comparison between paired biopsy group at first biopsy and validation group

** Comparison between paired biopsy group at second biopsy and validation group

paired biopsy group and 89 patients (10 %) in the validation group during the follow-up.

Prediction of HCC development by a single-point assessment in the paired biopsy group

The incidence of HCC development was compared between patients with F0–2 and F3–4 at the second liver biopsy. The 3-year, 5-year, and 7-year cumulative incidence of HCC was 13.3, 26.6, and 39.4 %, respectively, in patients with F3–4, which was significantly higher than those with F0–2 (1.7, 4.9, and 7.3 %, respectively; $p < 0.001$, Fig. 1a). Similarly, using the FIB-4 index at the second biopsy, the 3-year, 5-year, and 7-year cumulative incidence of HCC after interferon therapy was 1.0, 5.5 and 6.9 %, respectively, in patients with a FIB-4 index ≤ 3.25, whereas it was 11.9, 20.9, and 32.0 %, respectively, in those with a FIB-4 index >3.25 ($p < 0.001$, Fig. 1b).

Prediction of HCC development by time-course changes in FIB-4 index in the paired biopsy group

HCC development was compared with time-course changes in the fibrosis stage from repeated liver biopsies. For this analysis, 4 patients who were diagnosed as having cirrhosis at the first liver biopsy were excluded. The cumulative incidence of HCC was not significantly different between patients with fibrosis progression and those without (Fig. 1c). In contrast, when time-course changes in the FIB-4 index (ΔFIB-4/year) were considered, HCC developed more frequently in patients with large time-course changes in the FIB-4 index. Of 30 patients with HCC development, 28 patients developed HCC within 10 years. The AUROC of the ΔFIB-4 index/year for prediction of HCC development within 10 years was 0.61. Using a cut-off value for a ΔFIB-4/year of 0.3, the sensitivity and specificity for the prediction of HCC

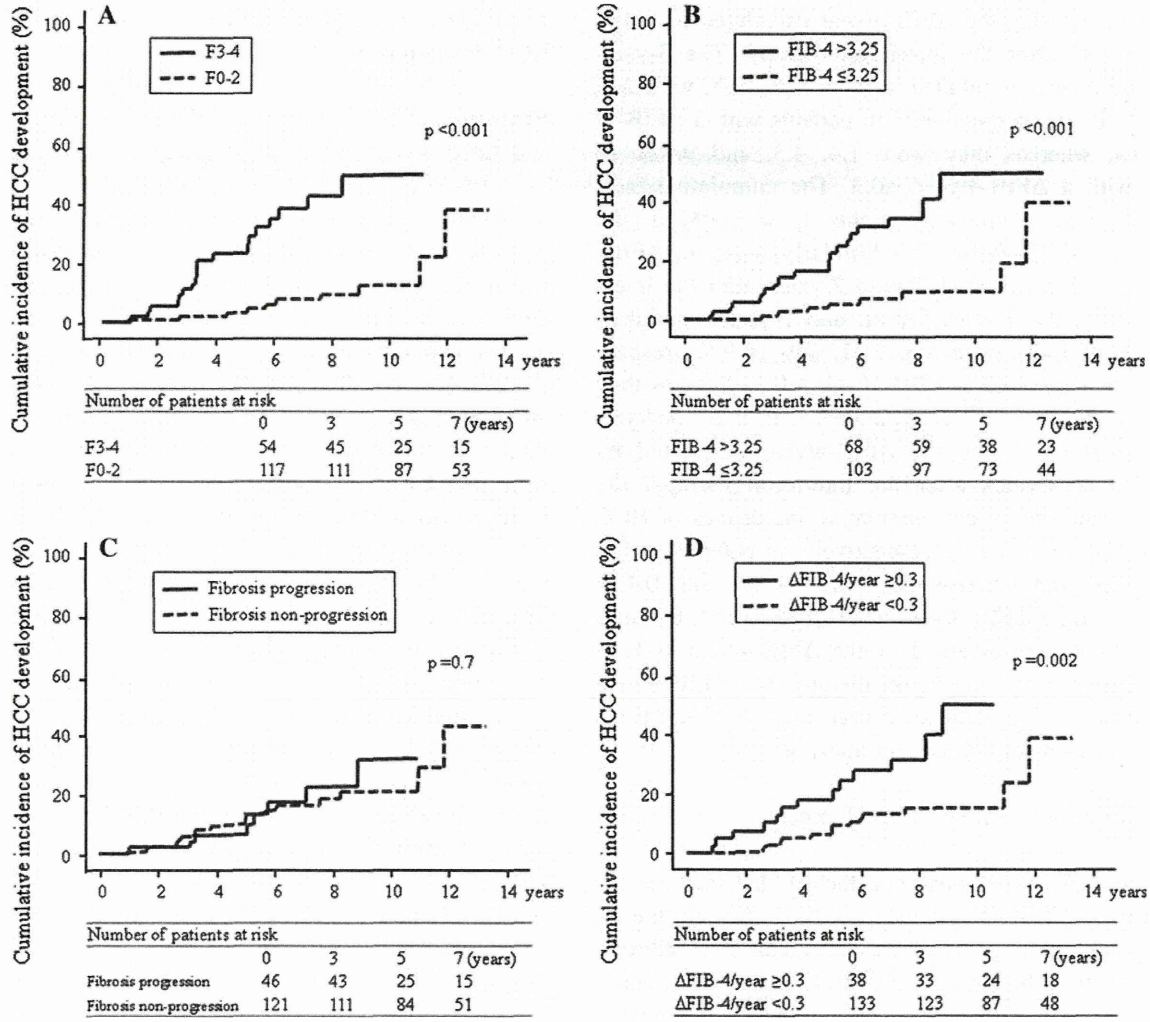

Fig. 1 Cumulative incidence of HCC development in the paired biopsy group. Patients were categorized into two groups according to **a** fibrosis stage, **b** FIB-4 index, **c** time-course change in fibrosis stage, and **d** time-course change in the FIB-4 index (ΔFIB-4/year)

development was 46 and 82 %, and the 3-year, 5-year and 7-year cumulative incidence of HCC was 13.2, 21.9, and 28.4 %, respectively, in patients with a ΔFIB-4/year ≥0.3, whereas it was 3.1, 9.6, and 13.1 % in those with a ΔFIB-4/year <0.3 ($p = 0.002$, Fig. 1d).

Validation by independent patients

The cumulative HCC incidence rate was similarly examined in the validation group using the FIB-4 index at the time of biopsy. In the group with a FIB-4 index >3.25, the 3-year, 5-year, and 7-year cumulative incidences of HCC were 3.9, 11.2, and 22.0 %, respectively, whereas, in the group with a FIB-4 index ≤3.25, the 3-year, 5-year, and 7-year cumulative incidences of HCC were 0.6, 4.0, and 6.4 %, respectively. The rate was significantly higher ($p < 0.001$) in the group with a FIB-4 index >3.25.

Time-course changes in the FIB-4 index and HCC incidence were examined with the cut-off value of a ΔFIB-4/year at 0.3. Cumulative incidence of HCC development was examined using the ΔFIB-4/year calculated by using data at 1 year after the interferon therapy. The 3-year, 5-year, and 7-year cumulative incidences of HCC were 2.8, 15.0, and 20.1 %, respectively, in patients with a ΔFIB-4/year ≥0.3, whereas they were 1.4, 4.3, and 9.8 % in patients with a ΔFIB-4/year <0.3. The cumulative incidence rate was significantly higher ($p = 0.008$) in the group with a ΔFIB-4/year ≥0.3. Similarly, using the ΔFIB-4/year calculated by using data at 2 years after the interferon therapy, the 3-year, 5-year, and 7-year cumulative incidences of HCC were 3.3, 11.1, and 16.9 %, respectively, in patients with a ΔFIB-4/year ≥0.3, whereas they were 1.4, 5.3, and 10.7 % in patients with a ΔFIB-4/year <0.3 ($p = 0.04$). Using the ΔFIB-4/year calculated by using data at 3 years after the interferon therapy, the 3-year, 5-year, and 7-year cumulative incidences of HCC were 4.4, 9.7, and 17.1 %, respectively, in patients with a ΔFIB-4/year ≥0.3, whereas they were 1.4, 5.0 and 10.4 % in patients with a ΔFIB-4/year <0.3 ($p = 0.005$). Because similar results were obtained by the ΔFIB-4/year at 1, 2, and 3 years after the interferon therapy, the ΔFIB-4/year calculated by using data at 1 year after the interferon therapy was used for subsequent analysis.

Factors associated with HCC development

Univariate analysis demonstrated factors that increase the hazard ratio (HR) for the development of HCC (Table 2). In the paired biopsy group for advanced liver fibrosis detected by liver biopsy, a high FIB-4 index level, and a ΔFIB-4/year ≥0.3 were risk factors for HCC development. Compared with patients with a FIB-4 index ≤3.25, the HR of those with a FIB-4 index >3.25 was 4.8 [95 % confidence interval (CI) 2.0–10.7, $p < 0.001$]. In terms of change in fibrosis stage, there was no significant difference between the progression and non-progression groups. In contrast, in terms of change in the FIB-4 index, compared with patients with a ΔFIB-4/year <0.3, the HR of those with a ΔFIB-4/year ≥0.3 was 3.1 (95 % CI 1.3–5.7, $p = 0.002$). Similar results were obtained in the validation group; a FIB-4 index >3.25 and a ΔFIB-4/year ≥0.3 were risk factors for HCC development (Table 2). These two groups of patients were combined and univariate and multivariate analysis were performed (Table 3). Because AST, ALT, platelets and age are contained in the FIB-4 index, these factors were excluded in the multivariate analysis. Multivariate analysis revealed that gender, fibrosis stage, the FIB-4 index and ΔFIB-4/year were independent factors associated with HCC development. The HR of HCC development with a FIB-4 index >3.25 and a ΔFIB-4/year ≥0.3 was 2.7 (95 % CI 1.7–4.2, $p < 0.001$) and 1.8 (95 % CI 1.2–2.6, $p = 0.003$), respectively. ΔAST/year, ΔALT/year, and ΔPlatelets/year were not associated with HCC development.

Evaluation of HCC risk by a combining the FIB-4 index and ΔFIB-4/year in the whole group

Multivariate analysis demonstrated that a high FIB-4 index level by single-point assessment and a time-course increase in the FIB-4 index were independent risk factors for HCC development. Their combined risk was examined in four groups, with the cut-off values of a FIB-4 index at 3.25 and a ΔFIB-4/year at 0.3. Patients with a FIB-4 index ≤3.25 and a ΔFIB-4/year <0.3 were defined as the low risk group. Patients with a FIB-4 index ≤3.25 and a ΔFIB-4/year ≥0.3 were defined as the intermediate risk-1 group. Similarly, patients with a FIB-4 index >3.25 and a ΔFIB-4/year ≥0.3 were defined as the high risk group. Patients with a FIB-4 index >3.25 and a ΔFIB-4/year <0.3 were defined as the intermediate risk-2 group. The 3-year, 5-year, and 7-year cumulative incidence of HCC in patients within the high risk group was 7.6, 21.0, and 30.0 %. Similarly, the 3-year, 5-year, and 7-year cumulative incidence of HCC was 4.1, 9.6, and 21.1 % in patients within the intermediate risk-2 group. It was 0.8, 10.1, and 13.8 % in the patients within the intermediate risk-1 group and 0.6, 2.9, and 4.8 % in patients within the low risk group ($p < 0.001$, Fig. 2). The HR of HCC development in patients at high risk was 7.3 (95 % CI 4.3–12.5, $p < 0.001$, Table 4). Sensitivity of prediction for HCC development by the liver biopsy and the FIB-4 index was 58 and 61 %, respectively. The combined risk classification by the FIB-4 index and the ΔFIB-4/year had higher sensitivity (72 %, Table 5).

Table 2 Factors associated with HCC development in the paired biopsy group and the validation group

Risk factor value	Paired biopsy group		Validation group	
	Hazard ratio (95 % CI)	*p* value	Hazard ratio (95 % CI)	*p* value
Risk factor at baseline[a]				
Age (by every 10 years)	1.3 (0.8–2.1)	0.3	1.9 (1.5–2.5)	<0.001
Gender				
Female	1		1	
Male	1.5 (0.7–3.2)	0.2	1.5 (0.9–2.2)	0.06
Fibrosis stage				
F0/F1/F2	1		1	
F3/F4	5.4 (2.5–11.3)	<0.001	4.7 (3.1–7.1)	<0.001
AST (by every 1× ULN)	1.3 (1.1–1.5)	0.01	1.4 (1.1–1.7)	<0.001
ALT (by every 1× ULN)	1.1 (1.0–1.3)	0.04	1.1 (0.9–1.2)	0.1
Platelets (10^9/L)				
≥150	1		1	
<150	3.0 (1.3–6.5)	0.006	2.6 (1.6–4.5)	<0.001
FIB-4 index				
≤3.25	1		1	
>3.25	4.8 (2.2–10.7)	<0.001	3.8 (2.4–5.8)	<0.001
Change of risk factor				
ΔAST/year (IU/L)				
<0	1		1	
≥0	0.9 (0.4–1.9)	0.8	1.4 (0.8–2.4)	0.2
ΔALT/year (IU/L)				
<0	1		1	
≥0	0.8 (0.4–1.8)	0.6	0.8 (0.4–1.6)	0.6
ΔPlatelets/year (10^9/L)				
>−0.5	1		1	
≤−0.5	2.4 (1.1–5.0)	0.01	0.7 (0.4–1.3)	0.3
ΔFIB-4/year				
<0.3	1		1	
≥0.3	3.1 (1.5–6.6)	0.002	1.8 (1.2–2.9)	0.008
Fibrosis stage change				
Non-progression	1			
Progression	1.2 (0.5–2.7)	0.7		

AST aspartate aminotransferase, *ALT* alanine aminotransferase

[a] Data at the second biopsy was used for the paired biopsy group

Discussion

Recently, non-invasive methods substituting liver biopsy for the diagnoses of liver fibrosis have been developed. It has been elucidated that non-invasive liver fibrosis markers are related to HCC development and mortality [23–25]. In addition, it was reported that after interferon therapy for chronic hepatitis C, some non-invasive liver fibrosis markers correlated with HCC development [26]. However, it remains unclear whether time-course changes in these markers correlate with HCC development and mortality.

Previously, we reported that time-course changes in the FIB-4 index correlated with liver fibrosis progression [20]. Because the FIB-4 index correlates with liver fibrosis, a risk factor for HCC development, and it considers age, another risk factor, it was presumed that the index could be closely correlated with HCC development. In this study, the significance of the FIB-4 index and time-course changes in the FIB-4 index were investigated in relation to HCC development.

The most important finding in this study was that it was possible to predict HCC development by time-course changes in the FIB-4 index. The cumulative HCC incidence rate was lower in patients with a ΔFIB-4/year <0.3 compared with those with a ΔFIB-4/year ≥0.3. It has been reported that a high level of AST and ALT levels correlate with progression of liver fibrosis, and improved levels prevent HCC development [27–29]. It is also known that liver fibrosis progression and the risk of HCC development is increased with a decrease in platelet count [30].

Table 3 Factors associated with HCC development in the combined population

Risk factor value	Univariate		Multivariate	
	Hazard ratio (95 % CI)	*p* value	Hazard ratio (95 % CI)	*p* value
Risk factor at baseline				
Age (by every 10 years)	1.8 (1.4–2.3)	<0.001		
Gender				
Female	1		1	
Male	1.5 (1.0–2.1)	0.03	2.0 (1.4–2.9)	<0.001
Fibrosis stage				
F0/F1/F2	1		1	
F3/F4	4.9 (3.5–7.2)	<0.001	3.0 (2.0–4.6)	<0.001
AST (by every 1× ULN)	1.4 (1.2–1.6)	<0.001		
ALT (by every 1× ULN)	1.1 (1.0–1.2)	0.04		
Platelets (10^9/L)				
≥150	1			
<150	2.7 (1.7–4.1)	<0.001		
FIB-4 index				
≤3.25	1		1	
>3.25	4.0 (2.8–5.9)	<0.001	2.7 (1.7–4.2)	<0.001
Change of risk factor				
ΔAST/year (IU/L)				
<0	1			
≥0	1.3 (0.8–2.1)	0.2		
ΔALT/year (IU/L)				
<0	1			
≥0	0.9 (0.6–1.6)	0.8		
ΔPlatelets/year (10^9/L)				
>−0.5	1			
≤−0.5	1.0 (0.6–1.6)	0.8		
ΔFIB-4/year				
<0.3	1		1	
≥0.3	2.1 (1.4–3.1)	<0.001	1.8 (1.2–2.6)	0.003

AST aspartate aminotransferase, *ALT* alanine aminotransferase

However, time-course changes of AST, ALT, and platelet count were not significantly associated with HCC development in this study. On the other hand, the FIB-4 index, which considers these factors together; had its time-course changes useful for real-time monitoring of disease progression. As the disease advances, the FIB-4 index deteriorates and the risk of HCC development increases.

One advantage of the FIB-4 index is the feasibility of repeated measurements for evaluating disease status. Needless to say, liver biopsy is the gold standard for diagnosis of liver fibrosis and is still important to predict the progression of liver disease. However, there are problems associated with liver biopsies including sampling errors and inter-observer variations [6, 7]. In addition, it is difficult to repeat biopsies, making it challenging to evaluate time-course changes because of the invasiveness of the procedure. In contrast, the FIB-4 index can be calculated using age and general biochemistry results and making it markedly easy to follow up time-course changes. In this study, changes in fibrosis stage between two liver biopsies failed to stratify HCC development. These results suggest that the FIB-4 index, rather than the liver biopsy, was more useful for real-time monitoring of disease advancement.

Correlations of non-invasive liver fibrosis markers including the FIB-4 index with HCC incidence risk have been reported previously [23–26, 31]. A similar result was shown in this study using a single-point assessment of the FIB-4 index. Since the FIB-4 index correlates with liver fibrosis, a high FIB-4 index indicates a high risk for HCC development similar to other liver fibrosis markers. Furthermore, an important fact in this study was that combining the FIB-4 index and time-course changes in the FIB-4 index could stratify patients with high risk of HCC development. A high FIB-4 index level by single-point assessment and a time-course increase in the FIB-4 index were independent risk factors for HCC development. Patients with a low baseline FIB-4 index and a time-course

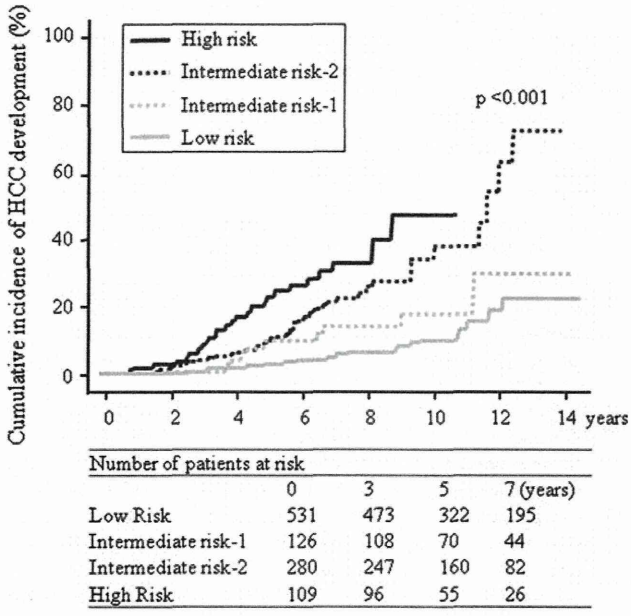

Fig. 2 Cumulative incidence of HCC development in the combined population. Patients were categorized into four groups using the FIB-4 index and time-course change in the FIB-4 index (ΔFIB-4/year). Low risk: FIB-4 index ≤3.25 and ΔFIB-4/year <0.3, intermediate risk-1: FIB-4 index ≤3.25 and ΔFIB-4/year ≥0.3, intermediate risk-2: FIB-4 index >3.25 and ΔFIB-4/year <0.3, high risk: FIB-4 index >3.25 and ΔFIB-4/year ≥0.3

Table 4 Evaluation of HCC risk by combining the FIB-4 and the ΔFIB-4/year

	Number of patients	Hazard ratio (95 % CI)	*p* value
Low risk	531	1	
Intermediate risk-1	126	2.1 (1.1–4.0)	0.03
Intermediate risk-2	280	4.1 (2.6–6.5)	<0.001
High risk	109	7.3 (4.3–12.5)	<0.001

Low risk: patients with a FIB-4 index ≤3.25 and a ΔFIB-4/year <0.3

Intermediate risk-1: patients with a FIB-4 index ≤3.25 and a ΔFIB-4/year ≥0.3

Intermediate risk-2: patients with a FIB-4 index >3.25 and a ΔFIB-4/year <0.3

High risk: patients with a FIB-4 index >3.25 and a ΔFIB-4/year ≥0.3

improvement in the FIB-4 index had a low risk of HCC development, whereas those with a high baseline FIB-4 index and worsening of the FIB-4 index had a markedly high risk for HCC development. In addition to the utility of predicting liver fibrosis and HCC development by single-point assessment, the combination with real-time monitoring enables stratification of a group with a high risk of HCC development, which is a great advantage of the FIB-4 index over a liver biopsy.

With regard to diagnosis capabilities for liver fibrosis, it has been reported that other non-invasive liver fibrosis markers have higher diagnostic capabilities than the FIB-4 index [32, 33]. However, the FIB-4 index has several advantages. Although, it has been reported that transient elastography has high diagnostic capabilities when it comes to liver fibrosis, measurements are sometimes impossible in patients with severe obesity [34]. Reproducibility of transient elastography was reportedly reduced in patients with steatosis, increased body mass index, and lower degrees of liver fibrosis [35]. Moreover, these modalities for measurement of elasticity of the liver using ultrasonography are not widely available, especially in countries where resources are limited. In contrast, the FIB-4 index can be determined by a general blood test, and it can be measured in almost all patients. The parameters required for calculation are only age, AST, ALT, and platelet count, which are measured during the routine examination of patients with liver disease. Therefore, additional blood collection is unnecessary, and the index can be calculated at no extra cost.

Table 5 Sensitivity of prediction for HCC development

	Patients with HCC development	Patients without HCC development
F0–2	50	724
F3–4	69	203
	Sensitivity: 58 %	Specificity: 78 %
FIB-4 index ≤3.25	42	615
FIB-4 index >3.25	77	312
	Sensitivity: 61 %	Specificity: 66 %
Low risk	29	502
Other risk	90	425
	Sensitivity: 72 %	Specificity: 54 %

Other risk: high risk, intermediate risk-2, and intermediate risk-1

In conclusion, it was possible to define a group with a high risk of HCC development by calculating the FIB-4 index and considering time-course changes in the FIB-4 index. Because measurement of the FIB-4 index is simple and easy to repeat, it is useful for non-invasive, real-time monitoring of HCC development.

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Conflict of interest The authors declare that they have no conflict of interest.

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Original Article

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

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

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

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

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

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

INTRODUCTION

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

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

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

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

METHODS

Patients

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

Histological evaluation

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

HCC surveillance and diagnosis

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

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

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

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

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

Clinical and biological data

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

Statistical analyses

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

RESULTS

Patient characteristics

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

Table 1 Patient characteristics

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

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

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

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

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

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

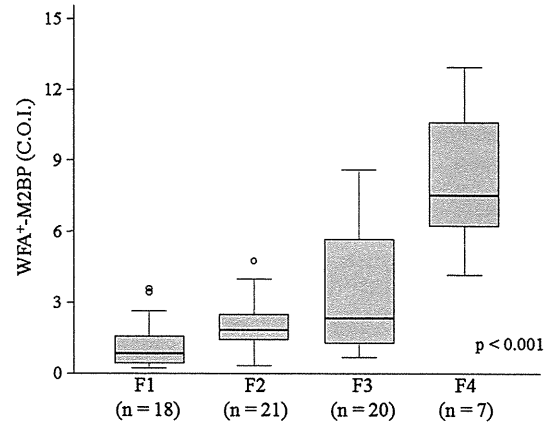


Figure 1 Correlation between WFA$^+$-M2BP and fibrosis stage. Box plot of WFA$^+$-M2BP is shown for each fibrosis stage. The box plot represents the 25th to 75th percentiles, giving the interquartile range. The line through the box indicates the median value, and an error bar indicates minimum and maximum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

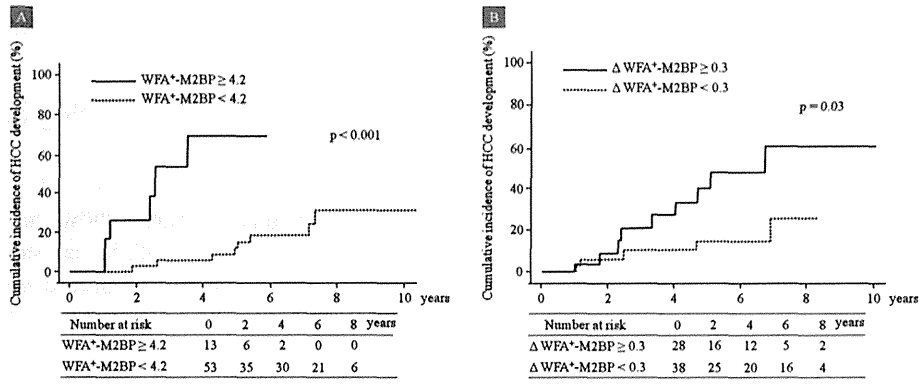


Figure 2 Cumulative incidence of hepatocellular carcinoma (HCC) development. Patients were categorized into two groups according to (a) WFA$^+$-M2BP and (b) time-course change in WFA$^+$-M2BP.

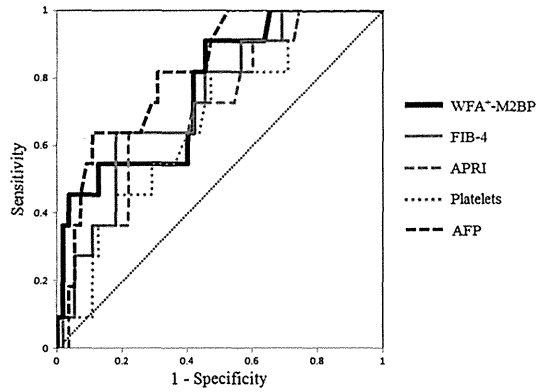


Figure 3 Receiver–operator curves of WFA+-M2BP, fibrosis markers and α-fetoprotein (AFP). APRI, aspartate aminotransferase-to-platelet ratio index.

Factors associated with HCC development

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

DISCUSSION

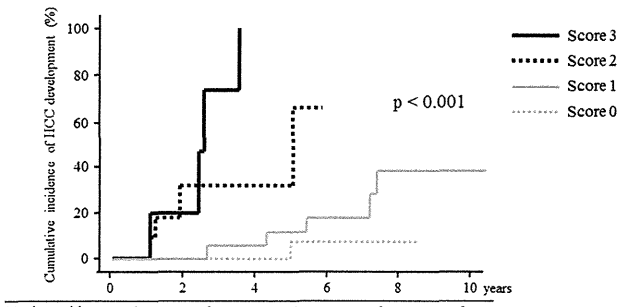
RECENTLY, SEVERAL NON-INVASIVE methods to evaluate liver fibrosis have been developed. The WFA$^+$-M2BP glycol marker test using sandwich immunoassay with WFA and anti-M2BP antibody has demonstrated utility as a liver fibrosis marker.[24,25] However, the relationship between WFA$^+$-M2BP and HCC development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

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

Table 2 Factors associated with HCC development

		Univariate		Multivariate	
		HR (95% CI)	*P*	HR (95% CI)	*P*
Age (every 10 years)		2.0 (0.8–5.0)	0.1		
Sex (male/female)	Male	1			
	Female	0.4 (0.1–1.2)	0.1		
AST (IU/L)	<40	1			
	≥40	2.5 (0.6–11)	0.2		
ALT (IU/L)	<40	1			
	≥40	1.3 (0.4–3.7)	0.6		
Bilirubin (mg/dL)		2.8 (0.3–24)	0.3		
Platelet counts ($\times 10^9$/L)	≥150	1			
	<150	2.4 (0.6–8.4)	0.2		
Fibrosis stage	F1/2	1		1	
	F3/4	3.9 (1.3–11)	0.01	1.8 (0.5–6.3)	0.3
AFP (ng/mL)	<10	1		1	
	≥10	5.8 (1.8–18)	0.003	4.7 (1.1–19)	0.03
WFA+-M2BP (COI)	<4.2	1		1	
	≥4.2	8.2 (2.6–26)	<0.001	4.1 (1.1–15)	0.04
ΔWFA+-M2BP/year	<0.3	1		1	
	≥0.3	3.1 (1.1–9.3)	0.04	5.5 (1.5–19)	0.008

AFP, α-fetoprotein; ALT, alanine aminotransferase; AST, aspartate aminotransferase; CI, confidence interval; COI, cut-off index; HCC, hepatocellular carcinoma; HR, hazard ratio.



Number at risk	0	2	4	6	8
Score 0	21	15	13	11	1
Score 1	28	19	16	10	5
Score 2	12	4	3	0	0
Score 3	5	3	0	0	0

Figure 4 Association between the risk score and cumulative incidence of hepatocellular carcinoma (HCC) development. WFA+-M2BP of 4.2 or more, ΔWFA+-M2BP/year of 0.3 or more and AFP of 10 ng/mL or more each contributed 1 point to the score. WFA+-M2BP of less than 4.2, ΔWFA+-M2BP/year of less than 0.3 and α-fetoprotein (AFP) of less than 10 ng/mL each contributed 0 points to the score. Patients were classified into four groups according to the total score of 0, 1, 2 or 3.

to be a significant predictive factor for HCC development. In a recent study, the significance of WFA+-M2BP for prediction of HCC was demonstrated in a large cohort study, further confirming the clinical impact of WFA+-M2BP.[28]

A new finding of our study was that time-course changes in WFA+-M2BP were associated with HCC development. An advantage of WFA+-M2BP testing over liver biopsy is that its non-invasiveness is suitable for repeated measurement. Liver biopsy is problematic to repeat to assess time-course changes because of its invasiveness.[29] WFA+-M2BP quantification can be used for real-time monitoring of liver disease, based on our finding that time-course changes were associated with HCC development. Furthermore, WFA+-M2BP and time-course changes in WFA+-M2BP were independent predictors of HCC development, and patients at high risk of HCC development could be identified using a combination of these factors. Therefore, single-point WFA+-M2BP assessment plus time-course changes in WFA+-M2BP are more useful to predict HCC development than a single-point liver biopsy.

WFA+-M2BP has some advantages over other serum fibrosis markers and elastography. Although APRI and FIB-4 serum fibrosis markers have demonstrated utility in predicting HCC development,[19–21] they are calculated using AST, ALT, platelet count and age. Hence, APRI and FIB-4 may not be appropriate in cases of advanced age, fatty liver or interferon therapy.[30] Furthermore, diagnostic accuracy of APRI and FIB-4 for HCC development was inferior to WFA+-M2BP in this study.

Liver elastography using ultrasonography has utility in predicting HCC development as well,[16] but these

modalities are not widely available, particularly in resource-constrained settings. Furthermore, measurements may be impossible in patients with severe obesity or ascites.[31] Reproducibility of transient elastography may be impaired in patients with steatosis, increased body mass index or less severe liver fibrosis.[32] In contrast, WFA$^+$-M2BP quantification requires a small blood sample and WFA$^+$-M2BP can be accurately measured without interference from the previously mentioned factors. WFA$^+$-M2BP quantification is entirely automated using the HISCL-2000i system and results can be acquired within 17 min. Because of these advantages, WFA$^+$-M2BP is more useful to predict liver fibrosis and HCC development than other serum fibrosis markers or elastography.

Our study was limited by the small number of patients and case–control pilot design. Patient characteristics between two groups were matched, but age, sex and fibrosis stage were biased nevertheless. A larger prospective study is needed to evaluate the utility of WFA$^+$-M2BP and time-course changes in WFA$^+$-M2BP as predictive factors of HCC development.

In conclusion, WFA$^+$-M2BP and time-course changes in WFA$^+$-M2BP were found to be independent predictive factors of HCC development, and patients at high risk of HCC development could be identified by combining these factors into a scoring system. Because WFA$^+$-M2BP quantification can be easily repeated, real-time monitoring of WFA$^+$-M2BP could be a novel predictor of HCC development.

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CONFLICT OF INTEREST

THE AUTHORS WHO have taken part in this study declare that they have no conflicts of interest to disclose.

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Risk Stratification of 7,732 Hepatectomy Cases in 2011 from the National Clinical Database for Japan

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BACKGROUND: There has been no report on risk stratification for hepatectomy using a nationwide surgical database in Japan. The objective of this study was to evaluate mortality and variables associated with surgical outcomes of hepatectomy at a national level.

STUDY DESIGN: We analyzed records of 7,732 patients who underwent hepatectomy for more than 1 segment (MOS) during 2011 in 987 different hospitals, as identified in the National Clinical Database (NCD) of Japan. The NCD captured 30-day morbidity and mortality as well as 90-day in-hospital mortality outcomes, which were submitted through a web-based data entry system. Based on 80% of the population, independent predictors for 30-day mortality and 90-day in-hospital mortality were calculated using a logistic regression model. The risk factors were validated with the remaining 20% of the cohort.

RESULTS: The median postoperative length of hospitalization was 16.0 days. The overall patient morbidity rate was 32.1%. Thirty-day mortality and 90-day in-hospital mortality rates were 2.0% and 4.0%, respectively. Totals of 14 and 23 risk factors were respectively identified for 30-day mortality and 90-day in-hospital mortality. Factors associated with risk for 90-day in-hospital mortality were preoperative condition and comorbidity, operative indication (emergency surgery, intrahepatic/perihilar cholangiocarcinoma, or gallbladder cancer), preoperative laboratory data, and extent and location of resected segments (segment 1, 7, or 8). As a performance metric, c-indices of 30-day mortality and 90-day in-hospital mortality were 0.714 and 0.761, respectively.

CONCLUSIONS: Here we report the first risk stratification analysis of hepatectomy using a Japanese nationwide surgical database. This system would predict surgical outcomes of hepatectomy and be useful to evaluate and benchmark performance. (J Am Coll Surg 2014;218:412–422. © 2014 by the American College of Surgeons)

The safety and efficacy of liver resection have improved dramatically in recent years, allowing broader indications for the procedure in both benign and malignant diseases.[1] Perioperative mortality rates in high volume cancer centers are reportedly 0% to 2%.[2-4] In contrast, population-based analyses using administrative data from Western countries have reported mortality rates of 5% to 10%,[4-7] indicating capacity for further improvement.

In 2006, the Japanese Society of Gastroenterological Surgery (JSGS) formed a committee to devise a database

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National Clinical Database (NCD) and the hospitals participating in NCD are the source of the data used herein and they have not verified and are not responsible for the statistical validity of the data analysis or the conclusions derived by the authors.

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