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Elevated Serum Levels of *Wisteria floribunda* Agglutinin-Positive Human Mac-2 Binding Protein Predict the Development of Hepatocellular Carcinoma in Hepatitis C Patients

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The *Wisteria floribunda* agglutinin-positive human Mac-2-binding protein (WFA^+-M2BP) was recently shown to be a liver fibrosis glycobiomarker with a unique fibrosis-related glycoalteration. We evaluated the ability of WFA^+-M2BP to predict the development of hepatocellular carcinoma (HCC) in patients who were infected with the hepatitis C virus (HCV). A total of 707 patients who had been admitted to our hospital with chronic HCV infection without other potential risk factors were evaluated to determine the ability of WFA^+-M2BP to predict the development of HCC; factors evaluated included age, sex, viral load, genotypes, fibrosis stage, aspartate and alanine aminotransferase levels, bilirubin, albumin, platelet count, alpha-fetoprotein (AFP), WFA^+-M2BP, and the response to interferon (IFN) therapy. Serum WFA^+-M2BP levels were significantly increased according to the progression of liver fibrosis stage ($P<0.001$). In each distinctive stage of fibrosis (F0-F1, F2, F3, and F4), the risk of development of HCC was increased according to the elevation of WFA^+-M2BP. Multivariate analysis identified age >57 years, F4, AFP >20 ng/mL, WFA^+-M2BP $\geq$4, and WFA^+-M2BP 1-4 as well as the response to IFN (no therapy vs. sustained virological response) as independent risk factors for the development of HCC. The time-dependent areas under the receiver operating characteristic curve demonstrated that the WFA^+-M2BP assay predicted the development of HCC with higher diagnostic accuracy than AFP. *Conclusion:* WFA^+-M2BP can be applied as a useful surrogate marker for the risk of HCC development, in addition to liver biopsy. (HEPATOLOGY 2014;60:1563-1570)

The annual incidence of hepatocellular carcinoma (HCC) in patients with hepatitis C virus (HCV)-related cirrhosis ranges from 1% to 7%.[1,2] Therefore, reliable methods for the early identification of liver fibrosis progression and compensated liver cirrhosis are an essential part of an efficient surveillance program for the detection of HCC.[3]

Until recently, liver biopsy was considered the gold standard for assessing the severity of liver fibrosis and cirrhosis.[4,5] Although liver biopsy is generally accepted

Abbreviations: Ab, antibody; AFP, alpha-fetoprotein; AIH, autoimmune hepatitis; ALT, alanine aminotransferase; ANOVA, analysis of variance; AST, aspartate aminotransferase; AUROC, area under the ROC; CT, computed tomography; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; IFN, interferon; MRI, magnetic resonance imaging; Peg-IFN, pegylated IFN; RBV, ribavirin; ROC, receiver operating characteristic; RT-PCR, reverse-transcriptase polymerase chain reaction; SVR, sustained virological response; US, ultrasound; WFA^+-M2BP, Wisteria floribunda *agglutinin-positive human Mac-2-binding protein.*

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to be a safe procedure, it can cause discomfort and carries a small risk of life-threatening complications.[6,7] Recently, an assay for *Wisteria floribunda* agglutinin-positive human Mac-2-binding protein (WFA^+-M2BP) was reported as a novel, noninvasive, and rapid bedside method to assess liver fibrosis.[8] M2BP has been shown to have multibranching and sialylated N-glycans. WFA is considered to recognize the GalNAc residue of N-glycans and O-glycans or the clustered LacNAc (Gal-GlcNAc) structure. Currently, we are analyzing the glycan structures of WFA^+-M2BP in detail using mass spectrometry–based technology.[9] Glycans can reflect the differentiation stage of cells, but not necessarily the level of cellular damage, and therefore they can be very effective markers for chronic disease. In the case of hepatitis, glycans are considered to reflect the progression of fibrosis more specifically than viral load. Several reports have identified M2BP as a potential marker of fibrosis progression in proteome study.[10-13] Kuno et al. were the first to report that a rapid, simple glycan-based immunoassay for WFA^+-M2BP can quantify fibrosis.[8,14]

On the other hand, we reported that alpha-fetoprotein (AFP) is a noninvasive predictive marker for the development of HCC in patients infected with HCV, which can be used to complement the information of fibrosis stage.[15]

In this report, we evaluated the utility of WFA^+-M2BP to predict the development of HCC in patients who were infected with HCV.

Patients and Methods

Patients. Between January 1992 and December 2003, 832 patients were determined to be positive for both anti-HCV by a second- or third-generation enzyme-linked immunoadsorbent assay and HCV RNA by polymerase chain reaction (PCR). They underwent liver biopsy guided by ultrasonography at the National Hospital Organization, Nagasaki Medical Center (Ōmura, Japan). Among them, 125 (15.0%) patients were excluded from enrollment in this retrospective analysis for the following reasons: (1) positivity for hepatitis B surface antigen (n = 12); (2) a heavy habitual drinking habit defined by an average daily consumption of >100 g of ethanol (n = 26); (3) autoimmune hepatitis (AIH), primary biliary cirrhosis, or idiopathic portal hypertension (n = 8); (4) positive antinuclear antibody (Ab; defined as titer >320×) without the diagnosis of AIH (n = 8); or (5) a short follow-up period <180 days (n = 71). The remaining 707 patients were analyzed retrospectively for the incidence of HCC.

For all patients in our cohort, a blood sample was taken on the day of the liver biopsy at our hospital. All samples were preceded to separate serum and stored at −20°C. At the time of blood withdrawal, all patients underwent liver biopsy. Their medical histories had been recorded, along with the results of routine tests for blood cell counts, liver biochemical parameters, and markers for HCV infection at the time of ultrasound (US)-guided liver biopsy and at regular intervals thereafter. Complete blood cell counts and biochemical tests were performed using automated procedures in the clinical pathological laboratories of our hospital.

Staging of Hepatic Fibrosis. Liver biopsies were taken by fine-needle aspiration (16G or 18G sonopsy) guided by US. Liver tissue specimens were fixed in 10% formalin, embedded in paraffin, and stained with hematoxylin and eosin. They were evaluated for the stage of hepatic fibrosis by a pathologist according to the criteria of Desmet et al.[16]

Measurement of WFA^+-M2BP. WFA^+-M2BP quantification was measured based on a lectin-Ab sandwich immunoassay using the fully automatic immunoanalyzer, HISCL-2000i (Sysmex Co., Hyogo, Japan).[8] The measured values of WFA^+-M2BP conjugated to WFA were indexed with the obtained values using the following equation:

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

where $[WFA^+\text{-M2BP}]_{sample}$ is the WFA^+-M2BP count of serum sample, PC is positive control, and NC is negative control. The positive control was supplied as

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a calibration solution preliminarily standardized to yield a COI value of 1.0.[14]

HCV RNA, HCV Core Antigen, and HCV Genotypes. HCV RNA was determined by reverse-transcriptase (RT)-PCR using a commercial kit (Amplicor HCV; Roche Diagnostic Systems, Basel, Switzerland). HCV core antigen was determined using the Lumispot Eiken HCV antigen assay (Eiken Chemicals, Tokyo, Japan). HCV core antigen levels were classified into low and high with a cutoff at 1,000 fmol/mL.[17] Genotypes of HCV were determined by RT-PCR with genotype-specific primers (HCV RNA core genotype; Roche Diagnostics, Tokyo, Japan).[18]

Interferon Therapy. During the observation period, 373 of the 707 (52.8%) patients received interferon (IFN) monotherapy, pegylated (Peg)-IFN monotherapy, or combination therapy with IFN plus ribavirin (RBV) or Peg-IFN plus RBV. Sustained virological response (SVR) was defined as the absence of detectable HCV RNA at the end of 6 months or more of treatment, whereas patients who failed to meet these criteria were judged as having non-SVR. There was no relapse of viremia after 6 months among the SVR patients.

Diagnosis of HCC. Patients were followed up by hematological and biochemical tests at an interval of 1-12 months. Diagnostic imaging by US, computed tomography (CT), and magnetic resonance imaging (MRI) were performed in most patients. HCC was diagnosed by typical vascular patterns on CT, MRI, and angiography or by fine-needle biopsy of space-occupying lesions detected in the liver.

Ethical Considerations. Informed consent was obtained from each patient included in the study, and the study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in the *a priori* approval by the institution's human research committee.

Statistical Analysis. Continuous variables (platelet counts, albumin, total bilirubin, aspartate aminotransferase [AST], alanine aminotransferase [ALT], AFP, HCV core antigen, and WFA^+-M2BP) were dichotomized with respect to the median value or clinically meaningful values in the multivariate analysis. To estimate the cumulative risk of developing HCC, Kaplan-Meier's method and the log-rank test were used. Cox's proportional hazards regression analysis was performed to evaluate risk factors for HCC. Regression analysis was performed to calculate Spearman's rank-correlation coefficient. Kruskal-Wallis' analysis of variance (ANOVA), followed by the Games-Howel's posthoc test, was used to assess whether there were any significant differences in terms of fibrosis stages (F0-F1, F2, F3, and F4). The diagnostic performances of WFA^+-M2BP and AFP for censored development of HCC were assessed by using time-dependent receiver operating characteristic (ROC) curves by examining the area under the ROC (AUROC).[19] Inclusion of variables was assessed using a step-wise selection method. Cochran-Armitage's test for trend was used in the categorical data analysis to assess for the presence of an association between a variable with two categories and a variable with more than three categories. A P value of 0.05 was considered statistically significant. Data analysis was performed with SPSS statistical software (version 22.0; (SPSS, Inc., Chicago, IL) and JMP 10 (SAS Institute Inc., Cary, NC).

Table 1. Demographic, Clinical, and Virological Characteristics of the 707 Patients Persistently Infected With HCV

Age, years	57.0 (19-79)
Male, N (%)	351 (49.6)
Observation period, years	8.2 ± 4.4*
IFN therapy	373 (52.8%)
Habitual alcohol intake	135 (19.1%)
Pathological findings	
Fibrosis (N) 0-1/2/3/4	274/193/120/120
Activity (N) 0-1/2/3	199/365/143
Platelet count, $\times 10^4/mm^3$	15.6 (3.0-39.1)
Albumin, g/dL	4.2 (2.7-5.3)
Bilirubin, mg/dL	0.7 (0.1-2.5)
AST, IU/mL	53 (11-422)
ALT, IU/mL	82 (1-1,057)
AFP, ng/mL	6 (0.7-510)
HCV core antigen ≥1,000 fmol/L (%)	539 (76.2)
HCV genotype, N (%) 1b	510 (72.1)
2a/2b	195 (27.6)
Unknown	2 (0.3)
WFA^+-M2BP	1.9 (0.2-19.2)

Values are the medians with ranges in parentheses.
*Results are expressed as the mean ± standard deviation.

Results

Characteristics at Enrollment. The baseline characteristics of the 707 patients at enrollment are summarized in Table 1. Median age was 57.0 years; 120 (17.0%) patients were diagnosed histologically with liver cirrhosis (fibrosis stage F4) and the remaining 587 had chronic hepatitis (fibrosis stage F0, F1, F2, or F3). The median value of AFP was 6 ng/mL. The median value of WFA^+-M2BP was 1.9 (range, 0.2-19.2). The average follow-up period was 8.2 years.

WFA^+-M2BP Value and Fibrosis Stage. The average values (mean ± 1 standard error) for each fibrosis stage were 1.3 ± 0.1 in F0-F1 (n = 274), 2.2 ± 0.1 in F2 (n = 193), 3.3 ± 0.2 in F3 (n = 120),

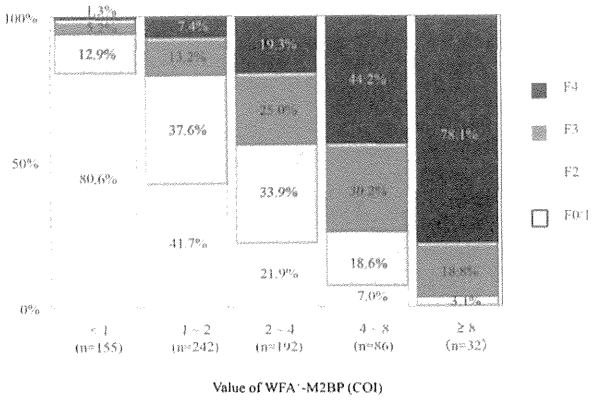

Fig. 1. Proportions of patients with different WFA$^+$-M2BP levels stratified by the fibrosis stage. The proportion of patients with F1 was diminished across increasing quintiles of WFA$^+$-M2BP level ($P < 0.0001$; Cochran-Armitage's trend test), whereas that with F4 was increased ($P < 0.0001$; Cochran-Armitage's trend test).

and 5.2 ± 0.3 in F4 (n = 120). The degree of fibrosis was positively correlated with the median value of WFA$^+$-M2BP ($P < 0.001$) by a nonparametric method (Kruskal-Wallis' one-way ANOVA). Games-Howel's test confirmed that the WFA$^+$-M2BP value increased significantly with increasing stage of liver fibrosis: $P < 0.0001$ (F0-F1, compared with F2, F3, and F4); $P < 0.0001$ (F2, compared with F3 and F4); and $P < 0.0001$ (F3, compared with F4).

We estimated the diagnostic accuracy of WFA$^+$-M2BP for detecting stage F3-F4 disease. The AUROC in the prediction of ≥F3 was 0.815 (range, 0.782-0.842). The desired specificity level of 95% was achieved for a 4.0 threshold, and the sensitivity was 40.0%.

We analyzed the proportions of the patients with different WFA$^+$-M2BP levels stratified by the fibrosis stage (Fig. 1). The proportion of patients with F1 was 125 cases (80.7%) in WFA$^+$-M2BP <1 (n = 155), 101 cases (41.7%) in WFA$^+$-M2BP ≤1 and <2 (n = 242), 42 cases (21.9%) in WFA$^+$-M2BP ≤2 and <4 (n = 192), 6 cases (7.0%) in WFA$^+$-M2BP ≤4 and <8 (n = 86), and 0 cases (0.0%) in WFA$^+$-M2BP ≥8 (n = 32). The proportion of patients with F1 was diminished across increasing quintiles of WFA$^+$-M2BP level ($P < 0.0001$; Cochran-Armitage's trend test). Conversely, the proportion of patients with F4 was 2 cases (1.3%) in WFA$^+$-M2BP <1 (n = 155), 18 cases (7.4%) in WFA$^+$-M2BP ≤1, and <2 (n = 242), 37 cases (19.3%) in WFA$^+$-M2BP ≤2 and <4 (n = 192), 38 cases (44.2%) in WFA$^+$-M2BP ≤4 and <8 (n = 86), and 25 cases (78.1%) in WFA$^+$-M2BP ≥8 (n = 32). The proportion of patients with F4 was increased with increasing quintiles of WFA$^+$-M2BP level ($P < 0.0001$; Cochran-Armitage's trend test).

Table 2. Step-wise Multiple Linear Regression Model to Identify Significant Independent Factors Affecting Serum WFA$^+$-M2BP Level

Final Fitted Model	Adjusted R^2	Standardized Coefficient β	*P* Value
Fibrosis stage		0.258	<0.001
AFP		0.187	<0.001
Albumin		−0.202	<0.001
AST (1: <53 IU/L; ≥2: 53 IU/L)		0.186	<0.001
Platelet	0.501	−0.147	<0.001
Sex (1: male; 2: female)		0.111	<0.001
HCV core antigen		−0.098	<0.001
Total bilirubin		0.091	0.001
Age		0.071	0.014

Relationship Between the WFA$^+$-M2BP Value and Baseline Biochemical Markers. To determine whether the WFA$^+$-M2BP value was associated with fibrosis stage, age, gender, platelet count, albumin, bilirubin, AST, ALT, AFP, HCV core antigen, HCV genotype, or histological grading, a step-wise multiple linear regression analysis was performed. Our results showed that independent variables, except for ALT, genotype, and histological grading, remained in the final equation (Table 2), suggesting that fibrosis stage was most closely associated with serum WFA$^+$-M2BP value (coefficient β, 0.258; $P < 0.001$).

Risk Factors for HCC. Cox's regression analysis was performed on several variables, including age, sex, alcohol consumption, IFN therapy during the observation period, biochemical and virological parameters, and serum WFA$^+$-M2BP level. The following factors were identified as posing an increased risk for HCC by the univariate analysis: age; response to IFN therapy (no therapy vs. SVR; $P < 0.001$); fibrosis stage (F3 and F4 vs. F0-F1; $P < 0.001$); platelet count (<15 × 10^4/mm^3 vs. ≥15 × 10^4/mm^3; $P < 0.001$); albumin (<4.2 vs. ≥4.2 g/mL; $P < 0.001$); AST (<53 vs. ≥53 IU/mL; $P < 0.001$), ALT (<82 vs. ≥82 IU/mL; $P = 0.035$), and AFP levels (≥20 and 6-20 vs. <6 ng/mL; $P < 0.001$); HCV genotype (1b vs. non-1b; $P = 0.025$); and serum WFA$^+$-M2BP level (≥4 and 1-4 vs. <1; $P < 0.001$). Multivariate analysis was performed on these factors (Table 3) and the following were identified as independent risk factors: fibrosis stage (F4); AFP (≥20 ng/mL); age (≥57 years); response to IFN therapy (no therapy vs. SVR); and WFA$^+$-M2BP (1-4 and ≥4).

Development of HCC. During the follow-up period, HCC developed in 110 (15.6%) patients. Of

Table 3. Factors Associated With Risk for HCC*

Features		HR (95% CI)	*P* Value
Fibrosis	F0-F1	1	
	F2	0.883 (0.411-1.897)	0.749
	F3	1.347 (0.624-2.906)	0.448
	F4	3.133 (1.536-6.390)	0.002
AFP	<6 ng/mL	1	
	6-20 ng/mL	1.710 (0.963-3.038)	0.067
	≥20 ng/mL	3.417 (1.807-6.460)	<0.001
Age	<57 years	1	
	≥57 years	2.039 (1.278-3.252)	0.003
IFN therapy	No therapy	1	
	Non-SVR	0.729 (0.467-1.137)	0.163
	SVR	0.089 (0.027-0.288)	<0.001
WFA^+-M2BP	<1	1	
	1-4	5.155 (1.180 – 22.500)	0.029
	≥4	8.318 (1.784 – 38.791)	0.007

Abbreviations: HR, hazard ratio; CI, confidence interval.
*Determined by multivariate analysis.

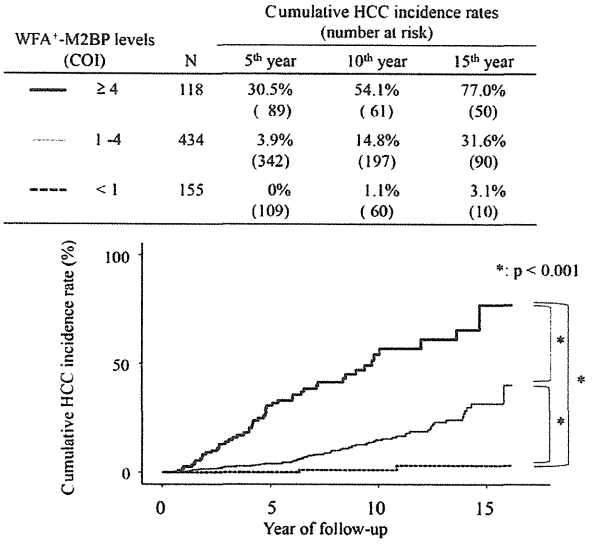

Fig. 2. Cumulative incidence of HCC according to WFA^+-M2BP level. Cumulative incidences of HCC according to the WFA^+-M2BP level were analyzed using Kaplan-Meier's method. Black solid, gray solid, and dotted lines indicate stratified WFA^+-M2BP level, ≥4, 1-4, and <1, respectively. Incidence rate differed significantly among the three groups ($P < 0.001$, by the log-rank test), increasing in accord with WFA^+-M2BP level.

the 110 patients with HCC, 58 (52.7%) were diagnosed with the disease by histological examination of biopsy-obtained or resected liver specimens. Of these 58 patients, 24 (41.3%) had hypovascular HCC.

Figure 2 shows the relation between Kaplan-Meier's estimates of the cumulative risk of HCC and the different WFA^+-M2BP levels at entry. The 10-year cumulative risk of HCC was 1.1% in the patients with WFA^+-M2BP <1 at entry, 14.8% among the patients with WFA^+-M2BP 1-4, and 54.1% in patients with WFA^+-M2BP >4. The incidence rate differed significantly among the three groups ($P < 0.001$, by the log-rank test), increasing in accord with WFA^+-M2BP level.

Figure 3 shows the relation between the cumulative incidence of HCC and WFA^+-M2BP levels, stratified by the fibrosis stage. In patients with fibrosis stage F0-F1, there were significant differences in HCC incidence between those with WFA^+-M2BP levels of 1-4 and those with levels of <1 ($P < 0.01$) and between those with WFA^+-M2BP levels of ≥4 and those with levels of <1 ($P < 0.01$). In patients with fibrosis stage F2-F3, there were significant differences in HCC incidence between those with WFA^+-M2BP levels of ≤1 and those with levels of >4 ($P < 0.01$) and between those with WFA^+-M2BP levels of 1-4 and those with levels of >4 ($P < 0.001$). In patients with fibrosis stage F4, there were significant differences in HCC incidence between those with WFA^+-M2BP levels of 1-4 and those with levels of >4 ($P < 0.05$). As with WFA^+-M2BP levels, incidence rates increased with fibrosis stage, and the change in incidence was significant for each fibrosis stage.

Predictive Accuracy of Cumulative Incidence of HCC Compared With WFA^+-M2BP and AFP. AUROC analyses for prediction of the development of HCC at 1, 2, 3, 5, 7, and 10 years (range) were 0.762 (0.553-0.971), 0.792 (0.669-0.915), 0.832 (0.751-0.914), 0.858 (0.805-0.911), 0.821 (0.767-0.876), and 0.800 (0.745-0.855) in WFA^+-M2BP and 0.791 (0.684-0.898), 0.790 (0.723-0.857), 0.772 (0.693-0.850), 0.800 (0.741-0.858), 0.796 (0.745-0.848), and 0.821 (0.773-0.868) in AFP, respectively. The WFA^+-M2BP assay was superior to AFP for predicting the development of HCC at 3, 5, and 7 years.

Discussion

Liver biopsy has long been considered the gold standard for assessment of hepatic fibrosis,[20-23] and the Metavir[24] and Desmet et al.[16] staging systems are most commonly used. A higher degree of liver fibrosis is known to be the strongest risk factor for hepatocarcinogenesis in hepatitis C patients.[1,20] However, it also has its limitations for the staging of fibrosis because of the heterogeneous distribution of fibrosis in the liver,[25] and liver biopsy is an invasive procedure with

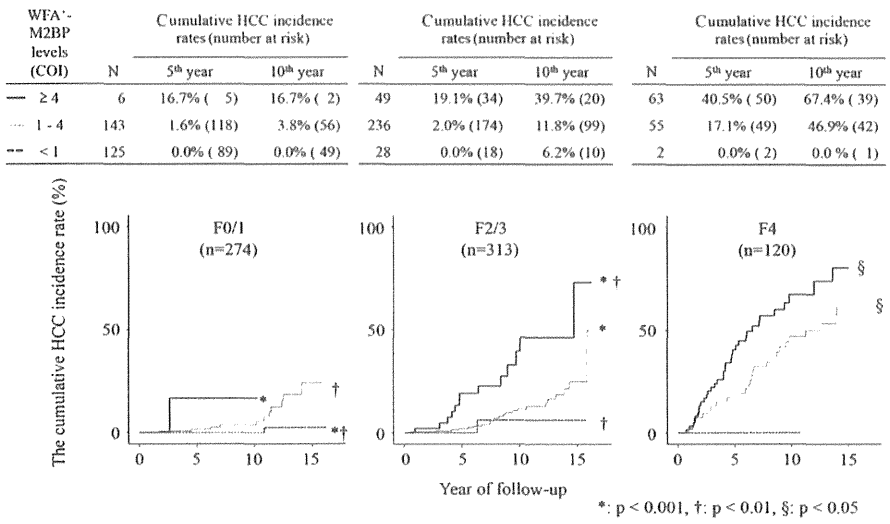

Fig. 3. Cumulative incidence of HCC according to WFA$^+$-M2BP levels, stratified by the fibrosis stage. Cumulative incidences of HCC, according to the WFA$^+$-M2BP level, stratified by the fibrosis stage were analyzed using Kaplan-Meier's method. Black solid, gray solid, and dotted lines indicate stratified WFA$^+$-M2BP level, ≥4, 1-4, and <1, respectively. Incidence rates increased in accord with WFA$^+$-M2BP level.

associated morbidity (pain, bleeding, or hemobilia).[26] For these reasons, patients are often reluctant to undergo this invasive procedure and instead choose one of several noninvasive methods available for assessing the degree of liver fibrosis.

Nevertheless, in the past, no significant progress was made in the development of noninvasive biomarkers to guide clinical usage. WFA$^+$-M2BP was recently validated as a liver fibrosis glycobiomarker with a fully automated immunoassay.[8] In the present study, we assessed the performance of the WFA$^+$-M2BP assay in comparison with liver fibrosis stage and several serum markers, and, based on the results, we estimated whether WFA$^+$-M2BP is a useful predictor of the development of HCC as well as liver biopsy stage.

The first main finding of our study was that there was a significant correlation between the WFA$^+$-M2BP value and the fibrosis stage (Fig. 1). Moreover, step-wise multiple linear regression analysis showed that liver fibrosis stage was most closely associated with serum WFA$^+$-M2BP level. In addition, the degree of necroinflammation had no apparent effect on the WFA$^+$-M2BP value. Based on these results, we proposed a clinical management algorithm using a WFA$^+$-M2BP assay to predict the fibrosis stage. This approach could be used reliably for the first-line pretherapeutic evaluation of fibrosis in HCV-infected patients. On the other hand, the most widely used noninvasive techniques have recently shifted to physical measurements, such as FibroScan,[27-30] acoustic radiation force impulse, and real-time strain elastography. FibroScan has the advantages of being rapid and technically simple; however, operator skill affects its diagnostic success rate. Also, stiffness measurements can be difficult to obtain in obese patients and impossible in patients who have ascites. This is regarded as a limitation of transient elastography.[27,28] Therefore, we suggest that FibroScan, in conjunction with an assay of serum fibrosis biomarkers, would improve the diagnostic accuracy.

The second main finding of our study was the significant association between the WFA$^+$-M2BP level and the risk of HCC development in hepatitis C patients (Figs. 2 and 3). The diagnostic performance of WFA$^+$-M2BP, based on the AUROC values, was superior to that of AFP for predicting the development of HCC at 3, 5, and 7 years. The WFA$^+$-M2BP value can be used as a noninvasive predictor of HCC development and can be considered a surrogate marker for liver fibrosis. Various risk factors have been reported for HCC development among patients with HCV, including older age,[1] male sex,[1] heavy alcohol consumption,[31] obesity,[32] cirrhosis,[1,33] lower platelet count,[34-36] high serum AFP level,[15,36-44] low serum albumin level,[31] and high serum ALT and AST level.[45-47] Our results were consistent with these findings. Among them, liver fibrosis stage was the strongest prognostic indicator of chronic hepatitis. However, liver biopsy has several disadvantages. In our study, we have shown that the WFA$^+$-M2BP value is also a significant risk factor of HCC development independent of these factors. However, even though WFA$^+$-M2BP can be considered a surrogate marker for liver fibrosis, a distinct advantage of WFA$^+$-M2BP over liver biopsy is its wider dynamic range for the evaluation of liver cirrhosis. In the Metavir and Desmet et al. scoring systems, cirrhosis is represented by a single category (F4). However, the degree of fibrosis may vary widely

among patients in this category, and the risk of HCC may not be uniform. In our study, the risk of HCC development increased with increasing WFA^+-M2BP level as well as with increasing fibrotic stage. According to the elevation of WFA^+-M2BP value, the risk of development of HCC was increased (Fig. 3). In other words, each fibrosis stage can be further stratified with clinical relevance based on the WFA^+-M2BP level.

In our study, multivariate analysis identified fibrosis stage, high AFP level, older age, SVR to IFN therapy (no therapy vs. SVR), and high WFA^+-M2BP value as independent predictors of HCC development. The stratified WFA^+-M2BP value was independently associated with HCC development. These results indicate that the correlation between high WFA^+-M2BP and HCC development remains significant, even if HCC develops from a noncirrhotic background. Tateyama et al.[15] reported that AFP was a noninvasive predictive marker for the development of HCC in this same cohort; furthermore, not only high AFP levels ($\geq$20 ng/mL), but also slightly elevated AFP levels of between 6 and 20 ng/mL could indicate substantial risks for the development of HCC, complementing the fibrosis stage. Our present study was redesigned by the addition of one parameter (WFA^+-M2BP). Multivariate analysis did not identify slightly elevated AFP levels (6-20 ng/mL) as an independent risk factor, but did identify both stratified WFA^+-M2BP levels (1-4 and $\geq$4) as independent risk factors. Also, the time-dependent AUROC analysis suggested that WFA^+-M2BP is superior to AFP as a predictor for the development of HCC. These results mean that the WFA^+-M2BP level is the most reliable noninvasive predictive marker for the development of HCC in patients infected with HCV.

One of the limitations of the present study is that this cohort of 707 patients was analyzed retrospectively. There is thus need of a future study to prospectively analyze the efficacy of WFA^+-M2BP as a predictor of HCC development.

Another limitation is that the hepatocarcinogenesis of the patients who underwent IFN therapy was not evaluated. In this study, among the patients who achieved SVR (n = 139), 3 cases developed HCC during the follow-up period. The WFA^+-M2BP titers were 6.4, 5.6, and 1.5, respectively, in the 3 patients. All 3 cases obtained titers higher than 1, and 2 cases obtained titers higher than 4. This result suggests that patients with a high WFA^+-M2BP value should be monitored for the development of HCC even after achieving SVR. However, future assessments of the WFA^+-M2BP values at IFN administration and at posttreatment will be needed to verify this recommendation.

In conclusion, this study revealed an association between WFA^+-M2BP and the risk of HCC development in chronic hepatitis C patients. The results suggested that the WFA^+-M2BP assay should not be limited to use as a surrogate for liver biopsy, but rather could be applied as dynamic indicator of the risk of HCC development.

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Genetic polymorphisms of *OCT-1* confer susceptibility to severe progression of primary biliary cirrhosis in Japanese patients

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Abstract
Background To identify the genetic factors involved in the pathogenesis of primary biliary cirrhosis (PBC), we focused on the organic cation transporter 1 (OCT1/SLC22A1), which is closely associated with phosphatidylcholine synthesis in hepatocytes.
Methods We selected four (rs683369, rs2282143, rs622342 and rs1443844) *OCT-1* single nucleotide polymorphisms (SNPs), and genotyped these SNPs using the TaqMan probe method in 275 Japanese PBC patients and 194 gender-matched, healthy volunteers as controls.
Results The Chi-square test revealed that the rs683369 variant allele (G) was associated with insusceptibility to PBC development [$P = 0.009$, odds ratio (OR) 0.60, 95 % confidence interval (CI) 0.40–0.88] in an allele model, and that the rs683369 variant allele (G) was associated with jaundice-type progression in a minor allele dominant genotype model ($P = 0.032$, OR 3.10, 95 % CI 1.05–9.14). The OCT-1 rs2282143 variant (T) and rs622342 variant (C) were also associated with jaundice-type progression in a minor allele recessive genotype model ($P = 0.0002$, OR 10.58, 95 % CI 2.36–47.54, and $P = 0.006$, OR 7.84, 95 % CI 1.39–44.36, respectively). Furthermore, the association of *OCT-1* rs683369 and rs622342 with susceptibility to jaundice-type progression was confirmed by a replication study with a distinct set of PBC patients who underwent liver transplantation.
Conclusions The present study is the first report on the association of *OCT-1* genetic polymorphisms with the overall development and jaundice-type progression of PBC.

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Keywords PBC · OCT-1 · Genetic polymorphism · Phosphatidylcholine · Hepatic failure · Progression

Introduction

Primary biliary cirrhosis (PBC) is a chronic and slowly progressive autoimmune liver disease characterized histopathologically by destruction of the intrahepatic small bile ducts with lymphocyte-predominant portal inflammation, resulting in cholestasis, further hepatic damage, fibrosis, cirrhosis, and eventually hepatic failure [1]. In addition to antimitochondrial antibodies (AMA), antinuclear antibodies (ANA) such as anti-gp210, anti-sp100, and anticentromere antibodies are detected in approximately 50–90 % of PBC patients [2–7].

At present, a majority of PBC patients undergoing treatment with ursodeoxycholic acid (UDCA) have a normal life expectancy without the need for additional therapeutic approaches. However, one-third of PBC patients show severe progression and require additional treatments [8]. Ultimately, a few percent of PBC patients who show resistance to UDCA undergo liver transplantation or die within a decade of diagnosis [9]. The patterns of PBC progression thus differ strikingly among individuals, but the genetic or environmental factors influencing these differences are largely unknown.

PBC is a multifactorial disorder, with multiple genetic and environmental factors contributing to its etiology [1, 10]. Since the concordance rate of PBC in monozygotic twins is very high (63 %) and familial clustering of PBC has been reported at high frequencies (1.0–7.1 %) [11, 12], strong genetic factors have been implicated in the pathogenesis of PBC. Many candidate gene-based association studies with single nucleotide polymorphisms (SNPs) have identified a number of susceptibility genes for PBC. However, most of these have not been replicated in different ethnicities except for *CTLA4* [13]. In addition, there have been several reports describing weak associations between PBC progression and genetic polymorphisms in *TNF alpha*, *eNOS*, *apo-E*, *SLC4A2/AE2*, *Keratin*, *CYP2E1*, *CTLA4*, *ITGAV*, and *IL-1* [14–21]. However, these results require further replication studies in multiple ethnicities.

Recent genome-wide association studies have identified more than 20 non-HLA susceptibility loci for PBC, including *TNFSF15*, *POU2AF1*, *IL12A*, *IL12RB2*, *STAT4*, *IRF5*, *IKZF3*, *MMEL1*, *SPIB*, *DENND1B*, *CD 80*, *IL7R*, *CXCR5*, *TNFRSF1A*, *CLEC16A*, *NFKB1*, *RAD51L1*, *MAP3K7IP1*, *PLCL2*, *RPS6KA4*, and *TNFAIP2* [22–27]. These results indicate the importance of IL-12 and TNF/TLR–NFκB signaling as well as B cell differentiation pathways in the development of PBC. However, these genome-wide association studies have not yet identified genetic loci associated with the progression of PBC.

Multidrug resistance protein 3 (MDR3) is a member of the superfamily of ATP-binding cassette (ABC) transporters, in which genetic polymorphisms are involved in cholestatic liver diseases such as intrahepatic cholestasis of pregnancy [28–30]. We previously reported that genetic polymorphism in MDR3 is associated with severe progression of PBC (i.e., jaundice-type progression) [31]. MDR3 functions as a transporter that is responsible for phosphatidylcholine (PC) secretion into bile. PC reduces bile acid toxicity by forming micelles with bile acid [32–35]. On the other hand, choline is used for the synthesis of PC in hepatocytes [36].

Organic cation transporter 1 (OCT-1) is a member of the solute carrier (SLC) transporter family, which transports choline into hepatocytes [37, 38]. Approximately 650 SNPs have been identified in the *OCT-1* gene, of which 69 are accompanied by amino acid changes. Among these nonsynonymous 69 SNPs, 12 have been found to be associated with alterations in OCT-1 expression and/or function in vitro [39]. OCT-1 also transports metformin into hepatocytes, and the genetic polymorphisms of *OCT-1* are known to influence the antihyperglycemic action of metformin in vivo [40]. These findings indicate that the transport of choline into hepatocytes is influenced by *OCT-1* genetic polymorphisms leading to altered synthesis of PC, which causes insufficient protection of bile ducts due to bile acid toxicity. Thus, we hypothesized that *OCT-1* genetic polymorphisms are potentially associated with greater severity of both bile duct damage and progression of PBC.

Methods

Subjects

The study subjects comprised 275 unrelated Japanese patients with PBC [39 males, 236 females, age 32–78 years, median 57 year, mean ± standard deviation (SD) 56.4 ± 10.4 years at the time of entry], and 194 gender-matched, unrelated, healthy Japanese volunteers as controls (Table 1). The PBC patients were registered in the PBC cohort study of the National Hospital Organization Study Group for Liver Disease in Japan (NHOSLJ) from August 1982 to August 2011. The time of entry was defined as the time of initial diagnosis of PBC. The observation period was defined as the time from the date of entry until the date of death, liver transplantation, last contact, death from non-liver-associated diseases, or end of follow-up, whichever came first.

Table 1 Characteristics of control subjects and PBC patients at each stage at the end of observation

Characteristics	Control subjects	PBC patients	Stage I	Stage II	Stage III
Total number of patients	194	275	194	66	15
Mean age (years)	39.8 ± 9.28	65.0 ± 11.7	63.5 ± 11.05	70.4 ± 9.62	59.8 ± 9.39
Observation period (months)	–	69.7 ± 52.5	63.8 ± 46.85	83.3 ± 59.9	85.7 ± 73.1
Male/female (%)	17/177 (8.8/91.2)	39/236 (14.1/85.9)	22/172 (11.3/88.7)	11/55 (16.7/83.3)	6/9 (40.0/60.0)
Anti-gp210 antibodies+ (%)	–	87 (31.6 %)	47 (24.2 %)	26 (39.4 %)	14 (93.3 %)
Anti-centromere antibodies+ (%)	–	66 (24.0 %)	38 (19.6 %)	25 (37.9 %)	3 (20 %)

PBC, primary biliary cirrhosis

Patients were diagnosed with PBC if they met at least two of the following internationally accepted criteria [41]: biochemical evidence of cholestasis, based mainly on alkaline phosphatase elevation; presence of serum anti-mitochondrial antibodies; histological evidence of non-suppurative destructive cholangitis; and destruction of interlobular bile ducts.

Patients with acute or autoimmune hepatitis (AIH; alanine aminotransferase >200 IU/l, aspartate aminotransferase >200 IU/l), given maintenance doses of prednisolone (PSL) higher than 5 mg/body for concomitant AIH, persistent hepatitis virus B or C infection, alcoholic liver disease, and other chronic liver diseases were excluded from this study.

Of the 275 patients in the study, 78 (26.4 %) had concomitant autoimmune diseases, as follows: Sjögren's syndrome ($n = 38$), systemic sclerosis ($n = 4$), Hashimoto's thyroiditis ($n = 8$), CREST syndrome ($n = 3$), rheumatoid arthritis ($n = 12$), Raynaud's disease ($n = 7$), interstitial pneumonitis ($n = 2$), polymyositis ($n = 1$), mixed connective tissue disease ($n = 1$), Basedow's disease ($n = 1$), and sarcoidosis ($n = 1$).

Patients were treated for PBC during the observation period as follows: UDCA (300–900 mg/day) alone ($n = 170$), bezafibrate (200–400 mg/day) alone ($n = 3$), PSL ($\leq$5 mg/day) alone ($n = 3$), UDCA + bezafibrate ($n = 64$), UDCA and/or bezafibrate + maintenance PSL ($\leq$5 mg/day) ($n = 29$), or no medication ($n = 1$).

Histological examination

Four-μm-thick formalin-fixed and paraffin-embedded sections of needle liver biopsy specimens (length ≥20 mm, a total of 210 samples from 210 different PBC patients) were routinely stained with H&E, Azan-Mallory, reticulin silver impregnation, and rhodamine. The histological variables included fibrosis (0–4), portal inflammation (0–3), interface hepatitis (0–3), lobular inflammation (0–3), copper-associated protein deposition (0–1), chronic non-suppurative destructive cholangitis (0–2), granuloma (0–2), ductal paucity (0–3), and ductal proliferation (0–3). Upon completion of the evaluation of each of these variables, a numerical necroinflammatory grade (A0–A3) and a histological stage using Scheuer's classification (stage 1–4) were determined [3, 7, 42]. Each biopsy specimen was analyzed by two independent observers (M.I. and Y.T.). In case of initial disagreement in the assessment, consensus was achieved by further review.

Clinical staging of PBC

PBC patients were assigned to one of the following three clinical stages based on liver biopsy findings and/or clinical manifestations: clinical stage I (early stage)—Scheuer's stage 1 or 2 or unknown histological stage without any signs indicating portal hypertension or liver cirrhosis; clinical stage II (late stage without jaundice)—Scheuer's stage 3 or 4 or any histological stage with signs indicating portal hypertension or liver cirrhosis, but without jaundice (total bilirubin <2 mg/dl); and clinical stage III (late stage with jaundice)—any Scheuer's stage with persistent jaundice (total bilirubin ≥ mg/dl). Clinical stages I + II were also defined as "non-jaundice stages," while stage III was defined as the "jaundice stage." Clinical stage I was defined as the "early stage," while stages II + III were defined as "late stages." In addition, the progression to clinical stage II was defined as "non-jaundice-type progression," while the progression to clinical stage III was defined as "jaundice-type progression."

Replication study in patients who underwent liver transplantation

A replication study for progression to clinical stage III (jaundice-type progression) was performed using a different set of 35 PBC patients [3 males (8.5 %), 32 females (91.5 %); age at liver transplantation 34–69 years, median 51.0 years, mean ± SD 51.3 ± 8.18 years] who underwent orthotopic liver transplantation in Kyushu University Hospital during the period from September 1999 to August 2007. The observation period from the initial diagnosis of PBC to liver transplantation ranged from 1 to 23 years (median 9.5 years, mean ± SD 9.2 ± 5.3 years).

Preparation of genomic DNA

Genomic DNA was extracted from whole blood samples using the NucleoSpin Blood L Kit (Macherey-Nagel, Duren, Germany) according to the manufacturer's protocol. Genomic DNA was also extracted from livers removed from PBC patients who underwent liver transplantation using the QuickGene DNA Whole Blood Kit S (Fujifilm, Tokyo, Japan) according to the manufacturer's protocol.

Selection of SNPs

Four SNPs—rs683369, rs2282143, rs622342, and rs1443844—were selected in the *OCT-1* gene (HGNC: 10963), referred to as *SLC22A1*, using the data available on the International HapMap website (http://www.HapMap.org/). These SNPs were previously reported to be associated with function and/or expression. The gene structure and positions of the four chosen SNP sites in the *OCT-1* gene are shown in Fig. 1.

Genotyping of four SNPs in OCT-1

We genotyped four candidate SNPs using TaqMan probes. In brief, the polymorphic region was amplified by PCR using a real-time PCR System (BIORAD, CA, USA) from 10 ng of genomic DNA in a 10-μl reaction mixture containing Premix EX Taq™ (Takara Biotechnology, Japan) and TaqMan® SNP Genotyping Assays (Applied Biosystems, CA, USA). The genotype was determined by detecting the fluorescence of FAM and VIC, according to the manufacturer's protocol. The fluorescence of FAM corresponded to the major allele while that of VIC corresponded to the minor allele.

Ethics board approval

The study protocol was approved by the Committee for Ethical Issues on Human Genome and Gene Analysis at the Clinical Research Center of the National Hospital

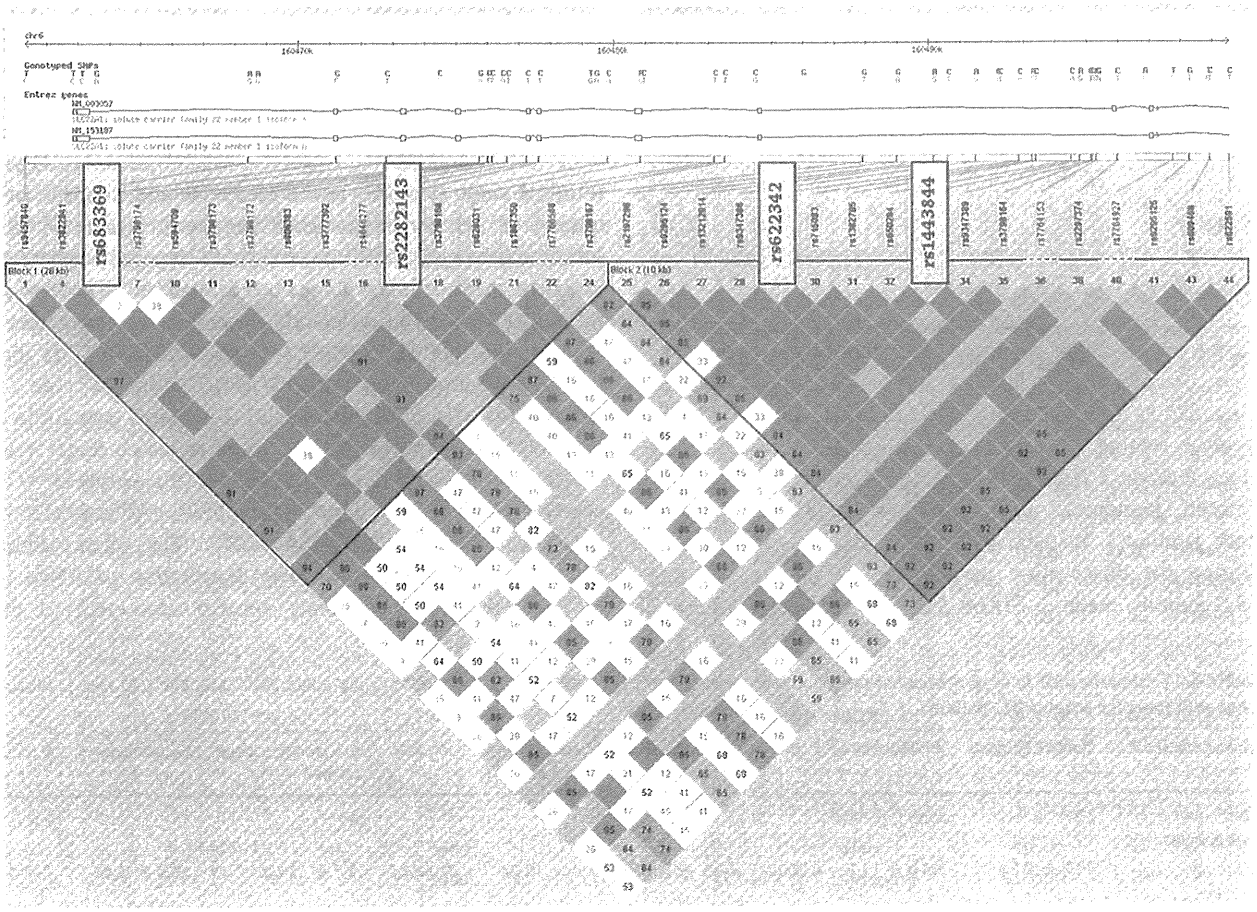

Fig. 1 Linkage disequilibrium (LD) plots for tag SNPs in *OCT1* using Haploview software version 4.2. The significant tag SNPs associated with the progression of PBC in the present study is highlighted in the *blue boxes*. Each *diamond* represents pairwise LD strength with color (D′/LOD method) and value (r^2). *Red diamonds* indicate high LD, *blue* indicate moderate, and *white* indicate low. *Empty diamonds* show r^2 value of 1.0. Haplotype blocks highlighted with *inverted triangles* were calculated using solid spine algorithm

Organization (NHO) Nagasaki Medical Center (Approval number 15005), Kyushu University Hospital (Approval number 449-00) and at every hospital participating in the clinical study. Written informed consent was obtained from each subject.

Statistical analysis

Data obtained are indicated as means ± SDs. Age and gender of PBC patients and control subjects were evaluated by the unpaired Student's *t* test and Chi-square test, respectively, using Prism 4 (GraphPad Software Inc., San Diego, CA, USA). Clinicopathological parameters were compared between subgroups of PBC patients using the unpaired Student's *t* test and Chi-square test, again with Prism 4. Expected allele frequencies were calculated from respective single allele frequencies according to the Hardy–Weinberg equilibrium. The observed and expected allele frequencies were compared by Chi-square test using SNP Alyze 6.0 standard software. The frequencies and distributions of alleles and genotypes were compared between PBC patients and control subjects, as well as between subgroups of PBC patients using the Chi-square test with Prism 4. A P value of <0.05 was considered to be statistically significant.

Results

Clinical course of PBC patients

At the beginning of the study, 182 liver biopsy specimens were classified as Scheuer's stage 1–2, while 28 were classified as Scheuer's stage 3–4. Based on our criteria for clinical staging, 238, 35, and 2 PBC patients were at clinical stage I (early stage), clinical stage II (late stage without jaundice), and clinical stage III (late stage with jaundice), respectively, at the time of study entry. During the observation period (13–306 months, median 54.0 months, mean ± SD 69.7 ± 52.5 months), of the 238 patients originally at clinical stage I, 37 progressed to clinical stage II while seven progressed to clinical stage III. Six of the 35 patients initially at clinical stage II progressed to clinical stage III. The two patients at clinical stage III at the time of entry received liver transplantations. At the end of the observation period, therefore, 194 patients were at clinical stage I and 66 were at clinical stage II. Of the 13 patients who progressed to clinical stage III during the observation period, six patients received liver transplantations, three remained alive without receiving liver transplantations, and four died of end-stage hepatic failure.

Comparison of clinicopathological parameters among PBC patients and between PBC patients and control subjects

There was a significant difference in mean age ($P = 0.0001$) but no difference in gender ($P = 0.475$) between PBC patients and control subjects (Table 2). The demographics of PBC patients at each stage are shown in Table 2. There was a significant difference in mean age ($P = 0.0002$) and observation period ($P = 0.0039$) between early-stage and late-stage PBC patients. Specifically, the observation period in early-stage PBC patients was approximately 2 years shorter than that in late-stage PBC patients. This finding implies that a few patients with early-stage PBC might progress to more advanced stages (clinical stage II or III) in the future. In addition, the frequency of males was significantly higher in jaundice-stage (clinical stage III) PBC patients as compared with non-jaundice-stage (clinical stages I + II) patients. There were no significant differences in age or observation period between non-jaundice-stage (clinical stages I + II) and jaundice-stage (clinical stage III) PBC patients (Table 2). These results imply that male PBC patients constitute a high-risk group for progression to the jaundice stage.

The following alleles are wild-types (major types): "C" at rs683369 SNP, "C" at rs2282143 SNP, "A" at rs622342 SNP, and "A" at rs1443844 SNP. The other alleles are variants (minor types). The distribution of SNPs in *OCT-1* among PBC patients corresponded well to the

Table 2 Comparison of demographics between control subjects and PBC patients, early-stage and late-stage PBC patients, and non-jaundice-stage and jaundice-stage PBC patients

Characteristics	Control subjects	PBC	*P* value	Early stage (stage I)	Late stage (stages II + III)	*P* value	Non-jaundice stage (stages I + II)	Jaundice stage (stage III)	*P* value
Total number of patients	194	275		194	81		260	15	
Mean age (years)	39.8 ± 9.28	65.0 ± 11.7	0.0001	63.5 ± 11.05	68.4 ± 10.38	0.0002	65.4 ± 11.10	59.8 ± 9.39	0.070
Observation period (months)	–	–	–	63.8 ± 46.85	83.7 ± 62.12	0.0039	68.7 ± 51.69	85.7 ± 73.13	0.223
Male/female (%)	17/177 (8.8/91.2)	39/236 (14.1/85.9)	0.074	22/172 (11.3/88.7)	17/64 (20.9/79.1)	0.065	33/227 (12.7/87.3)	6/9 (40.0/60.0)	0.011

Hardy–Weinberg equilibrium, implying that our sample had a homogeneous genetic background.

OCT-1 genetic polymorphisms that confer susceptibility to the development and progression of PBC in an allele model

A Chi-square test revealed that the frequency of the "G" variant allele at rs683369 SNP was significantly decreased in PBC patients as compared to healthy control subjects (16.0 vs. 10.0 %; $P = 0.009$, OR 0.60, 95 % CI 0.40–0.88), as shown in Table 3. As shown in Table 5, this test also showed that the frequency of the following alleles tended to be higher in jaundice-stage PBC patients as compared to non-jaundice-stage PBC patients: the "G" variant allele at rs683369 SNP (20.0 vs. 9.0 %; $P = 0.054$), the "T" variant allele at rs2282143 SNP (30.0 vs. 18.0 %; $P = 0.084$), and the "C" variant allele at rs622342 SNP (27.0 vs. 14.0 %; $P = 0.068$). There was no allele that showed significant increase in late-stage as compared to early-stage PBC patients (Table 4).

OCT1 genetic polymorphisms that confer susceptibility to the development and progression of PBC in minor allele dominant or recessive genotype models

The distributions and frequencies of genotypes at the four SNP sites were compared between patients with early- and late-stage PBC and those at the non-jaundice and jaundice stages, as well as between PBC patients and healthy control subjects. A Chi-square test revealed several findings. First, the frequency of the "C/G or G/G" genotypes at rs683369 SNP was significantly higher in healthy control subjects as compared to PBC patients (18.9 vs. 27.3 %; $P = 0.031$, OR 0.62, 95 % CI 0.40–0.96) (Table 3). Moreover, as shown in Table 3, "G/G" genotypes at rs683369 SNP were significantly more frequent in healthy control subjects as compared to PBC patients (0.7 vs. 3.7 %; $P = 0.025$, OR 0.20, 95 % CI 0.04–0.95). Second, the frequency of the "C/G or G/G" genotypes at rs683369 SNP were significantly higher in jaundice-stage PBC patients as compared to non-jaundice-stage PBC patients (40.0 vs. 17.7 %; $P = 0.032$, OR 3.10, 95 % CI 1.05–9.14) (Table 5). Third, the frequency of the "T/T" genotype at rs2282143 SNP was significantly higher in jaundice-stage PBC patients as compared to non-jaundice-stage PBC patients (20.0 vs. 2.4 %; $P = 0.0002$, OR 10.58, 95 % CI 2.36–47.54) (Table 5). Finally, as shown in Table 5, the frequency of the "C/C" genotype at rs622342 SNP was significantly higher in jaundice-stage PBC patients as compared to non-jaundice-stage PBC patients (13.3 vs. 1.9 %; $P = 0.006$, OR 7.84, 95 % CI 1.39–44.4).

Replication study in PBC patients undergoing liver transplantation

OCT-1 rs683369, rs2282143, and rs622342 SNPs were significantly associated with the progression of PBC. In

Table 3 Allele and genotype comparisons of tag SNPs in three inheritance models: control subjects vs. PBC patients

Gene symbol	Tag SNP (major > minor)	Genotype	Number of genotypes (%)		Inheritance model	*P* value	OR	95 % CI
			Control subjects	PBC				
OCT-1/SLC22a1	rs683369 (C > G)	MAF	0.16	0.10	Allele	0.009	0.60	0.40–0.88
		C/C	141 (72.6)	223 (81.1)	Dominant	0.031	0.62	0.40–0.96
		C/G	46 (23.7)	50 (18.2)				
		G/G	7 (3.7)	2 (0.7)	Recessive	0.025	0.20	0.04–0.95
	rs2282143 (C > T)	MAF	0.16	0.17	Allele	0.502	1.13	0.79–1.58
		C/C	138 (71.1)	184 (66.9)	Dominant	0.331	1.22	0.81–1.82
		C/T	48 (24.7)	82 (29.8)				
		T/T	8 (4.2)	9 (3.3)	Recessive	0.526	0.73	0.27–1.93
	rs622342 (A > C)	MAF	0.18	0.15	Allele	0.270	0.82	0.57–1.16
		A/A	133 (68.6)	199 (72.4)	Dominant	0.372	0.83	0.55–1.24
		A/C	53 (27.3)	69 (25.1)				
		C/C	8 (4.1)	7 (2.5)	Recessive	0.339	0.61	0.21–1.70
	rs1443844 (A > G)	MAF	0.29	0.30	Allele	0.905	1.02	0.76–1.35
		A/A	97 (50.0)	135 (49.1)	Dominant	0.359	1.19	0.82–1.70
		A/G	79 (40.7)	115 (41.8)				
		G/G	18 (9.3)	25 (9.1)	Recessive	0.944	0.98	0.51–1.84

Allele, allele model; Dominant, minor allele dominant model; Recessive, minor allele recessive model

Abbreviations: MAF, minor allele frequency; OR, odds ratio; CI, confidence interval

Table 4 Allele and genotype comparisons of tag SNPs in three inheritance models: PBC progression in early- vs. late-stage patients

Gene symbol	Tag SNP (major > minor)	Genotype	Number of genotypes (%)		Inheritance model	*P* value	OR	95 % CI
			Early stage	Late stage				
OCT-1/SLC22a1	rs683369 (C > G)	MAF	0.10	0.10	Allele	0.976	1.01	0.55–1.87
		C/C	157 (80.9)	66 (81.5)	Dominant	0.914	0.96	0.50–1.88
		C/G	36 (18.6)	14 (17.3)				
		G/G	1 (0.5)	1 (1.2)	Recessive	0.522	2.41	0.14–39.07
	rs2282143 (C > T)	MAF	0.17	0.20	Allele	0.389	1.23	0.77–1.95
		C/C	132 (68.0)	52 (64.2)	Dominant	0.537	1.19	0.69–2.04
		C/T	57 (29.4)	25 (30.9)				
		T/T	5 (2.6)	4 (4.9)	Recessive	0.316	1.96	0.51–7.51
	rs622342 (A > C)	MAF	0.15	0.16	Allele	0.685	1.11	0.67–1.84
		A/A	141 (72.7)	58 (71.6)	Dominant	0.856	1.05	0.59–1.88
		A/C	49 (25.3)	20 (24.7)				
		C/C	4 (2.1)	3 (3.7)	Recessive	0.401	1.83	0.40–8.35
	rs1443844 (A > G)	MAF	0.30	0.29	Allele	0.744	0.94	0.63–1.40
		A/A	95 (49.0)	40 (49.4)	Dominant	0.950	0.98	0.58–1.65
		A/G	80 (41.2)	35 (43.2)				
		G/G	19 (9.8)	6 (7.2)	Recessive	0.649	0.74	0.28–1.91

Allele, allele model; Dominant, minor allele dominant model; Recessive, minor allele recessive model

Abbreviations: MAF, minor allele frequency; OR, odds ratio; CI, confidence interval

Table 5 Allele and genotype comparisons of tag SNPs in three inheritance models: PBC progression in non-jaundice vs. jaundice-stage patients

Gene symbol	Tag SNP (major > minor)	Genotype	Number of Genotypes (%)		Inheritance model	*P* value	OR	95 % CI
			Non-jaundice stage	Jaundice stage				
OCT-1/SLC22a1	rs683369 (C > G)	MAF	0.09	0.20	Allele	0.054	2.46	0.95–6.31
		C/C	214 (82.3)	9 (60.0)	Dominant	0.032	3.10	1.05–9.14
		C/G	44 (16.9)	6 (40.0)				
		G/G	2 (0.8)	0 (0.0)	Recessive	0.733	3.34	0.15–72.58
	rs2282143 (C > T)	MAF	0.18	0.30	Allele	0.084	2.02	0.89–4.55
		C/C	175 (67.3)	9 (60.0)	Dominant	0.558	1.37	0.47–3.98
		C/T	79 (30.3)	3 (20.0)				
		T/T	6 (2.4)	3 (20.0)	Recessive	0.0002	10.58	2.36–47.54
	rs622342 (A > C)	MAF	0.14	0.27	Allele	0.068	2.16	0.92–5.02
		A/A	190 (73.1)	9 (60.0)	Dominant	0.271	1.81	0.62–5.27
		A/C	65 (25.0)	4 (26.7)				
		C/C	5 (1.9)	2 (13.3)	Recessive	0.006	7.84	1.39–44.36
	rs1443844 (A > G)	MAF	0.30	0.23	Allele	0.413	0.70	0.29–1.65
		A/A	126 (48.5)	9 (60.0)	Dominant	0.384	0.63	0.22–1.81
		A/G	110 (42.3)	5 (33.3)				
		G/G	24 (9.2)	1 (6.7)	Recessive	0.737	0.70	0.08–5.57

Allele, allele model; Dominant, minor allele dominant model; Recessive, minor allele recessive model

Abbreviations: MAF, minor allele frequency; OR, odds ratio; CI, confidence interval

order to verify the reproducibility of this result, we performed a replication study in PBC patients who underwent liver transplantation. A Chi-square test revealed that the frequencies of the "C/G or G/G" genotypes at rs683369 SNP were significantly higher in liver transplantation cases as compared to non-jaundice-stage PBC patients (34.3 vs. 17.7 %; $P = 0.021$, OR 2.42, 95 % CI 1.12–5.23) (Table 6). This was also the case for the "C/C" genotype at

Table 6 Replication study in PBC patients who underwent liver transplantation

Gene symbol	Tag SNP (major > minor)	Genotype	Number of Genotypes (%)		Inheritance model	*P* value	OR	95 % CI
			Non-jaundice stage	Liver transplantation				
OCT-1/SLC22a1	rs683369 (C > G)	MAF	0.09	0.20	Allele	0.0058	2.46	1.28–4.74
		C/C	214 (82.3)	23 (65.7)	Dominant	0.021	2.42	1.12–5.23
		C/G	44 (16.9)	10 (28.6)				
		G/G	2 (0.8)	2 (5.7)	Recessive	0.017	7.82	1.06–57.40
	rs2282143 (C > T)	MAF	0.18	0.19	Allele	0.825	1.08	0.56–2.04
		C/C	175 (67.3)	22 (62.9)	Dominant	0.599	1.22	0.58–2.53
		C/T	79 (30.3)	13 (37.1)				
		T/T	6 (2.4)	0 (0.0)	Recessive	0.364	–	–
	rs622342 (A > C)	MAF	0.14	0.24	Allele	0.033	1.90	1.05–3.46
		A/A	190 (73.1)	23 (65.7)	Dominant	0.361	1.42	0.67–3.00
		A/C	65 (25.0)	7 (20.0)				
		C/C	5 (1.9)	5 (14.3)	Recessive	0.0001	8.50	2.32–31.08
	rs1443844 (A > G)	MAF	0.30	0.29	Allele	0.890	0.92	0.53–1.59
		A/A	126 (48.5)	16 (45.7)	Dominant	0.760	1.12	0.54–2.26
		A/G	110 (42.3)	18 (51.4)				
		G/G	24 (9.2)	1 (2.9)	Recessive	0.203	0.29	0.04–2.21

Allele, allele model; Dominant, minor allele dominant model; Recessive, minor allele recessive model

Abbreviations: MAF, minor allele frequency; OR, odds ratio; CI, confidence interval

Table 7 Combined analysis of jaundice-stage patients in the initial cohort and PBC patient who underwent liver transplantation in the replication cohort

Gene symbol	Tag SNP (major > minor)	Genotype	Number of genotypes (%)		Inheritance model	*P* value	OR	95 % CI
			Non-jaundice stage	Jaundice stage				
OCT-1/SLC22a1	rs683369 (C > G)	MAF	0.09	0.20	Allele	0.0016	2.46	1.39–4.36
		C/C	214 (82.3)	32 (64.0)	Dominant	0.0034	2.62	1.35–5.06
		C/G	44 (16.9)	16 (32.0)				
		G/G	2 (0.8)	2 (4.0)	Recessive	0.063	5.38	0.74–39.11
	rs2282143 (C > T)	MAF	0.18	0.22	Allele	0.286	1.33	0.79–2.25
		C/C	175 (67.3)	31 (62.0)	Dominant	0.467	1.26	0.67–2.36
		C/T	79 (30.3)	16 (32.0)				
		T/T	6 (2.4)	3 (6.0)	Recessive	0.154	2.70	0.65–11.19
	rs622342 (A > C)	MAF	0.14	0.25	Allele	0.0084	1.98	1.18–3.31
		A/A	190 (73.1)	32 (64.0)	Dominant	0.192	1.52	0.81–2.89
		A/C	65 (25.0)	11 (22.0)				
		C/C	5 (1.9)	7 (14.0)	Recessive	0.0001	8.30	2.52–27.36
	rs1443844 (A > G)	MAF	0.30	0.27	Allele	0.498	0.85	0.52–1.37
		A/A	126 (48.5)	25 (50.0)	Dominant	0.842	0.94	0.51–1.72
		A/G	110 (42.3)	23 (46.0)				
		G/G	24 (9.2)	2 (4.0)	Recessive	0.222	0.41	0.09–1.79

Allele, allele model; Dominant, minor allele dominant model; Recessive, minor allele recessive model

Abbreviations: MAF, minor allele frequency; OR, odds ratio; CI, confidence interval

rs622342 SNP (14.3 vs. 1.9 %; $P = 0.0001$, OR 8.50, 95 % CI 2.32–31.08) (Table 6). When the NHOSLJ cohort was combined with the replication cohort, "C/G or G/G" genotypes at rs683369 SNP were significantly higher in jaundice-stage PBC patients ($P = 0.0034$, OR 2.62, 95 % CI 1.35–5.06) as compared to non-jaundice-stage PBC patients (Table 7). This was also the case for "C/C" genotype at rs622342 SNP ($P = 0.0001$, OR 8.30, 95 % CI 2.52–27.36).

Multivariate analysis

Since the presence of anti-gp210 antibodies is a strong risk factor for the progression to jaundice-stage in PBC, the three *OCT-1* SNPs ("C/G" or "G/G" genotype at rs683369, "T/T" genotype at rs2282143 and "C/C" genotype at rs622342), which revealed significant risk factors for the progression to jaundice-stage in the present study, were independently evaluated by multivariate analysis in conjunction with anti-gp210 antibodies-status and male sex (Table 8). While the positive anti-gp210 antibodies status revealed the most significant risk factor for the progression to jaundice-stage in each analysis ($P < 0.0001$, OR 12.84–13.75), only "C/C" genotype at rs622342 remained a significant risk factor for the progression to jaundice-stage ($P = 0.010$, OR 10.144, 95 % CI 1.75–58.71). The "C/G" or "G/G" genotype at rs683369 and "T/T" genotype at rs2282143 showed a trend of risk for jaundice-stage progression.

Discussion

The present study is the first to demonstrate the association of *OCT-1* genotypes with the progression of PBC in the Japanese population. The results showed that the rs683369 genotype "C/G or G/G" and the rs622342 genotype "C/C" in *OCT-1* were closely associated with the susceptibility to severe progression (especially jaundice-type) of PBC in the Japanese population. Conversely, genotypes "C/C" in rs683369 and "A/A or A/C" in rs622342 were associated with insusceptibility to the progression of PBC. These findings suggest that *OCT-1* is one of the genetic determinants for the predisposition to severe progression of PBC in the Japanese population. However, it remains to be confirmed whether this association is reproducible in a larger number of Japanese PBC patients, as well as in other ethnic populations.

Table 8 Multivariate analysis for the progression to jaundice stage

Factor	*P* value	OR	95 % CI
rs683369 (risk genotype)[a]	0.087	2.29	0.89–5.92
gp210 positive	0.0001	12.84	3.71–44.51
Sex (male)	0.312	1.74	0.59–5.08
rs2282143 (risk genotype)[a]	0.062	4.97	0.92–26.73
gp210 positive	0.0001	12.97	3.73–45.06
Sex (male)	0.335	1.71	0.57–5.10
rs622342 (risk genotype)[a]	0.010	10.14	1.75–58.71
gp210 positive	0.0001	13.75	3.86–49.04
Sex (male)	0.799	1.17	0.34–3.99

Abbreviations: OR, odds ratio; CI, confidence interval

[a] rs683369 (C/G, G/G), rs2282143 (T/T), rs622342 (C/C)

OCT-1 is one of the most abundant transporters responsible for the uptake of choline from sinusoidal blood across the basolateral membrane of hepatocytes. Following the synthesis of PC from choline in hepatocytes, PC is secreted into bile ducts via MDR3 [32, 33, 38, 43]. Secreted PC reduces the cytotoxic effects of bile acids by combining with them to form micelles [34–37]. In addition, some reports have shown that the amount of OCT-1 protein in hepatocytes is decreased during cholestasis [39, 44, 45]. It has been reported that 69 of approximately 650 SNPs identified in the *OCT-1* gene are accompanied by amino acid changes. Approximately 15 % of these non-synonymous 69 SNPs are reportedly associated with alterations in OCT-1 expression and/or function in vitro [39]. Collectively, these reports indicate that genetic polymorphisms of *OCT-1* are potentially involved in the pathogenesis of cholestatic liver diseases, including PBC. Our own results are consistent with this suggestion, demonstrating for the first time that variant genotypes of rs683369 and rs622342 contribute to the more severe progression of PBC (i.e., jaundice-type progression).

In addition to choline, several drugs, including metformin, amantadine, and levodopa are substrates of OCT-1 [46–48]. Several reports have investigated the relation between *OCT-1* genetic polymorphisms and the effects of these drugs. For example, the effect of metformin is decreased in patients with the rs622342 SNP genotype "C/C" [40]. Since metformin must be transported into hepatocytes in order to exert its antihyperglycemic effect, it is possible that this transport does not occur at sufficient levels in patients with the rs622342 SNP genotype "C/C". Thus, it is reasonable to speculate that patients with the rs622342 SNP genotype "C/C" who have altered function of OCT-1 show more severe PBC progression due to decreased supply of choline into hepatocytes, resulting in insufficient secretion of PC into bile ducts. In fact, an insufficient supply of choline into hepatocytes is observed in PBC livers at the mRNA and protein levels (manuscript in preparation). *OCT-1* rs683369 is a non-synonymous SNP (OCT-1 Phe160Leu) that influences the mRNA expression of OCT-1, but not its transporter activity or its affinity to its substrates (e.g., metformin, 1-methyl-4-phenylpyridinium) [39, 49, 50]. On the other hand, the rs622342 SNP is located in an intron, and its exact influence on OCT-1 expression and function is unknown. *OCT-1* rs683369 and rs622342 SNPs are in linkage disequilibrium (linkage disequilibrium coefficient, $D' = 0.826$, $r^2 = 0.365$). In addition, new genetic polymorphisms that influence the expression, location, and function of OCT-1 have recently been reported in East Asian populations. However, the degree of their linkage

disequilibrium with the rs683369 and rs622342 SNPs is still unknown [51]. Collectively, these findings suggest the possibility that other still unidentified genetic polymorphisms in linkage disequilibrium with the rs683369 and/or rs622342 SNPs contribute to the severe progression of PBC.

Since the presence of anti-gp210 antibodies is the strongest risk factor so far identified for the progression to jaundice-stage in PBC [2, 5, 52], the risk of three *OCT-1* SNPs ("C/G" or "G/G" genotype at rs683369, "T/T" genotype at rs2282143 and "C/C" genotype at rs622342) for the progression to jaundice-stage were evaluated by multivariate analysis in conjunction with anti-gp210 antibodies-status. While the positive anti-gp210 antibodies-status revealed the most significant risk factor for the progression to jaundice-stage, only the "C/C" genotype at rs622342 remained a significant risk factor for this progression. The "C/G" or "G/G" genotype at rs683369 and "T/T" genotype at rs2282143 showed a trend of risk for jaundice-stage progression. These results indicate that the three SNPs (*OCT-1* rs683369, rs2282143 and rs622342) might be risk factors for jaundice-stage progression, independent from anti-gp210 antibodies-status.

In conclusion, our results clearly indicate that *OCT-1* genetic polymorphisms are closely associated with the severe progression (i.e., jaundice-type progression) of PBC. This implies that the genotyping of *OCT-1* could be potentially useful for DNA-based diagnosis in Japanese patients with PBC as a genetic biomarker for predicting the progression and prognosis.

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

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