Table 2. Variables Associated with the Presence of Significant Fibrosis (F2-4) and Severe Fibrosis (F3-4) by Univariate and Multivariate Analysis

Features	No Significant Fibrosis (n = 89)	Significant Fibrosis (n = 94)	P Value (Univariate)	Odds Ratio (95% CI) (Multivariate)	No Severe Fibrosis (n = 135)	Severe Fibrosis (n = 48)	P Value	Odds Ratio (95% CI) (Multivariate)
Age (years)	54.7 ± 11.8	60.5 ± 10.4	0.001		55.8 ± 11.9	62.9 ± 7.8	0.001	1.15 (1.02-1.31)
Male sex (%)	30 (33.7)	45 (47.9)	0.051		52 (38.5)	23 (47.9)	0.255	
AST (IU/L)	45.7 ± 41.6	68.3 ± 43.5	<0.0001		49.7 ± 40.1	79.1 ± 47.4	<0.0001	
ALT (IU/L)	51.0 ± 56.6	74.0 ± 54.9	<0.0001		55.9 ± 54.9	82.5 ± 57.9	<0.0001	
GGT (IU/L)	40.6 ± 61.7	62.1 ± 63.1	<0.0001		45.5 ± 67.1	65.8 ± 46.7	<0.0001	
Bilirubin (mg/dL)	0.6 ± 0.3	0.7 ± 0.4	0.014		0.6 ± 0.3	0.8 ± 0.4	0.005	
Albumin (g/L)	4.2 ± 0.3	4.0 ± 0.5	<0.001		4.2 ± 0.3	3.8 ± 0.5	<0.0001	
Cholinesterase (IU/L)	329.2 ± 76.0	247.2 ± 96.9	<0.0001		312.4 ± 84.4	217 ± 91.9	<0.0001	
Cholesterol (mg/dL)	181.0 ± 31.5	167.5 ± 36.2	0.005		178.1 ± 34.1	162.4 ± 33.5	0.016	
Platelets (10^9/L)	186 ± 53	142 ± 52	<0.0001	0.87 (0.77-0.99)	180 ± 52	119 ± 46	<0.0001	0.74 (0.58-0.94)
Prothrombin time (%)	94.7 ± 33.4	80.1 ± 32.1	0.0001		89.5 ± 36.2	80.8 ± 23.2	<0.001	
α2-MG (g/L)	326 ± 117.7	389.2 ± 141.1	0.002		331.1 ± 122.5	423.9 ± 137.5	<0.0001	
HA (μg/L)	85.6 ± 154.3	318.7 ± 556.1	<0.0001	1.01 (1.01-1.02)	115.4 ± 201.1	458.2 ± 711.0	<0.0001	
TIMP1 (pg/ml)	183.5 ± 53.3	238.6 ± 106.1	<0.0001		189.7 ± 64.5	263.9 ± 113.8	<0.0001	
AOL/DSA	1.4 ± 1.2	10.9 ± 15.9	<0.0001	1.51 (1.07-2.15)	2.0 ± 2.6	18.3 ± 19.3	<0.0001	
MAL/DSA	10.6 ± 1.7	7.5 ± 3.4	<0.0001		10.2 ± 2.0	5.6 ± 3.4	<0.0001	0.52 (0.37-0.76)

95% CI: 0.77-0.99), HA (OR: 1.01, 95% CI: 1.01-1.02), and AOL/DSA (OR: 1.51, 95% CI: 1.07-2.15) were independently associated with the presence of significant fibrosis.

Comparison of Variables Associated with the Presence of Severe Fibrosis by Univariate and Multivariate Analysis. Variables associated with the presence of severe fibrosis were assessed by univariate and multivariate analysis (Table 2). The variables of age ($P = 0.001$), AST ($P < 0.0001$), ALT ($P < 0.0001$), GGT ($P < 0.0001$), bilirubin ($P = 0.005$), α2-MG ($P < 0.0001$), HA ($P < 0.0001$), TIMP1 ($P < 0.0001$), and AOL/DSA ($P < 0.0001$) were significantly higher in the severe fibrosis group than in the no severe fibrosis group. The variables albumin ($P < 0.0001$), cholinesterase ($P < 0.0001$), cholesterol ($P = 0.016$), platelets ($P < 0.0001$), prothrombin time ($P < 0.001$), and MAL/DSA ($P < 0.0001$) were significantly lower in the severe fibrosis group than in the no severe fibrosis group. Multivariate analysis showed that age (OR: 1.15, 95% CI: 1.02-1.31), platelets (OR: 0.74, 95% CI: 0.58-0.94), and MAL/DSA (OR: 0.52, 95% CI:

Table 3. Variables Associated with the Presence of Cirrhosis (F4) by Univariate and Multivariate Analysis

Features	No Cirrhosis (n=157)	Cirrhosis (n = 26)	P Value	Odds Ratio (95% CI) (Multivariate)
Age (years)	56.6 ± 11.7	63.8 ± 7.3	0.0016	
Male sex (%)	60 (38.2)	15 (57.7)	0.061	
AST (IU/L)	54.6 ± 41.7	74.9 ± 53.7	0.016	
ALT (IU/L)	62.1 ± 58.1	67.2 ± 48.2	0.446	
GGT (IU/L)	48.5 ± 63.9	64.9 ± 53.8	0.0031	
Bilirubin (mg/dL)	0.6 ± 0.3	1.0 ± 0.5	<0.0001	
Albumin (g/L)	4.2 ± 0.4	3.6 ± 0.5	<0.0001	
Cholinesterase (IU/L)	305.3 ± 83.9	181.7 ± 90.1	<0.0001	
Cholesterol (mg/dL)	178.4 ± 33.3	146.9 ± 29.8	<0.0001	
Platelets (10^9/L)	172 ± 54	106 ± 36	<0.0001	0.76 (0.58-0.99)
Prothrombin time (%)	88.7 ± 35.5	79.2 ± 16.1	0.0004	
α2-MG (g/L)	346.2 ± 131.6	416.9 ± 127.8	0.019	
HA (μg/L)	137.1 ± 215.7	617.4 ± 915.1	<0.0001	
TIMP1 (pg/ml)	196.4 ± 70.4	287.3 ± 126.6	<0.0001	
AOL/DSA	3.4 ± 7.1	24.0 ± 20.4	<0.0001	
MAL/DSA	9.8 ± 2.4	4.2 ± 2.8	<0.0001	0.67 (0.49-0.90)

0.37-0.76) were independently associated with the presence of severe fibrosis.

Comparison of Variables Associated with the Presence of Cirrhosis by Univariate and Multivariate Analysis. Variables associated with the presence of cirrhosis were assessed by univariate and multivariate analysis (Table 3). Age ($P = 0.0016$), AST ($P = 0.016$), GGT ($P = 0.0031$), bilirubin ($P < 0.0001$), α2-MG ($P = 0.019$), HA ($P < 0.0001$), TIMP1 ($P < 0.0001$), and AOL/DSA ($P < 0.0001$) were significantly higher in the cirrhosis group than in the no cirrhosis group. Albumin ($P < 0.0001$), cholinesterase ($P < 0.0001$), cholesterol ($P < 0.0001$), platelets ($P < 0.0001$), prothrombin time ($P = 0.0004$), and MAL/DSA ($P < 0.0001$) were significantly lower in the cirrhosis group than in the no cirrhosis group. Multivariate analysis showed that platelets (OR: 0.76, 95% CI: 0.58-0.99) and MAL/DSA (OR: 0.67, 95% CI: 0.49-0.90) were independently associated with the presence of cirrhosis.

Evaluation of the Two Glyco-Parameters AOL/DSA and MAL/DSA for Estimating the Progression of Liver Fibrosis. To assess the correlation of the two obtained glyco-parameters with the progression of fibrosis, we analyzed the data of triple lectins from HISCL measurements on the 183 CHC patients. The boxplots of AOL/DSA and MAL/DSA in relation to the fibrosis staging are shown in Fig. 1A,B, respectively. The AOL/DSA values gradually increased with the progression of fibrosis and Pearson's correlation efficient was R = 0.61. On the other hand, the MAL/DSA values gradually decreased with the progression of fibrosis and Pearson's correlation efficient was R = −0.69. Both parameters fitted the quantification of the progression of fibrosis from F2 to F4.

LecT-Hepa, Combined with Two Glyco-Parameters, Was Evaluated in the Diagnosis of Significant Fibrosis, Severe Fibrosis, and Cirrhosis. LecT-Hepa was calculated using two glyco-parameters (AOL/DSA and MAL/DSA). The boxplots of LecT-Hepa in relation to the fibrosis staging are shown in Fig. 2. The LecT-Hepa values gradually increased with the progression of fibrosis. Pearson's correlation coefficient between LecT-Hepa and liver fibrosis was very high (R = 0.72), and was superior to those for AOL/DSA (R = 0.61) and MAL/DSA (R = −0.69). We next examined AUC to characterize the diagnostic accuracy of LecT-Hepa at each stage of fibrosis, i.e., significant fibrosis (F2/F3/F4), severe fibrosis (F3/F4), and cirrhosis (F4). For the prediction of significant fibrosis, AUC (95% CI), sensitivity, specificity, PPV, NPV, LR (+), and LR (−) of the test were 0.802 (0.738-0.865), 59.6%, 89.9%, 85.7%, 66.7%, 5.89, and 0.45,

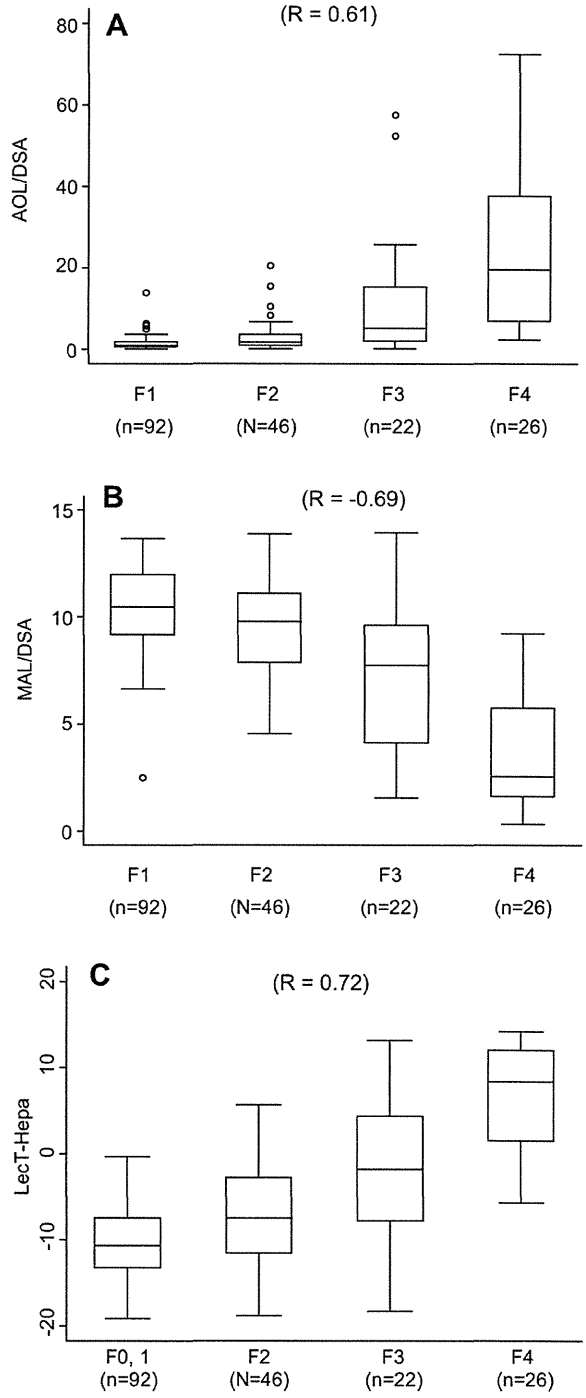
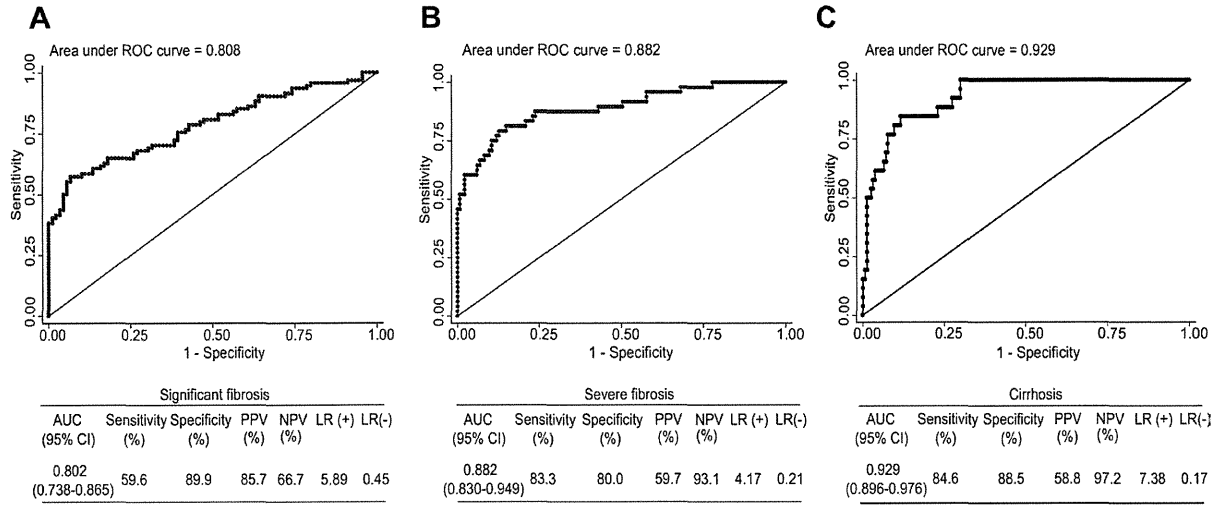


Fig. 1. Boxplot of (A) AOL/DSA, (B) MAL/DSA, and (C) LecT-Hepa in relation to the fibrosis score. The box represents the interquartile range. The whiskers indicate the highest and lowest values, and the dots represent outliers. The line across the box indicates the median value. Correlation of AOL/DSA, MAL/DSA, and LecT-Hepa was measured by HISCL with the progression of liver fibrosis. R: Pearson's correlation coefficient.



Significant fibrosis						
AUC (95% CI)	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	LR (+)	LR(-)
0.802 (0.738-0.865)	59.6	89.9	85.7	66.7	5.89	0.45

Severe fibrosis						
AUC (95% CI)	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	LR (+)	LR(-)
0.882 (0.830-0.949)	83.3	80.0	59.7	93.1	4.17	0.21

Cirrhosis						
AUC (95% CI)	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	LR (+)	LR(-)
0.929 (0.896-0.976)	84.6	88.5	58.8	97.2	7.38	0.17

Fig. 2. ROC curves of LecT-Hepa to distinguish between significant fibrosis and no significant fibrosis in patients with chronic hepatitis C (A); severe fibrosis and no severe fibrosis (B); cirrhosis and no cirrhosis (C). AUC: area under the receiver operating characteristic curve; PPV: positive predictive values; NPV: negative predictive values; LR (+): positive likelihood ratio; LR (−): negative likelihood ratio.

respectively (Fig. 3A). For the prediction of severe fibrosis, AUC (95% CI), sensitivity, specificity, PPV, NPV, LR (+), and LR (−) were 0.882, 83.3%, 80.0%, 59.7%, 93.1%, 4.17, and 0.21, respectively (Fig. 3B). For the prediction of cirrhosis, AUC (95% CI), sensitivity, specificity, PPV, NPV, LR (+), and LR (−) were 0.929 (0.896-0.976), 84.6%, 88.5%, 58.8%, 97.2%, 7.38, and 0.17, respectively (Fig. 3C).

Comparison of AUC, Sensitivity, Specificity, PPV, and NPV for Predicting the Diagnosis of Significant Fibrosis, Severe Fibrosis, and Cirrhosis. ROC curves of LecT-Hepa, HA, TIMP1, platelets, APRI, Forns index, Fib-4 index, and Zeng's score for predicting significant fibrosis, severe fibrosis, and cirrhosis were plotted, as shown in Fig. 3A-C. The AUC of LecT-Hepa for predicting significant fibrosis (0.802) was superior to HA (0.756), TIMP1 (0.697), platelets (0.729), APRI (0.777), Fib-4 index (0.747), Forns index (0.783), and Zeng's score (0.791). For predicting severe fibrosis, AUC of LecT-Hepa (0.882) was superior to HA (0.839), TIMP1 (0.753), platelet count (0.821), APRI (0.840), Fib-4 index (0.811), Forns index (0.861), and Zeng's score (0.863). For predicting cirrhosis, AUC of LecT-Hepa (0.929) was superior to HA (0.866), TIMP1 (0.783), platelets (0.851), APRI (0.787), Fib-4 index (0.856), Forns index (0.887), and Zeng's score (0.853). Sensitivity, specificity, PPV, and NPV by eight noninvasive tests and markers are shown in Table 4. In general, indicators of LecT-Hepa were superior to other noninvasive tests and markers. Specificity and PPV used to distinguish significant fibrosis in LecT-Hepa were superior to those in other tests and markers, although sensitivity and NPV by LecT-Hepa (59.6% and 66.7%, respectively) to distinguish significant fibrosis were inferior to those in other tests and markers. When distinguishing severe fibrosis, the categories of sensitivity (83.3%), specificity (80.0%), PPV (59.7%), and NPV (93.1%) for LecT-Hepa were superior to those in other tests and markers, except for specificity (82.2%) and PPV (61.0%) in HA. When distinguishing cirrhosis, the categories of sensitivity (84.6%), specificity (88.5%), PPV (58.8%), and NPV (97.2%) in LecT-Hepa were superior to those in other tests and markers, except for sensitivity by HA (88.5%), Forns index (84.6%), and Zeng's score (92.3%) and NPV by Zeng's score (98.3%).

Discussion

Our results showed that the LecT-Hepa test, calculated by combining two glyco-parameters (AOL/DSA and MAL/DSA), had higher sensitivity and specificity for diagnosing severe fibrosis and cirrhosis compared to other noninvasive tests and markers for these conditions. The new glyco-marker we have developed is based on the glyco-alteration on the AGP, which is mainly synthesized in the liver. AGP has been considered one of the best candidates for glyco-markers in liver fibrosis or HCC. This is because it is a well-characterized glycoprotein with five highly branched, complex-type *N*-glycans, whose alteration (e.g., desialylation, increased branching, and increased fucosylation) occurs during the progression of liver fibrosis and carcinogenesis.[24] It has already been reported that an

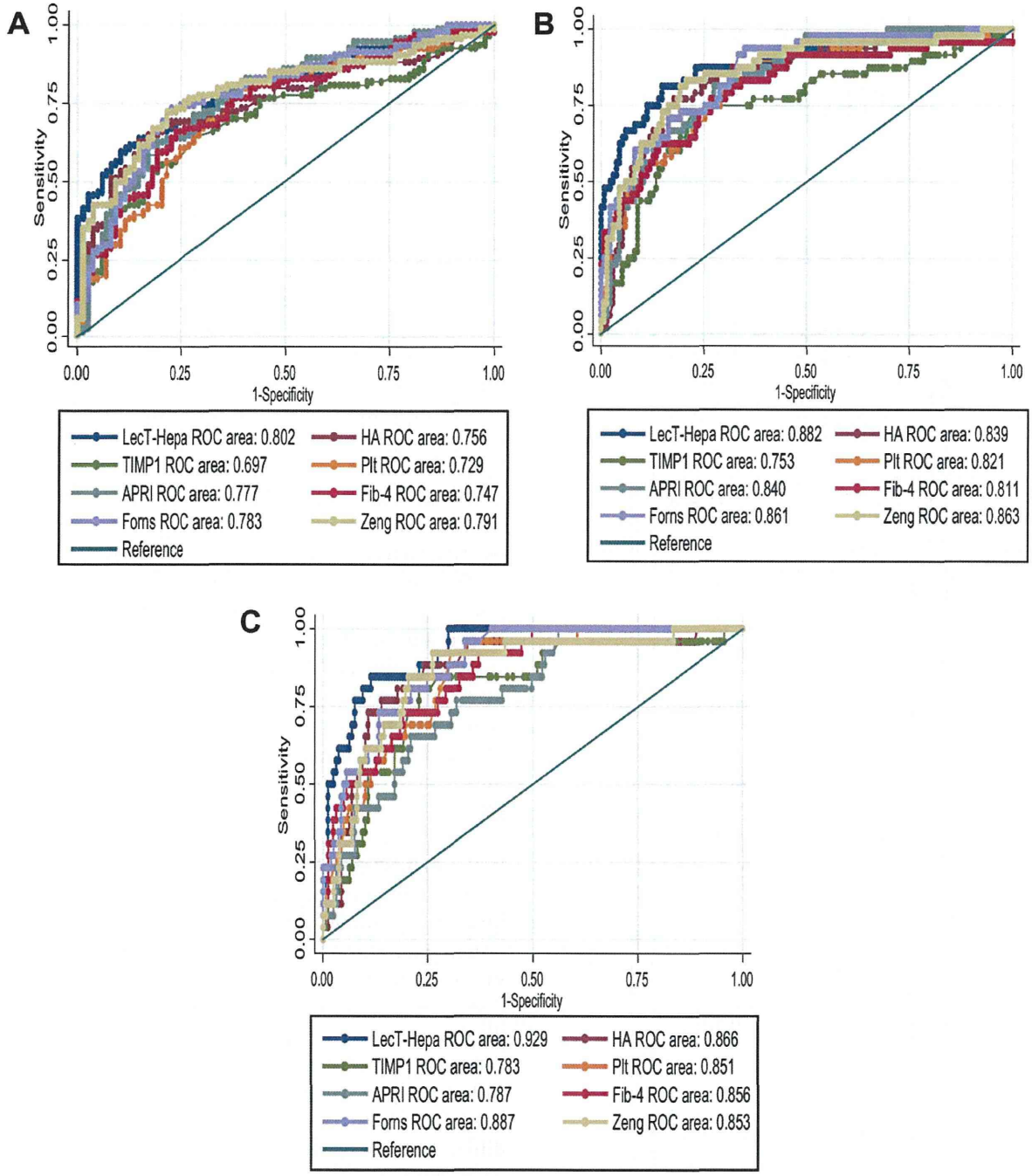


Fig. 3. Comparison of ROC curves in the performance of LecT-Hepa, HA, TIMP1, Plt, APRI, Fib-4 Index, Forns index, Zeng's score for the diagnosis of significant fibrosis (A), severe fibrosis (B), and cirrhosis (C). ROC: receiver operating characteristic curve; TIMP1: tissue inhibitors of metalloproteinases 1; Plt: platelet count; HA: hyaluronic acid.

increased degree of fucosylation was detected in cirrhosis patients using a fucose-binding lectin (AAL)-antibody sandwich ELISA and an automated analyzer.[24] The detection of asialo-AGP using lactosamine-recognition lectin RCA120 has also been reported as an alternative method for finding cirrhosis.[25] Meanwhile, we detected many other aspects of glyco-alteration of AGP using a multiplex sandwich immunoassay with a 43-lectin microarray,[26] resulting in the selection of three lectins—MAL, AOL, and DSA—to serve, collectively, as a fibrosis indicator and a signal normalizer.[14] Since two glyco-parameters (AOL/DSA and MAL/

Table 4. Diagnostic Performance of Biochemical Markers and Scores by Stage of Fibrosis

	No Significant Fibrosis (F0-1) vs. Significant Fibrosis (F2-4)				No Severe Fibrosis (F0-2) vs. Severe Fibrosis (F3-4)				No Cirrhosis (F0-3) vs. Cirrhosis (F4)			
	AUC (95% CI)	Se (%)	Sp (%)	PPV (%)	NPV (%)	AUC (95% CI)	Se (%)	Sp (%)	PPV (%)	NPV (%)	AUC (95% CI)	Se (%)	Sp (%)	PPV (%)	NPV (%)
LecT-Hepa	0.802 (0.738-0.865)	59.6	89.9	85.7	66.7	0.882 (0.830-0.949)	83.3	80	59.7	93.1	0.929 (0.896-0.976)	84.6	88.5	58.8	97.2
HA	0.756 (0.684-0.827)	68.1	78.7	77.8	69.6	0.839 (0.771-0.908)	77.1	82.2	61	90.3	0.866 (0.790-0.942)	88.5	75.8	37.3	96.8
TIMP1	0.697 (0.619-0.774)	65.9	71.9	70.4	60.7	0.753 (0.665-0.841)	75	76.3	53	88.9	0.783 (0.710-0.887)	80.8	74.5	27.8	94.6
Platelets	0.729 (0.656-0.803)	78.7	61.9	68.5	73.5	0.821 (0.751-0.891)	81.3	70.4	49.4	91.3	0.851 (0.785-0.918)	84.6	70.7	32.3	95.8
APRI	0.777 (0.709-0.844)	71.3	71.9	72.2	68.8	0.840 (0.780-0.900)	81.3	72.6	50.6	91.5	0.787 (0.703-0.871)	76.9	68.2	27.9	93.9
Fib-4	0.747 (0.671-0.818)	65.9	76.4	74.7	68	0.811 (0.733-0.889)	77.1	73.3	50	89.2	0.856 (0.788-0.924)	73.1	80.9	37.5	94.1
Forns	0.783 (0.716-0.852)	73.4	77.5	77.5	73.4	0.861 (0.802-0.920)	81.3	71.1	50	91.4	0.887 (0.831-0.943)	84.6	75.2	36.1	96.7
Zeng	0.791 (0.723-0.858)	82.9	70.7	75	79.7	0.863 (0.799-0.925)	81.3	79.8	59.5	92.8	0.853 (0.783-0.933)	92.3	73.9	36.9	98.3

AUC, area under the ROC curve; CI, confidence interval; Se, sensitivity; Sp, specificity; PPV, positive predictive values; NPV, negative predictive values.

DSA) on AGP are normalized by an internal standard lectin (DSA), LecT-Hepa is not influenced by the amount of AGP. We confirmed that the use of this lectin set was statistically superior to the previously selected lectins (AAL and RCA120).

This triplex-sandwich immunoassay employing DSA/MAL/AOL lectins and an anti-AGP antibody from the lectin microarray has already been converted to a fully automated immunoassay analyzer (HISCL-2000i) for clinical use.[15] Pretreatment requires 3 hours, and quantifying the two glyco-parameters for the LecT-Hepa to use this automated analyzer takes 17 minutes. Currently, we can obtain data from LecT-Hepa to predict liver fibrosis on the same day of blood sample collection. This simple and reliable glycomarker may be suitable for clinical use, and may substitute for liver biopsy in some cases.

We are confident that our study samples are representative of most patients. The AUC scores for distinguishing significant fibrosis, severe fibrosis, and cirrhosis by APRI, HA, Fib-4 index, Forns index, and Zeng's score were not significantly different from those in previous studies.[11,27,28] Every serum sample in this study was obtained from a patient immediately before or no more than 2 months after liver biopsy. As many serum samples as possible were collected from each liver center to eliminate a selection bias in any center. Since we could not perform liver biopsy on the patients who had a tendency to develop hemorrhages, fewer samples of severe fibrosis and cirrhosis were collected than those of milder fibrosis. In fact, the population of fibrosis staging in this study was similar to that of a previous, large prospective study evaluating noninvasive fibrosis markers.[29] In addition, we did not include patients with obvious decompensated cirrhosis. This is because inclusion of patients with severe liver disease would have artificially improved the predictive values of the logistic function. On the other hand, we included many patients with mild histological features (48.6% with F0-1). Sampling variation poses potential difficulties, especially in the early stages of disease, when fibrosis might be unevenly distributed.

There are several advantages in using reliable noninvasive markers for assessing liver fibrosis. First, they can be used to accurately determine the appropriate time for initiating IFN treatment in CHC patients. These markers can also help monitor and assess the therapeutic efficacy of IFN treatment in improving liver function in cases of liver fibrosis and cirrhosis. Finally, these markers will be essential in the development of new, antifibrotic treatments. Recently, many directed or targeted therapies against liver fibrosis,

such as anti-transforming growth factor beta and anti-tumor necrosis factor alpha compounds have been developed.[30,31] To evaluate these new drugs, reliable and simple noninvasive fibrosis markers are needed. LecT-Hepa appears to be one of the most prominent candidates to serve as a marker for developing antifibrotic drugs.

In conclusion, both glyco-parameters (AOL/DSA and MAL/DSA) using lectins in a bedside, clinical chemical analyzer succeeded in the quantification of the progression of liver fibrosis. Using LecT-Hepa, the combination score of both AOL/DSA and MAL/DSA is a reliable method for determining fibrosis staging and can be a good substitute for liver biopsy.

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Data mining model using simple and readily available factors could identify patients at high risk for hepatocellular carcinoma in chronic hepatitis C

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Background & Aims: Assessment of the risk of hepatocellular carcinoma (HCC) development is essential for formulating personalized surveillance or antiviral treatment plan for chronic hepatitis C. We aimed to build a simple model for the identification of patients at high risk of developing HCC.

Methods: Chronic hepatitis C patients followed for at least 5 years (n = 1003) were analyzed by data mining to build a predictive model for HCC development. The model was externally validated using a cohort of 1072 patients (472 with sustained virological response (SVR) and 600 with nonSVR to PEG-interferon plus ribavirin therapy).

Results: On the basis of factors such as age, platelet, albumin, and aspartate aminotransferase, the HCC risk prediction model identified subgroups with high-, intermediate-, and low-risk of HCC with a 5-year HCC development rate of 20.9%, 6.3–7.3%, and 0–1.5%, respectively. The reproducibility of the model was confirmed through external validation ($r^2 = 0.981$). The 10-year HCC development rate was also significantly higher in the high- and intermediate-risk group than in the low-risk group (24.5% *vs.* 4.8%; $p < 0.0001$). In the high-and intermediate-risk group, the incidence of HCC development was significantly reduced in patients with SVR compared to those with nonSVR (5-year rate, 9.5% *vs.* 4.5%; $p = 0.040$).

Conclusions: The HCC risk prediction model uses simple and readily available factors and identifies patients at a high risk of HCC development. The model allows physicians to identify patients requiring HCC surveillance and those who benefit from IFN therapy to prevent HCC.

Keywords: Decision tree; Prediction; Pegylated interferon; Ribavirin; Risk.
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Introduction

Hepatocellular carcinoma (HCC) is the sixth most common cancer worldwide [1] and its incidence is increasing in many countries [2]. Chronic viral hepatitis is responsible for 80% of all HCC cases [2]. The need to conduct HCC surveillance should be determined according to the risk of HCC development because this surveillance is cost-effective only in populations with an annualized cancer development rate of $\geqslant 1.5\%$ [3]. The annualized rate of developing HCC from type C liver cirrhosis is 2–8% [4–6], indicating that this population with type C liver cirrhosis needs surveillance. However, the annualized rate of HCC development is <1.5% in patients with chronic hepatitis C but without cirrhosis and the benefit of surveillance for all patients with chronic hepatitis has not yet been established [3]. HCC surveillance may be needed for patients with advanced fibrosis because the risk of HCC development increases in parallel with the progression of liver fibrosis [7,8]. Liver biopsy is the most accurate means of diagnosing fibrosis, but a single liver biopsy cannot indicate long-term prognosis because liver fibrosis progresses over time. Serial liver biopsies are not feasible because of the procedure's invasiveness. Moreover, factors other than fibrosis, such as advanced age, obesity, sex, lower albumin, and low platelet counts, also contribute to the development of HCC from chronic hepatitis C [8–11]. Therefore, these factors must be considered while assessing the risk of HCC development.

A meta-analysis of controlled trials [12] has shown that interferon (IFN) therapy reduced the rate of HCC development in patients with type C liver cirrhosis. However, there was a marked heterogeneity in the magnitude of the prevention effect

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of IFN on HCC development among the studies, probably due to the large differences in the baseline rate of HCC development among the different trials [12]. Whether the incidence of HCC development could be reduced in all patients with chronic hepatitis C, especially in those without liver cirrhosis, remains to be elucidated.

Data mining analysis, unlike conventional statistical analysis, is performed in an exploratory manner without considering a predefined hypothesis. Decision tree analysis, the major component of data mining analysis, is used to extract relevant factors from among various factors. These relevant factors are then combined in an orderly sequence to identify rules for predicting the incidence of the target outcome [13]. Data mining analysis has been used to define prognostic factors in various diseases [14–20]. In the field of hepatic diseases, data mining analysis has proven to be a useful tool for predicting early response [21], sustained virological response (SVR) [22–25], relapse [26], and adverse events [27] in patients with chronic hepatitis C treated with pegylated interferon (PEG-IFN) plus ribavirin (RBV). The findings of data mining analysis are expressed as flowcharts and are therefore easily understood [28] and readily available for clinical use, even by physicians without a detailed understanding of statistics.

In the present study, data mining analysis was used to identify risk factors for HCC development in a cohort of patients with chronic hepatitis C who had been followed for at least 5 years. An HCC risk prediction model was constructed on the basis of simple and generally available tests because the goal was to make the model easy to use in the clinic. The suitability, reproducibility, and generalizability of the results were validated using the data of an external cohort that was independent of the model derivation cohort.

Cancer

Materials and methods

Patients

The model derivation cohort consisted of 1003 chronic hepatitis C patients without cirrhosis who had a non-sustained virological response (nonSVR) to previous IFN administered at the Musashino Red Cross Hospital and were followed for at least 5 years. Patients who had SVR or those who were followed for less than 5 years were not included. An analytical database on age, body mass index, albumin, aspartate aminotransferase (AST) levels, alanine aminotransferase (ALT) levels, γ-glutamyltransferase (GGT) levels, total bilirubin levels, total cholesterol levels, hemoglobin levels, and platelet count at the start of the observation was created. Histological data such as fibrosis stage, activity grade, or degree of steatosis was not included in the database because the goal of the present study was to make the model on the basis of simple and generally available tests. The patients who developed HCC more than 5 years after the start of the observation were considered not to have developed HCC by the 5-year point because the model was intended to predict HCC development within 5 years. The 1072 chronic hepatitis C patients included in the external validation cohort were treated with PEG-IFN and RBV at the University of Yamanashi, Tokyo Medical and Dental University, Osaka University, Osaka City University, Nagoya City University, or Toranomon Hospital and followed for at least 5 years. Among them, 600 had nonSVR and 472 had SVR. Data from nonSVR patients in this external cohort were used for external validation of the HCC prediction model. To assess the preventive effect of PEG-IFN plus RBV therapy on HCC development, the cumulative HCC development rate was compared between SVR and nonSVR patients in the external validation cohort after stratification by the risk of HCC development as determined by data mining analysis. 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 review committees of all concerned hospitals.

HCC surveillance and diagnosis

HCC surveillance was conducted by performing abdominal ultrasonography every 4–6 months. Contrast-enhanced computer tomography, magnetic resonance imaging, or angiography were performed when abdominal ultrasonography suggested a new lesion suspicious for HCC. Classical HCC was diagnosed for tumors showing vascular enhancement with washout on at least two types of diagnostic imaging. Tumor biopsy was used to diagnose tumors with non-classical imaging findings.

Statistical analysis

The IBM-SPSS Modeler 13 (IBM SPSS Inc., Chicago, IL, USA) was used for decision tree analysis. The statistical methods used have been described previously [21,22,24–27]. In brief, the software searched the analytical database for the factor that most effectively predicted HCC development and for its cutoff value. The patients were divided into two groups according to that predictor. Each divided group was repeatedly assessed and divided according to this 2-choice branching method. Branching was stopped when the number of patients decreased to $\leqslant 20$ to avoid over fitting. Finally, an HCC risk prediction model was created through this analysis. The model classified patients into subgroups with different HCC development rates in a flowchart form. For model validation, nonSVR patients from an external cohort were individually fitted into the model and classified into the subgroups and the HCC development rates of those subgroups were then calculated. The suitability and reproducibility of the model were validated by comparing the subgroup HCC development rates of the model derivation group to those of the validation group.

On univariate analysis, Student's *t*-test was used for continuous variables and Fisher's exact test was used for categorical data. Logistic regression was used for multivariate analysis. A log-rank test for Kaplan–Meier analysis was used to statistically test HCC development rates over time. *p*-Values of <0.05 were considered significant. SPSS Statistics 18 (IBM SPSS Inc.) was used for these analyses.

Results

Univariate and multivariate analysis of factors associated with HCC development

The baseline characteristics of patients are shown in Table 1. The 5-year HCC development rate in the model derivation group was 6.2%, which did not differ significantly from the rate of 6.0% in the nonSVR group of the external cohort, but the rate of 2.0% in the SVR group of the external cohort was significantly lower than that in the model derivation group ($p = 0.0003$) and the nonSVR group of the external cohort ($p = 0.0012$). On univariate analysis, the factors found to be associated with HCC development in the model derivation cohort were age, AST levels, albumin levels, total cholesterol levels, and platelet count. On multivariate analysis, age (odds ratio 1.086), albumin levels (odds ratio 0.248), and platelet count (odds ratio 0.842) were significant predictors of HCC development (Table 2).

HCC risk prediction model by data mining analysis

The results of decision tree analysis are presented in Fig. 1. Age was selected as the first predictor. The 5-year HCC development rate was 3.4% in younger patients (<60 years) and 8.6% in older patients ($\geqslant$60 years). The second predictor for younger patients (<60 years) was platelet count. The HCC development rate was 6.9% in patients with a lower platelet count ($<150 \times 10^9$/L) and 0.8% in patients with a higher count ($\geqslant 150 \times 10^9$/L). The second predictor for older patients ($\geqslant$60 years) was also platelet count. The HCC development rate was 13.1% in patients with a lower platelet count ($<150 \times 10^9$/L) and 1.8% in patients with a higher count ($\geqslant 150 \times 10^9$/L). The third predictor was albumin levels,

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Table 1. Baseline characteristics of patients for model deviation and external validation.

	Model derivation (n = 1003)	External cohort, non-SVR (n = 600)	External cohort, SVR (n = 472)
Sex: Male/Female*	463 (46%)/540 (54%)	306 (51%)/294 (49%)	299 (63%)/173 (37%)
Age (yr)	57.3 (11.1)	55.9 (9.6)	51.4 (10.6)
Body mass index (kg/m^2)	23.5 (3.2)	23.4 (3.3)	23.3 (3.1)
Albumin (g/dl)	4.1 (0.3)	4.0 (0.4)	4.0 (0.3)
AST (IU/L)	64.2 (36.5)	67.3 (43.8)	62.5 (48.3)
ALT (IU/L)	80.6 (55.1)	81.2 (62.3)	88.6 (82.1)
GGT (IU/L)	59.3 (50.5)	67.6 (65.1)	55.7 (71.2)
Total cholesterol (mg/dl)	172.1 (31.5)	168.2 (31.0)	174.3 (33.7)
Platelet (10^9/L)	154.0 (53.0)	153.7 (53.2)	176.6 (49.7)
Hemoglobin (g/dl)	13.3 (1.5)	14.2 (1.5)	14.4 (1.4)
HCC development within 5 years: n (%)*	62 (6.2%)	36 (6.0%)	10 (2.0%)

Data expressed as mean (standard deviation) unless otherwise indicated.
AST, aspartate aminotransferase; ALT, alanine aminotransferase; GGT, gamma-glutamyltransferase; HCC, hepatocellular carcinoma; SVR, sustained virological response.
*Data expressed as number of patients (percentage).

whose cutoff value was 3.75 g/dl in patients with a higher platelet count ($\geqslant 150 \times 10^9$/L). The HCC development rate was 6.3% when albumin levels were lower (<3.75 g/dl) and 1.5% when levels were higher ($\geqslant$3.75 g/dl). The cutoff value for albumin levels was 4.0 g/dl in patients with a lower platelet count ($<150 \times 10^9$/L). The HCC development rate was 20.9% when albumin levels were lower (<4.0 g/dl) and 6.4% when levels were higher ($\geqslant$4.0 g/dl). The fourth and final predictor was AST levels. The HCC development rate was 7.3% when AST levels were at least 40 IU/L and 0% when the levels were <40 IU/L. On the basis of this analysis, seven subgroups with a 5-year HCC development rate of 0–20.9% were identified. The area under the receiver operating characteristic curve according to the HCC risk prediction model was 0.817.

External validation of the HCC risk prediction model with an independent external cohort

Six hundred nonSVR patients from an external cohort were fitted into the HCC risk prediction model and classified into the seven subgroups. The 5-year HCC development rate of these subgroups was 0–17.9%. The HCC development rate in the individual subgroups of the model derivation group was closely correlated to that in the corresponding subgroups of the external validation group (Fig. 2; correlation coefficient $r^2 = 0.981$). The HCC development rate in the subgroup of patients with the highest risk of HCC development (high-risk group) according to the model older age ($\geqslant$60 years) with a lower platelet count ($<150 \times 10^9$/L) and lower albumin levels (<4.0 g/dl) was 20.9% in the model derivation group and 17.9% in the external validation group. The intermediate-risk group or the patients with an HCC development rate of at least 5% consisted of the following three subgroups: (1) older age ($\geqslant$60 years), lower platelet count ($<150 \times 10^9$/L), higher albumin levels ($\geqslant$4.0 g/dl), and higher AST levels ($\geqslant$40 IU/L); (2) older age ($\geqslant$60 years), higher platelet count ($\geqslant 150 \times 10^9$/L), and lower albumin levels (<3.75 g/dl); and (3) younger age (<60 years) and lower platelet count ($<150 \times 10^9$/L). In these intermediate-risk groups, the 5-year HCC development rate was 6.3–7.3% in the model derivation group and 5.3–7.9% in the external validation group. The low-risk group consisted of the following three subgroups: (1) younger age (<60 years) and higher platelet count ($\geqslant 150 \times 10^9$/L); (2) older age ($\geqslant$60 years), lower platelet count ($<150 \times 10^9$/L), higher albumin levels ($\geqslant$4.0 g/dl), and lower AST levels (<40 IU/L); and (3) older age ($\geqslant$60 years), higher platelet count ($\geqslant 150 \times 10^9$/L), and higher albumin levels ($\geqslant$3.75 g/dl). In these low-risk groups, the 5-year HCC development rate was 0–1.5% in the model derivation group and 0–2.9% in the external validation group.

Table 2. Multivariable analysis of factors associated with subsequent development of HCC within 5 years.

	Odds ratio	95% CI	*p* value
Age	1.086	1.029-1.146	0.003
Albumin	0.248	0.100-0.613	0.003
Platelet	0.842	0.769-0.921	<0.0001

CI, confidence interval.

Predictability of the HCC risk prediction model on HCC development rate beyond 5 years

Cumulative HCC development rates in the high-, intermediate-, and low-risk groups were compared over time using the Kaplan–Meier method. The 10-year rates were 28.9% in the high-risk group, 22.9% in the intermediate-risk group, and 4.8% in the low-risk group (Fig. 3A). The high and intermediate-risk group created by pooling data from the high- and intermediate-risk groups had a significantly higher cumulative HCC development rate than the low-risk group beyond 5 years (Fig. 3B; 5-year rate, 11.6% *vs.* 1.0%; 10-year rate, 24.5% *vs.* 4.8%; p <0.0001).

Effect of response to PEG-IFN plus RBV therapy in the reduction of HCC development: analysis stratified by the HCC risk prediction model

The 600 nonSVR patients and 472 SVR patients in the external cohort were fitted into the HCC risk prediction model and

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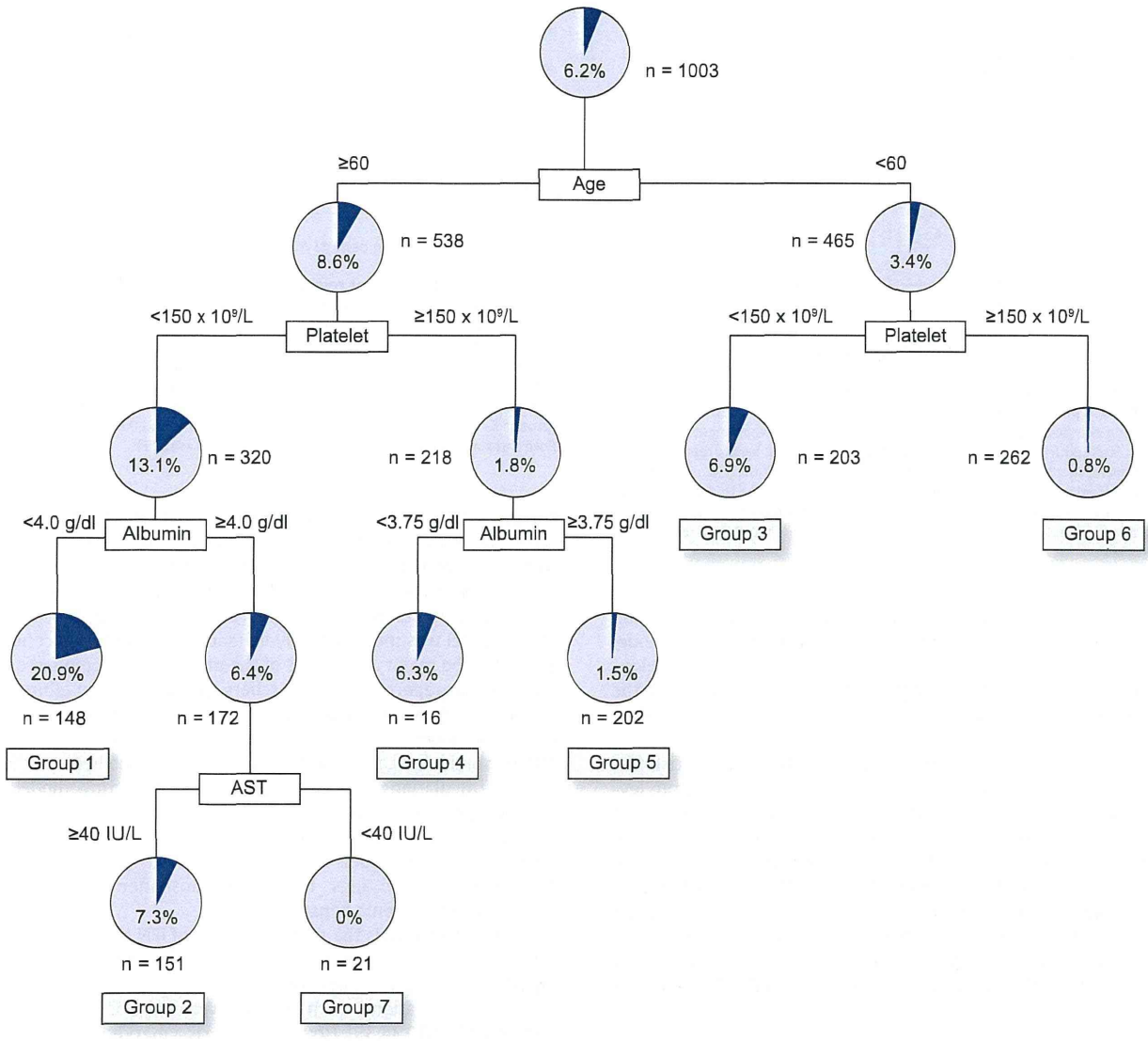

Fig. 1. The decision tree model of HCC development within 5 years. Boxes indicate the factors used to differentiate patients and the cutoff values for those different groups. Pie charts indicate the HCC development rate within 5 years for each group of patients after differentiation. Terminal groups of patients differentiated by analysis are numbered from 1 to 7.

classified into the high-and intermediate-risk group or the low-risk group, as defined above. The HCC development rate was significantly lower in SVR patients than in nonSVR patients in the high-and intermediate-risk group (5-year HCC rate, 9.5% *vs.* 4.5%; $p = 0.040$, log-rank test). In the low-risk group, the 5-year rate was 1.8% in nonSVR patients and 0.9% in SVR patients. Both rates were low and not significantly different ($p = 0.331$, log-rank test) (Fig. 4).

Discussion

An awareness of the risk of HCC development in the context of routine care for chronic hepatitis C is essential for formulating an HCC surveillance plan personalized for individual patients. The risk of developing HCC from chronic hepatitis is lower than that from cirrhosis [7]; therefore, across-the-board surveillance for chronic hepatitis C is not recommended [3]. A method to easily determine this risk, without performing serial liver biopsies, would be extremely significant clinically. In the present study, an HCC risk prediction model that included the factors such as age, platelet count, albumin levels, and AST levels was constructed. The model was found to have excellent reproducibility when validated with an external cohort. This model could identify subgroups of chronic hepatitis C patients at high risk of HCC development; the 5-year HCC development rate for the high- and intermediate-risk groups was 11.6%, yielding an annual incidence of 2.3%. This HCC risk prediction model requires only

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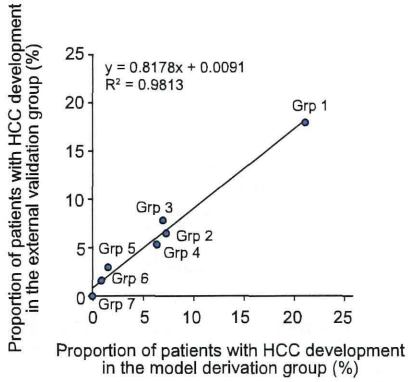

Fig. 2. External validation of the decision tree model with an independent cohort. Each patient in the external validation group was allocated to groups 1–7 following the flowchart of the decision tree. The HCC development rates were then calculated for each group and the graph plotted. The *x*-axis represents the HCC development rate in the model derivation group, and the *y*-axis represents the HCC development rate in the external validation group. The HCC development rates in each subgroup of patients are closely correlated between the model derivation group and the external validation group (correlation coefficient: $R^2 = 0.981$).

simple test values that are readily obtained in routine care and can therefore be easily used at the patient bedside. The model can be used to identify patients with a high risk of HCC development and therefore requiring surveillance, thereby allowing the formulation of surveillance plans personalized for individual patients.

Advanced fibrosis has been reported as independent risk factors for HCC development [7,8]. Platelet counts and albumin levels, which were factors selected for discrimination of the risk of HCC development, are closely related to the stage of fibrosis. Their correlation with the HCC risk has been repeatedly demonstrated [9–11,29–31]. The present study confirmed the impact of old age and advanced fibrosis, as reflected by low platelet counts and albumin levels. These results are consistent with our previous report [32]. What is unique to the present study was the study design to build a simple and reliable model for

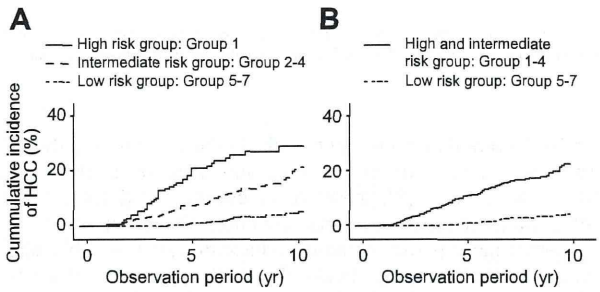

Fig. 3. Cumulative incidence of HCC development beyond 5 years in subgroups of patients defined by the decision tree model. Cumulative incidences of HCC in the groups classified by the decision tree model are compared. (A) The cumulative HCC development rate beyond 5 years is higher in the high- (group 1) and intermediate-risk (groups 2–4) groups compared to the low-risk group (groups 5–7). (B) The high and intermediate-risk group created by pooling data from the high- and intermediate-risk groups has a significantly higher cumulative HCC development rate than the low-risk group (5-year rate, 11.6% *vs.* 1.0%; 10-year rate, 24.5% *vs.* 4.8%; $p < 0.0001$).

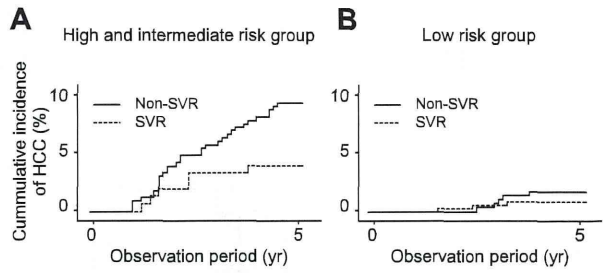

Fig. 4. Sustained virological response to PEG-IFN plus RBV therapy reduces the incidence of HCC development after stratification by the HCC risk. The 600 nonSVR patients and the 472 SVR patients in the external cohort were fitted into the HCC risk prediction model and classified into the high and intermediate-risk group or the low-risk group. The HCC development rate is significantly lower in SVR patients than in nonSVR patients in the high and intermediate-risk group (groups 1–4) (5-year HCC rate, 9.5% *vs.* 4.5%; $p = 0.040$). In the low-risk group (groups 5–7), the 5-year rate is 1.8% in nonSVR patients and 0.9% in SVR patients. Both rates are low and not significantly different ($p = 0.331$).

the prediction of HCC development that could be easily used in the clinic. For this purpose, a novel statistical method was used, histological factors were excluded in the analysis, the model derivation cohort was restricted to those who had nonSVR and had a long follow-up period duration (5 years), and the reproducibility of the model was independently validated by an external cohort. These are the major differences of the present study compared to our previous report. Many researchers have put a lot of efforts to formulate regression models for HCC prediction [9,10,33]. These prediction models are useful for identifying high-risk patients but are somewhat complicated to use at the bedside because they require calculations to be performed. Our prediction model is used simply by incorporating patients' data obtained through simple tests into the decision tree and following the flowchart. These prediction models based on factors easily accessible in routine clinical settings help physicians identify high-risk patients out of chronic hepatitis.

Viral eradication is the short-term goal of IFN therapy, but the ultimate goal is the prevention of HCC occurrence. Previous reports have shown that SVR to IFN therapy suppresses HCC occurrence in patients with type C liver cirrhosis and chronic hepatitis [7,12,30,34,35]. However, there is a marked heterogeneity in the magnitude of the treatment effect on the risk of HCC among studies, probably due to differences in the baseline risk of HCC among different trials [12]. Thus, the question remains whether the preventive effect of IFN therapy on HCC development could apply to all patients with chronic hepatitis C, especially those without liver cirrhosis. The result of the present study indicated that among high- and intermediate-risk patients, as assessed with our HCC risk prediction model, the cumulative HCC development rate was significantly reduced in SVR patients compared with nonSVR patients. This finding suggests that patients with chronic hepatitis, in whom disease has not yet progressed to hepatic cirrhosis but who are at a high risk of HCC development, benefit from antiviral treatment. The preventive effect of IFN on HCC development was not evident in low-risk patients within 5 years of observation. A longer observation term may be required to analyze the possible effect of antiviral therapy in these patients. Application of the present model on treatment decision may have limitations in that effect to prevent HCC development may differ in newer therapeutic agents such as protease

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inhibitors [36,37], and that low-risk patients may also benefit from therapy after a longer term observation period such as 15–20 years.

Patients with chronic hepatitis often have no subjective symptoms accompanying their disease and therefore have a low consciousness of the disease. The broad array of adverse reactions and the high cost of IFN therapy are frequent hurdles in motivating patients to undergo therapy. However, patients may be convinced to undergo therapy or remain motivated for continued therapy if they are made aware of their risk of HCC development and the preventive effect of IFN on HCC development.

In conclusion, a reproducible HCC risk prediction model, which includes the factors such as age, platelet count, albumin levels, and AST levels, was constructed to predict the 5-year HCC development rate in patients with chronic hepatitis C. The model requires only a combination of readily available test values and can therefore be easily used at the bedside. The information provided by the model allows the physician to identify patients requiring IFN therapy for the prevention of HCC and formulate plans for imaging HCC surveillance.

Conflict of interest

The authors who have taken part in this study declared that they do not have anything to disclose regarding funding or conflict of interest with respect to this manuscript.

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Amino Acid Substitutions in the Hepatitis C Virus Core Region Are Associated With Postoperative Recurrence and Survival of Patients With HCV Genotype 1b-Associated Hepatocellular Carcinoma

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Objective: We researched the molecular marker for prognosis of postoperative patients with hepatocellular carcinoma (HCC).

Background: The association of amino acid substitutions in the hepatitis C virus (HCV) core region and hepatocarcinogenesis has recently been explored. We investigated if these amino acid substitutions are associated with recurrence or survival in patients with HCC after attempted curative treatment by hepatectomy.

Methods: A total of 163 patients infected with HCV genotype 1b who previously underwent hepatectomy for primary, not recurrent HCC were analyzed. Amino acid substitutions in the HCV core region were measured by direct sequencing. Postoperative recurrence or survival rates were compared according to tumor characteristics, tumor markers, and amino acid substitutions in the core region.

Results: Recurrence rates after hepatectomy were higher in patients bearing a methionine at residue 91 of the HCV core region than in patients with leucine ($P = 0.0002$). Survival was also decreased in patients with methionine at this residue from that seen in patients with leucine at this position ($P = 0.0061$). The associations between amino acid substitutions at residue 91 of the HCV core region and either recurrence or survival rates were independent of liver function, progression of HCC, or tumor marker levels.

Conclusions: Amino acid substitutions at residue 91 of the HCV core region are associated with postoperative recurrence or survival in patients infected with HCV genotype 1b who developed HCC and treated by hepatectomy. This factor should be taken into consideration for the postoperative management of patients with HCC.

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Hepatocellular carcinoma (HCC) is the sixth most common cancer worldwide and the third most common cause of cancer-related death.[1,2] In Japan, HCC is the third and fifth most common causes of death from cancer in men and women, respectively.[3] One of the most important risk factors for the development of HCC[4,5] is chronic viral hepatitis. Hepatitis C virus (HCV) infection, one of main causes of chronic viral hepatitis, can result in liver cirrhosis and HCC.[6] The majority of patients in Japan with HCC exhibit chronic HCV infection.[7]

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Amino acid substitutions in the HCV core region, especially at residues 70 and/or 91 have been associated with poor responses to antiviral therapy with peginterferon and ribavirin as curative therapy for HCV (eradication of HCV) in patients infected with HCV genotype 1b.[8,9] More recently, an association between substitutions in this region and aspects of hepatocarcinogenesis, including the incidence of HCC and patient prognosis, has been reported.[10–13] In previous reports, researchers demonstrated a difference in the incidence of HCC according to amino acid differences in residues 70 and/or 91 of the HCV core region.[10–12] In addition, Ogura et al[13] reported that the mortality of patients with HCC was affected by amino acid substitutions at residue 91 in the HCV core region as well as the type of initial treatment or preservation of liver function. This study, however, included patients who underwent a variety of treatments, both surgical and nonsurgical, and only survival rate was analyzed. It remains unclear if amino acid substitutions in the HCV core region affect the prognosis of patients with HCC who have been treated with curative intent.

In this study, we evaluated the impact of amino acid substitutions in the HCV core region (residues 70 and 91) on the survival and recurrence rates in patients with HCC after hepatectomy with curative intent.

METHODS

Patients

A total of 969 patients were diagnosed with primary, not recurrent HCC between January 1999 and December 2008 at Ogaki Municipal Hospital. Of these patients, 331 patients were treated with hepatectomy. Decisions regarding individual treatments were made based on the treatment guidelines for HCC in Japan.[14] In all patients, HCC tumors were resected with ample margins; enucleation of tumors without margins was not performed. HCV infection was confirmed in 229 of the 331 patients by positive serum HCV RNA at the time of HCC diagnosis using PCR-based detection. HCV genotype was assessed in 209 patients with PCR amplifying the core gene sequences using genotype-specific primers,[15] determining that 166 patients had infections with HCV genotype 1b. We excluded 3 patients who had coinfection with hepatitis B virus to avoid a possible impact of HBV on outcomes, resulting in a final study population of 163 patients (Supplemental Digital Content 1, available at: http://links.lww.com/SLA/A144). A diagnosis of HCC was confirmed by pathologic diagnosis of resected specimens.

After hepatectomy, all patients were followed for 1.65 to 128.9 months (median follow-up period, 48.3 months) at our institution with US and CT or MRI performed every 3 to 6 months. Regular monitoring of serum tumor markers (alpha-fetoprotein [AFP], *lens culinaris* agglutinin-reactive AFP [AFP-L3], and des-gamma-carboxy prothrombin [DCP]) was performed every 3 months. When an elevation of tumor markers was detected, additional imaging examination (usually by CT or MRI) was performed to check for a

recurrence of HCC. If the presence of a recurrence was confirmed, patients underwent treatment for recurrent HCC based on the treatment guidlines. Recurrent HCC was categorized into intrahepatic metastasis and multicentric recurrence according to the previous study.[16] Intrahepatic metastasis was defined as recurrent tumors consisting of moderately or poorly differentiated HCC with the same or lower degree of differentiation compared with the differentiation of the primary tumors. Multicentric recurrence was defined according to previously reported criteria with some modifications[17] as follows: (1) The recurrent tumors consisted of well-differentiated HCC occurring in a different hepatic segment, than even moderately or poorly differentiated preexisting HCCs; (2) Both the primary and recurrent tumors were well-differentiated HCCs; and (3) The recurrent tumor contained regions of dysplastic nodule in peripheral areas.

The entire protocol was approved by the hospital institutional review board and carried out in compliance with the Helsinki Declaration.

Measurements of Amino Acid Substitutions of HCV Core Region

Amino acid substitutions in the core region of HCV were analyzed by direct sequencing of amino acids 1–191 of genotype 1b[8] using stored serum samples. HCV RNA was extracted from serum samples and were PCR amplified using the following primer pairs:

5'-GCCATAGTGGTCTGCGGAAC-3' (CC11: outer, sense primer)

5'-GGAGCAGTCCTTCGTGACATG-3' (e14: outer, antisense primer),

5'-GCTAGCCGAGTAGTGTT-3' (CC9: inner, sense primer), and

5'-GGAGCAGTCCTTCGTGACATG-3' (e14: inner, antisense primer).

Amplified PCR products were purified and used for direct sequencing. Sequencing results were used to detect substitutions of arginine or glutamine at amino acid 70 and leucine or methionine at amino acid 91.

Measurement of the Tumor Markers for HCC

Three tumor markers for HCC, AFP, AFP-L3, and DCP, were measured in serum samples taken at the time of HCC diagnosis. Serum AFP levels were determined by enzyme-linked immunosorbent assay using a commercially available kit (ELISA-AFP, International Reagents, Kobe, Japan). A cut-off value of 20 ng/mL AFP was used to define AFP positivity, as proposed by Oka *et al.*[18] Serum AFP-L3, expressed as the percentage of total AFP (AFP-L3 level/total AFP level × 100), was measured by lectin-affinity electrophoresis followed by antibody-affinity blotting (AFP Differentiation Kit L, Wako Pure Chemical Industries, Ltd., Osaka, Japan). The cut-off value used to establish AFP-L3 positivity was 10%, as proposed by Shimizu *et al.*[19] The serum DCP level was determined by a specific enzyme immunoassay (Eitest PIVKA-II kit, Eisai Laboratory, Tokyo, Japan) according to the manufacturer's instructions. The cut-off value used to establish DCP positivity was 40 mAU/mL, as proposed by Okuda *et al.*[20]

Statistical Analyses

Differences in percentages between groups were analyzed by the χ^2 test. Differences in mean quantitative values were analyzed by the Mann–Whitney *U* test. The date of treatment (hepatectomy) was defined as time zero for calculations of patient recurrence and survival rates. In the analysis of recurrence rate, patients in whom HCC recurred were noncensored, and those in whom HCC did not recur were censored. In the analysis of cancer specific survival rates, patients who died from HCC-related cause were noncensored and the other patients were censored. In the analysis of overall survival rate, all patients who died were not censored and surviving patients were censored. The Kaplan–Meier method[21] was used to calculate survival rates, whereas the log-rank test[22] was used to analyze differences in survival.

The Cox proportional hazards model[23] was used for univariate and multivariate analyses of factors related to recurrence and survival. Variables analyzed included patient age and gender, Child-Pugh class (A/B), tumor size (≤2 cm / >2 cm and ≤5 cm / >5 cm), number of tumors (single/multiple), macroscopic portal vein invasion (absent/present), amino acid substitutions of the HCV core region (at residue 70: arginine/glutamine, at residue 91: leucine/methionine), pretreatment serum AFP level (<20 ng/mL/≥20 ng/mL), pretreatment AFP-L3 proportion (<10%/≥10%), and pretreatment serum DCP level (<40 mAU/mL/≥40 mAU/mL). Data analyses were performed using the JMP statistical software, version 6.0 (Macintosh version; SAS Institute, Cary, NC, USA). All *P* values were derived from 2-tailed tests, with a $P < 0.05$ accepted as statistically significant.

RESULTS

Patients Characteristics

Table 1 summarizes the pretreatment characteristics of the study patients. This population was composed of 121 males and

TABLE 1. Characteristics of Study Patients (n = 163)

Age (mean ± SD, years) (range)	67.4 ± 7.1 (47–83)
Sex ratio (female/male)	42 (25.8)/121 (74.2)
Surveillance state at diagnosis (our institution/ others/ none)*	114 (69.9)/ 45 (27.6)/ 4 (2.4)
HCV infection	163 (100)
Child-Pugh class (A/B)†	152 (93.3)/ 11 (6.7)
Albumin (mean ± SD, g/dL)	3.85 ± 0.43
Total bilirubin (mean ± SD, mg/dL)	0.74 ± 0.35
15-minute retention rate of ICG (%)	15.5 ± 7.3
Prothrombin (%)	90.4 ± 14.3
Platelet (×1000/μL)	124 ± 52
Tumor size (mean ± SD, cm) (range)	2.93 ± 2.02 (0.6-7.6)
≤ 2 cm / > 2 cm and ≤ 5 cm / > 5 cm	63 (38.6)/ 80 (49.1)/ 20 (12.3)
Tumor number (single/multiple)	137 (84.0)/ 26 (16.0)
Macroscopic-portal vein invasion (absent/present)	143 (87.7)/ 20 (12.3)
Microscopic-portal vein invasion (absent/present)	133 (81.6)/ 30 (18.4)
AFP (median, ng/mL) (range)‡	18.0 (0.8–5280)
≥20 ng/mL / <20 ng/mL	84 (52.2)/ 77 (47.8)
AFP-L3 (median, %) (range)‡	0.5 (0–87.2)
≥ 10% / <10%	125 (82.8) /26 (17.2)
DCP (median, mAU/mL) (range)‡	42.0 (10–36,164)
≥ 40 mAU/mL / <40 mAU/mL	78 (49.4)/ 80 (50.6)
Interferon therapy after hepatectomy (no/yes)	139 (85.3)/ 24 (14.7)

Percentages were in parentheses.

HCV indicates hepatitis C virus; ICG, indocyanine green test; AFP, alpha-fetoprotein;AFP-L3, *lens culinaris* agglutinin-reactive AFP; DCP, des-gamma-carboxy prothrombin.

*Our institution, patients had been under surveillance at our institution before the detection of HCC; others, patients had been under surveillance at family physician before the detection of HCC; none, patients had not beem under surveillance and had admitted to our institution with symptoms.

†Category of Child-Pugh class A includes patients without cirrhosis.

‡AFP, AFP-L3, and DCP were not measured in 2, 12, and 5 patients, respectively.

42 females with a mean age of 67.4 ± 7.1 years. HCC was detected under surveillance at our institution in 69.9% of patients, and 93.3% of patients exhibited Child-Pugh class[24] A liver function. Multiple tumors were present in 16.0% of patients. Macroscopic portal vein invasion was observed in 11.7% of patients, whereas microscopic portal vein invasion was observed in 18.4% of patients. Pretreatment AFP, AFP-L3, and DCP were above the specified cut-off levels in 47.8%, 17.2%, and 50.6% of patients, respectively. Antiviral therapy with interferon was performed in 24 (14.7%) patients after hepatectomy.

Recurrence Rate after Hepatectomy Based on Amino Acid Substitutions of the HCV Core Region and Pretreatment Serum Tumor Markers

Sequencing of the HCV core region failed in 6 patients, preventing detection of amino acid substitutions at residues 70 and 91. At residue 70, 87 of 157 patients (55.4%) possessed arginine, whereas 70 patients (44.6%) had glutamine at that position. At residue 91, 97 of 157 patients (61.8%) had leucine and 60 patients (38.2%) had methionine.

We determined the rates of recurrence for patients after attentive curative treatment with hepatectomy based on the amino acid residue at position 91 of the HCV core region (Fig. 1). The recurrence rate for patients with methionine at this position was significantly higher than that of patients bearing a leucine ($P = 0.0002$). In contrast, we found no difference in recurrence according to amino acid substitutions at residue 70 (Supplemental Digital Content 2, available at: http://links.lww.com/SLA/A145). When we analyzed recurrence rates according to pretreatment tumor markers, there was no difference in recurrence rate according to pretreatment serum AFP, AFP-L3, or DCP levels (Supplemental Digital Content 3, available at: http://links.lww.com/SLA/A146). Univariate analysis identified tumor size (>5 cm), macroscopic portal vein invasion, and amino acid substitution at residue 91 of the HCV core region as factors that significantly associated with recurrence rate after hepatectomy. By multivariate analysis, these 3 factors were also selected as independent factors associated with increased recurrence rates (Table 2). Recurrent HCC was categorized into multicentric recurrence in 25 of 55 patients (45.5%) with recurrence bearing a leucine and in 33 of 48 patients (68.8%) with methionine, the prevalence being higher in patients with methionin ($P = 0.0292$).

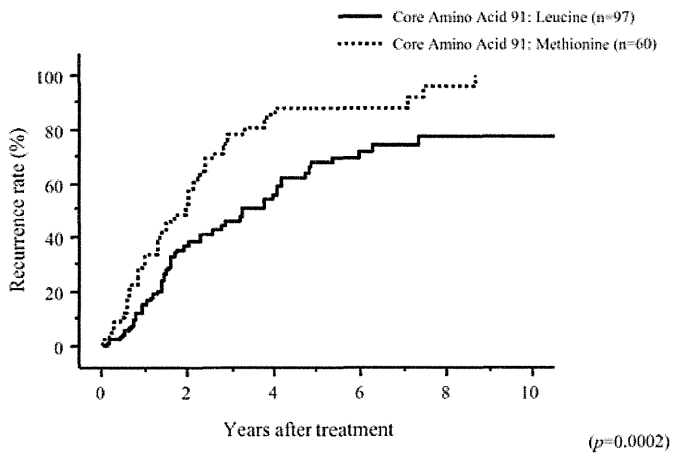

FIGURE 1. Recurrence rates after hepatectomy in patients bearing leucine (solid line) or methionine (dotted line) at residue 91 of the HCV core region. Recurrence rates were significantly higher in patients with methionine than in patients with leucine at residue 91 of the HCV core region ($P = 0.0002$).

Comparison of the characteristics of patients according to amino acid substitution at residue 91 of the HCV core region did not reveal differences in patient age, gender, liver function, the progression of HCC, or pretreatment AFP, AFP-L3, and DCP levels between patients with leucine and methionine at residue 91 (Table 3). The rate of patients who underwent postoperative interferon therapy and the rate of patients who achieved the eradication of HCV by interferon therapy were not different between these 2 groups.

Cancer Specific and Overall Survival Rates after Hepatectomy According to Amino Acid Substitutions of the HCV Core Region and Pretreatment Serum Tumor Markers

We determined the cancer specific and overall survival rates of patients after hepatectomy as a function of amino acid substitution at residue 91 of the HCV core region. The cancer specific survival of patients bearing methionine at residue 91 was significantly lower than that of patients with leucine at residue 91 ($P = 0.0010$, Supplemental Digital Content 4, available at: http://links.lww.com/SLA/A147) and consequently, overall survival of patients with methionine was significantly lower than that of patients with leucine ($P = 0.0061$, Fig. 2). In contrast, we did not find a difference in survival correlating with the amino acid substitution at residue 70 (Supplemental Digital Content 5, available at: http://links.lww.com/SLA/A148). When we analyzed patient survival rates according to pretreatment tumor markers, there was no difference in patient survival according to pretreatment serum AFP or DCP levels. We did identify a significant difference in survival associated with pretreatment AFP-L3 proportions ($P = 0.0473$) (Supplemental Digital Content 6, available at: http://links.lww.com/SLA/A150). We found significantly higher survival rate of patients who underwent postoperative antiviral therapy with interferon than that of patients who did not ($P = 0.0065$, Supplemental Digital Content 7, available at: http://links.lww.com/SLA/A151). Univariate analysis identified patient age, tumor number, macroscopic portal vein invasion, amino acid substitution at residue 91 of the HCV core region, and postoperative interferon therapy as factors significantly associated with survival after hepatectomy. By multivariate analysis, tumor number, portal vein invasion, amino acid substitution at residue 91 of the HCV core region, and postoperative interferon therapy were selected as independent factors associated with patient survival (Table 4).

DISCUSSION

The results of this study demonstrated that amino acid substitutions at residue 91 in the HCV core region were associated with recurrence and survival rates for patients with HCC bearing HCV genotype 1b infection who underwent hepatectomy with curative intent. Comparison of patient background characteristics, including liver function at diagnosis and HCC tumor progression (size, number, and portal vein invasion), did not reveal any difference between HCV genotype 1b-positive patients with leucine at residue 91 and those with methionine at that position, indicating that the differences in recurrence and survival seen for different amino acid substitutions at residue 91 were not due to the differences in liver function or tumor progression before treatment. Multivariate analyses identified that methionine substitution at this position was an independent factor associated with increased recurrence and decreased survival after hepatectomy. In previous reports, Akuta et al[10] and Nakamoto et al[12] studied the association between the incidence of primary, not recurrent HCC and amino acid substitutions in the HCV core region, combining substitutions at residues 70 and 91. They

TABLE 2. Univariate and Multivariate Analyses for Factors Associated with Postoperative Recurrence in HCC Patients Infected with HCV Genotype 1b (n = 157)

Factor		Univariate analysis	Multivariate analysis	Risk ratio (95% confidence interval)
Age		0.0731	–	
Sex	Male			
	Female	0.0892	–	
Child-Pugh class	A			
	B	0.7315	–	
Tumor size	≤2 cm			1
	>2 cm and ≤ 5 cm	0.1061	–	
	>5 cm	0.0018	0.0165	1.5254 (1.0869–2.0542)
Tumor number	Single			
	Multiple	0.5379	–	
Macroscopic-PV invasion	Absent			1
	Present	0.0011	0.0011	1.8240 (1.2972–2.4585)
Core-70 amino acid	Arginine			
	Glutamine	0.1130	–	
Core-91 amino acid	Leucine			1
	Methionine	0.0023	0.0207	1.2878 (1.0399–1.5895)
Pretreatment AFP	<20 ng/mL			
	≥20 ng/mL	0.6394	–	
Pretreatment AFP-L3	<10%			
	≥10%	0.0763	–	
Pretreatment DCP	<40 mAU/mL			
	≥40 mAU/mL	0.0643	–	
IFN therapy after hepatectomy	No			
	Yes	0.1859	–	

PV indicates portal vein; IFN, interferon; AFP, alpha-fetoprotein; AFP-L3, *lens culinaris* agglutinin-reactive AFP; DCP, des-gamma-carboxy prothrombin.

*Category of Child-Pugh class A includes patients without cirrhosis.

TABLE 3. Comparison of Clinical Characteristics of Study Patients Based on the Amino Acid Substitutions at Residue 91 of the HCV Core Region (n = 157)

	Core Amino Acid 91: Leucine (n = 97)	Core Amino Acid 91: Methionine (n = 60)	*P* value
Age (mean ± SD, years) (range)	66.8 ± 6.9 (47-79)	68.7 ± 7.3 (49-83)	0.0737
Sex ratio (female/male)	28 (28.9)/ 69 (71.1)	14 (23.3)/ 46 (76.7)	0.5642
Child-Pugh class (A/B)*	93 (95.9)/ 4 (4.1)	54 (90.0)/ 6 (10.0)	0.2581
Albumin (mean ± SD, g/dL)	3.85 ± 0.42	3.83 ± 0.45	0.5523
Total bilirubin (mean ± SD, mg/dL)	0.73 ± 0.33	0.76 ± 0.37	0.5546
15-minute retention rate of ICG (%)	15.7 ± 6.7	15.4 ± 8.4	0.8114
Prothrombin (%)	90.4 ± 14.2	90.1 ± 14.6	0.6752
Platelet (× 1000/μL)	127 ± 55	117 ± 46	0.1679
Tumor size (mean ± SD, cm) (range)	2.86 ± 2.25 (0.6-17.6)	2.93 ± 1.64 (0.7-11.0)	0.2020
≤2 cm/>2 cm and ≤5 cm/>5 cm	37 (38.2)/ 46 (47.4)/ 14 (14.4)	25 (41.7)/ 30 (50.0)/ 5 (8.3)	0.5047
Tumor number (single/multiple)	82 (84.5)/ 15 (15.5)	50 (83.3)/ 10 (16.7)	0.8414
Macroscopic-portal vein invasion (absent/present)	84 (86.6)/ 13 (13.4)	54 (90.0)/ 6 (10.0)	0.7003
Microscopic-portal vein invasion (absent/present)	77 (79.4)/ 20 (20.6)	51 (85.0)/ 9 (15.0)	0.5021
AFP (median, ng/mL) (range)†	16.0 (0.8-5280)	22.6 (0.8-3480)	0.1780
≥ 20 ng/mL / <20 ng/mL	53 (55.2)/ 43 (44.8)	27 (45.8)/ 32 (54.2)	0.3280
AFP-L3 (median, %) (range)†	0.5 (0-87.2)	0.5 (0-65.7)	0.0893
≥ 10% / <10%	77 (87.5)/11 (12.5)	43 (75.4)/ 14 (24.6)	0.0980
DCP (median, mAU/mL) (range)†	45.5 (10-36164)	40.0 (10-11638)	0.7514
≥ 40 mAU/mL / <40 mAU/mL	45 (47.9)/ 49 (52.1)	30 (51.7)/ 28 (48.3)	0.7684
Interferon therapy after hepatectomy (no/yes)	85 (87.6)/ 12 (12.4)	53 (88.3)/ 7 (11.7)	0.8954
Eradication of HCV by interferon therapy (no/yes)	94 (96.9)/ 3 (3.1)	57 (95.0)/ 3 (5.0)	0.8571

Percentages were in parentheses.

ICG indicates indocyanine green test; AFP, alpha-fetoprotein; AFP-L3, *lens culinaris* agglutinin-reactive AFP; DCP, des-gamma-carboxy prothrombin; HCV, hepatitis C virus.

*Category of Child-Pugh class A includes patients without cirrhosis.

†AFP, AFP-L3, and DCP were not measured in 2, 12, and 5 patients, respectively.

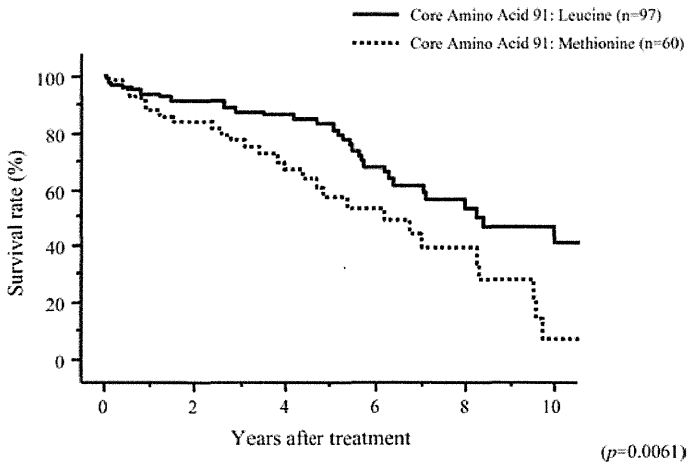

FIGURE 2. Overall survival rates after hepatectomy in patients bearing leucine (solid line) or methionine (dotted line) at residue 91 of the HCV core region. Survival rates were significantly lower in patients with methionine at residue 91 of the HCV core region than those with leucine at residue 91 ($P = 0.0061$).

TABLE 4. Univariate and Multivariate Analyses for Factors Associated with Postoperative Survival in HCC Patients Infected with HCV Genotype 1b (n = 157)

Factor		Univariate analysis	Multivariate analysis	Risk ratio (95% confidence interval)
Age		0.0198	0.1698	
Sex	Male			
	Female	0.0934	–	
Child-Pugh class	A			
	B	0.3398	–	
Tumor size	≤2 cm			
	>2 cm and ≤5 cm	0.3474	–	
	>5 cm	0.0898	–	
Tumor number	Single			1
	Multiple	0.0190	0.0434	1.3841 (1.0102–1.8466)
Macroscopic-PV invasion	Absent			1
	Present	0.0022	0.0031	1.8280 (1.2460–2.5621)
Core-70 amino acid	Arginine			
	Glutamine	0.1483	–	
Core-91 amino acid	Leucine			1
	Methionine	0.0063	0.0076	1.4517 (1.1063–1.8994)
Pretreatment AFP	<20 ng/mL			
	≥20 ng/mL	0.3632	–	
Pretreatment AFP-L3	<10%			
	≥10%	0.0617	–	
Pretreatment DCP	<40 mAU/mL			
	≥40 mAU/mL	0.5713	–	
IFN therapy after hepatectomy	No			1
	Yes	0.0013	0.0203	0.5052 (0.2036–0.9141)

PV indicates portal vein; IFN, interferon; AFP, alpha-fetoprotein; AFP-L3, *lens culinaris* agglutinin-reactive AFP; DCP, des-gamma-carboxy prothrombin.
*Category of Child-Pugh class A includes patients without cirrhosis.

reported that lower incidence of the development of HCC in patients bearing an arginine at residue 70 and a leucine at residue 91 (double-wild type) than in patients with other substitutions at these positions (nondouble-wild type). We also found significant difference in both recurrence and survival rates after hepatectomy between these 2 groups ($P = 0.0219$ and 0.0384, Supplemental Digital Content 8 and 9, available at: http://links.lww.com/SLA/A152 and http://links.lww.com/SLA/A153). However, the differences in recurrence and survival rates after hepatectomy were more marked when stratifying patients according to amino acid substitutions at residue 91 in the HCV core region, indicating different effects of the amino acid substitutions of the HCV core region between de novo HCC cases and recurrences after curative resection.

Pretreatment elevations of tumor markers for HCC, especially AFP-L3 and DCP, have been reported to indicate malignant potential of HCC tumor and be associated with higher recurrence rates and lower survival rates.[25,26] In this study, however, we were unable to identify differences in recurrence or survival according to pretreatment elevations of tumor markers, except for a mild association of AFP-L3 elevation and decreased survival rates. This may be due to a focus in our study on patients who underwent hepatectomy as a curative radical treatment and our exclusion of patients who underwent nonsurgical treatment or no treatment. Hepatectomy may overcome the malignant potential of HCC tumor associated with a pretreatment elevation of tumor markers.

Postoperative interferon therapy has been reported to decrease recurrence rate[27] and increase survival rate[28] after hepatectomy, especially in patients who achieved the eradication of HCV by the therapy. We found higher survival rates in patients who underwent postoperative interferon therapy than in those who did not. The effect

of interferon therapy to improve liver function might have contributed to the increased survival[28] in our study patients. In contrast, we failed to find the effect of interferon on the suppression of recurrence of HCC after hepatectomy. Recent studies revealed that the efficacy of interferon therapy is strongly associated with amino acid substitutions in the HCV core region,[8] mainly with amino acid substitutions at residue 70.[9,29] In contrast, our results showed the association between amino acid substitutions at residue 91 and postoperative recurrence and survival rates of patients with HCC after hepatectomy. Together with previous reports and our results, it seems that postoperative interferon therapy did not play a role in the association between postoperative recurrence and survival of patients with HCC and amino acid substitutions in the HCV core region observed in this study.

Previous studies examining the patterns of HCC recurrence in patients with HCV-related HCC reported that intrahepatic metastases are predominant within 2 to 3 years of treatment; multicentric recurrence of HCC becomes predominant after that period.[16] A comparison of the recurrence curve for patients with leucine at residue 91 of the HCV core region with that for patients bearing methionine at that position (Fig. 1) revealed that the difference in recurrence rates became marked 2 years after hepatectomy and suggests that amino acid substitution at residue 91 is associated with multicentric recurrence of HCC in patients with HCV genotype 1b infection. Indeed, the prevalence of multicentric recurrence was significantly higher in patients bearing a methionine at residue 91 in the HCV core region than in patients with leucine.

There are several limitations to this study. The study population included only patients infected with HCV genotype 1b; our study did not examine the association between amino acid substitutions at residue 91 and recurrence or survival rates in patients with other HCV genotypes. All patients were Mongoloid Japanese; these results, therefore, should be evaluated in other ethnicities to demonstrate the generalization of our findings. In addition, the percentage of macroscopic and microscopic portal vein invasion in the study patients was lower in comparison to HCC patients from Japanese general population.[30] This will be because of the high rate of patients in whom HCC was detected under surveillance at our liver center in this study; HCC was diagnosed in early stage in most of these patients.[31] Furthermore, as none of our patients were treated with liver transplantation, we have no data on the effect of amino acid substitutions at residue 91 on recurrence or survival in this subpopulation. Finally, the mechanism underlying the effect of this amino acid substitution on patient recurrence and survival remains unknown, which we hope will be investigated in the future to shed light on potential treatment approaches.

In conclusion, our examination of 163 patients treated by hepatectomy with curative intent revealed that amino acid substitution at residue 91 of the HCV core region influenced recurrence and survival after hepatectomy; patients bearing methionine at this position demonstrated higher recurrence and lower survival rates. Further studies will be needed to confirm this association in other population and elucidate the mechanism underlying this effect.

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