reported using this method, leading to misdiagnosis of cirrhosis.[8] Therefore, finding a noninvasive method for diagnosing liver fibrosis is an emerging issue in the care of patients with CHC.

Several methods have been studied for the noninvasive diagnosis of hepatic fibrosis or cirrhosis, including clinical[9] or blood markers,[10,11] and signal analysis (ultrasonography, magnetic resonance imaging, and elastography).[12,13] Although each method can play a substantial role in the diagnosis of cirrhosis, it is evident that the best way of monitoring hepatitis progression employs an accurate serological method for the quantitative evaluation of fibrosis. We developed a new glyco-marker using multiple lectins that performed well in estimating liver fibrosis in a single-center study.[14,15]

Recent progress in glycoproteomics has had a great influence on work toward ideal, disease-specific biomarkers for a number of conditions. Glycoproteins that exhibit disease-associated glyco-alteration and are present in serum or other fluids have the potential to act as biomarkers for the diagnosis of a target disease,[16] because the features of glycosylation depend on the extent of cell differentiation and the stage of the cell. Detecting hepatic disease-associated glyco-markers for clinical applications has been a continuous challenge since the early 1990s, because increased fucosylation on complex-type *N*-glycans has been frequently detected in glycoproteins from patients with hepatocellular carcinoma (HCC) and cirrhosis.[17,18] Of all the alpha-fetoprotein (AFP) glycoforms, more than 30% have been found to react to a fucose-binding lectin, *Lens culinaris* agglutinin. This fraction, designated AFP-L3, was approved by the U.S. Food and Drug Administration (FDA) in 2005 for the diagnosis and prognosis of HCC.[19] We have found that two fibrosis-indicator lectins (*Aspergillus oryzae* lectin [AOL] and *Maackia amurensis* lectin [MAL]) together with an internal, standard lectin (*Datura stramonium* lectin [DSA]) on an alpha 1-acid glycoprotein (AGP) could, using lectin microarray, clearly distinguish between cirrhosis and chronic hepatitis patients.[14] We have further simplified this quantitative method so that it could be performed using bedside, clinical chemistry analyzers.[15]

The aim of the current study was to evaluate this new glyco-marker (LecT-Hepa) using multiple lectins and bedside clinical chemistry analyzers for use in the assessment of liver fibrosis. In this multicenter study we compared the method's efficiency in estimating liver fibrosis with other noninvasive fibrosis markers and tests.

Materials and Methods

Study Population. This study included 183 consecutive adult patients with CHC who had undergone percutaneous liver biopsy at one of the following institutions: Hokkaido University Hospital, Musashino Red Cross Hospital, National Center for Global Health and Medicine, Hyogo College of Medicine Hospital, or Nagoya City University Hospital in Japan. A diagnosis of CHC was defined as detectable serum anti-hepatitis C virus (HCV) antibody and HCV-RNA, found using polymerase chain reaction assays, of at least 2 points. Exclusion criteria were coinfection with hepatitis B virus or human immunodeficiency virus (HIV), and other disorders that commonly cause liver diseases. Informed consent was obtained from each patient who participated in the study. This study was conducted in accordance with the provisions of the Declaration of Helsinki and was approved by our Institutional Review Board.

Histological Staging. Ultrasonography-guided liver biopsy was performed according to a standardized protocol. Specimens were fixed, paraffin-embedded, and stained with hematoxylin-eosin and Masson's trichrome. A minimum of six portal tracts in the specimen were required for diagnosis. All liver biopsy samples were independently evaluated by two senior pathologists who were blinded to the clinical data. Liver fibrosis stages were assessed using METAVIR fibrosis (F) staging.[20] Significant fibrosis was defined as METAVIR F $\geq$2, severe fibrosis as METAVIR F $\geq$3, and cirrhosis as METAVIR F4. Two patients were excluded from the study because of inadequate histological samples.

Clinical and Biological Data. The age and sex of the patients were recorded. Serum samples were collected immediately before or no more than 2 months

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after liver biopsy and were stored at −80°C until analysis. The concentrations of the following variables were obtained by analyzing the serum samples: aspartate aminotransferase (AST), alanine aminotransferase (ALT), gamma-glutamyltransferase (GGT), total bilirubin, albumin, cholinesterase, total cholesterol, platelet count (platelets), prothrombin time, haptoglobin, hyaluronic acid (HA), α2-macroglobulin (α2-MG), tissue inhibitors of metalloproteinases 1 (TIMP1). The aspartate aminotransferase-to-platelet ratio index (APRI), Fib-4 index, Forns index, and Zeng's score were calculated according to published formulae appropriate to each measure.[2,7,21,22]

Rapid Lectin-Antibody Sandwich Immunoassay Using HISCL. Fibrosis-specific glyco-alteration of AGP was qualified from simultaneous measurements of the lectin-antibody sandwich immunoassays using three lectins (DSA, MAL, and AOL). In principle, the glycan part of the AGP was captured by the lectin immobilized on the magnetic beads, and the captured AGP was then quantified by an antihuman AGP mouse monoclonal antibody probe that was cross-linked to an alkaline phosphatase (ALP-αAGP). The assay manipulation was fully automated using a chemiluminescence enzyme immunoassay machine (HISCL-2000i; Sysmex, Kobe, Japan). We used the following criterion formula, named the "LecT-Hepa Test," to enhance the diagnostic accuracy by combining two glyco-parameters (AOL/DSA and MAL/DSA) as described before: $F = \mathrm{Log}_{10}[\mathrm{AOL/DSA}]*8.6-[\mathrm{MAL/DSA}]$.[15]

Statistical Analyses. Quantitative variables were expressed as the mean ± standard deviation (SD) unless otherwise specified. Categorical variables were compared using a chi-squared test or Fisher's exact test, as appropriate, and continuous variables were compared using the Mann-Whitney U test. $P < 0.05$ was considered statistically significant. A multivariate forward stepwise logistic regression analysis was performed to determine the independent predictors of the absence or presence of significant fibrosis, severe fibrosis, and cirrhosis, respectively. Pearson's correlation coefficient was used as necessary. To assess the classification efficiencies of various markers for detecting significant fibrosis, severe fibrosis, and cirrhosis,[23] and to determine area under the curve (AUC) values, receiver-operating characteristic (ROC) curve analysis was also carried out. Diagnostic accuracy was expressed as the diagnostic specificity (specificity), diagnostic sensitivity (sensitivity), positive predictive values (PPV), negative predictive values (NPV), positive likelihood ratio (LR [+]), negative likelihood ratio (LR [−]), and AUC (95% confidence interval [95% CI]). We performed statistical analyses using STATA v. 11.0 (StataCorp, College Station, TX).

Table 1. Baseline Characteristics of the 183 Patients with Chronic Hepatitis C at the Time of Liver Biopsy

Features	Total (n = 183)
Age (years)	57.6 ± 11.4
Male sex	75 (41.0)
AST (IU/L)	57.4 ± 43.9
ALT (IU/L)	62.8 ± 56.8
GGT (IU/L)	51.1 ± 62.6
Bilirubin (mg/dL)	0.7 ± 0.4
Albumin (g/L)	4.1 ± 0.4
Cholinesterase (IU/L)	283.5 ± 97.0
Cholesterol (mg/dL)	174.1 ± 35.5
Platelets (10^9/L)	163 ± 57
Prothrombin time (%)	87.2 ± 33.4
α2-MG (g/L)	356.8 ± 133.1
HA (μg/L)	205.3 ± 428.0
TIMP1 (pg/ml)	210.6 ± 87.7
AOL/DSA	6.3 ± 12.3
MAL/DSA	9.0 ± 3.1
Fibrosis stage (%):	
F0-1	89 (48.6)
F2	46 (25.1)
F3	22 (12.0)
F4	26 (14.2)

Results

Baseline Characteristics of the 183 Patients with Chronic Hepatitis C at the Time of Liver Biopsy. Patient characteristics at the time of liver biopsy are shown in Table 1. The mean age of the 183 patients was 57.6 ± 11.4 years, and 75 (41%) of them were men. F0-F1 was diagnosed in 89 cases (48.6%), F2 in 46 (25.1%), F3 in 22 (12.0%), and F4 (cirrhosis) in 26 (14.2%).

Comparison of Variables Associated with the Presence of Significant Fibrosis by Univariate and Multivariate Analysis. Variables associated with the presence of significant 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 ($P = 0.014$), α2-MG ($P = 0.002$), HA ($P < 0.0001$), TIMP1 ($P < 0.0001$), and AOL/DSA ($P < 0.0001$) were significantly higher in the significant fibrosis group than in the not significant fibrosis group. The variables albumin ($P < 0.001$), cholinesterase ($P < 0.0001$), cholesterol ($P = 0.005$), platelets ($P < 0.0001$), prothrombin time ($P = 0.0001$), and MAL/DSA ($P < 0.0001$) were significantly lower in the significant fibrosis group than in the not significant fibrosis group. Multivariate analysis showed that platelets (odds ratio [OR]: 0.87,

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,

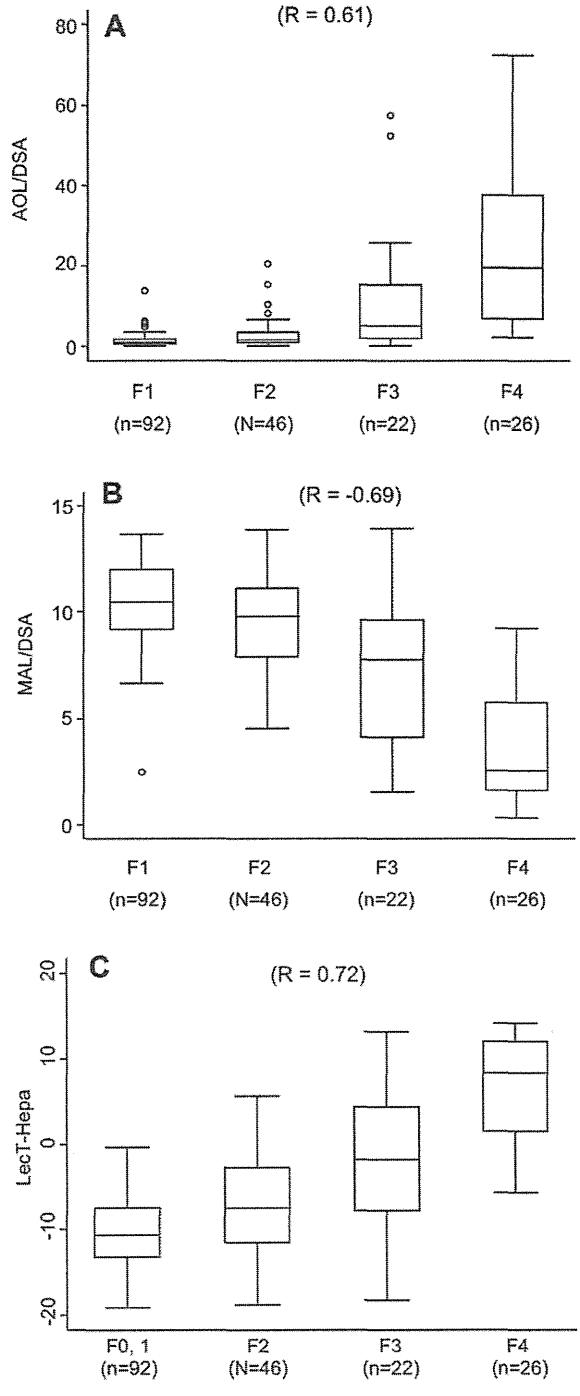
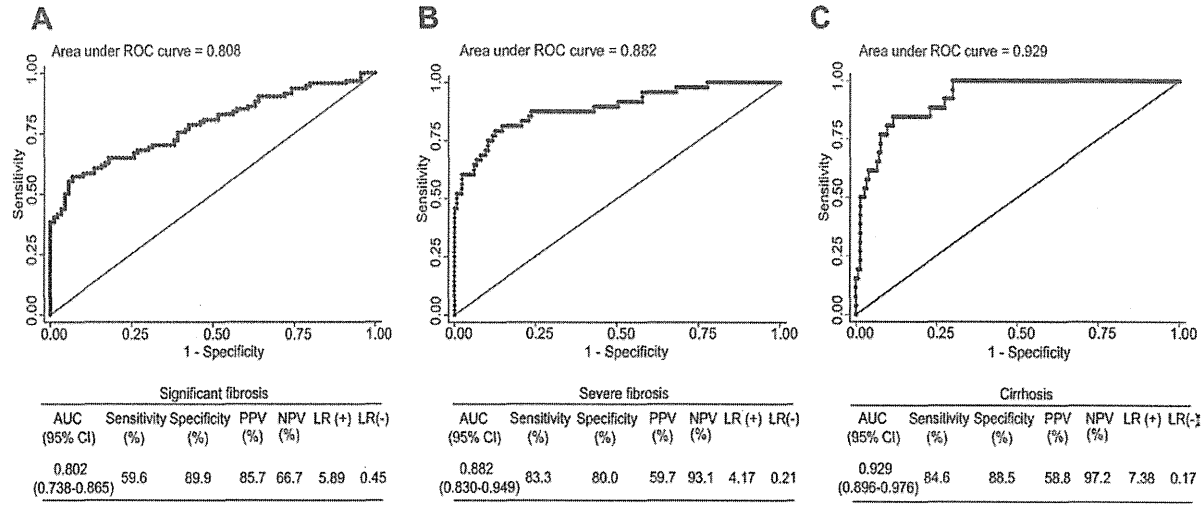


Fig. 1. Boxplot of (A) AOL/DSA, (B) MAL/DSA, and (C) LecT-Hepa in relation to the fibrosis score. The box represents the interquartile range. The whiskers indicate the highest and lowest values, and the dots represent outliers. The line across the box indicates the median value. Correlation of AOL/DSA, 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

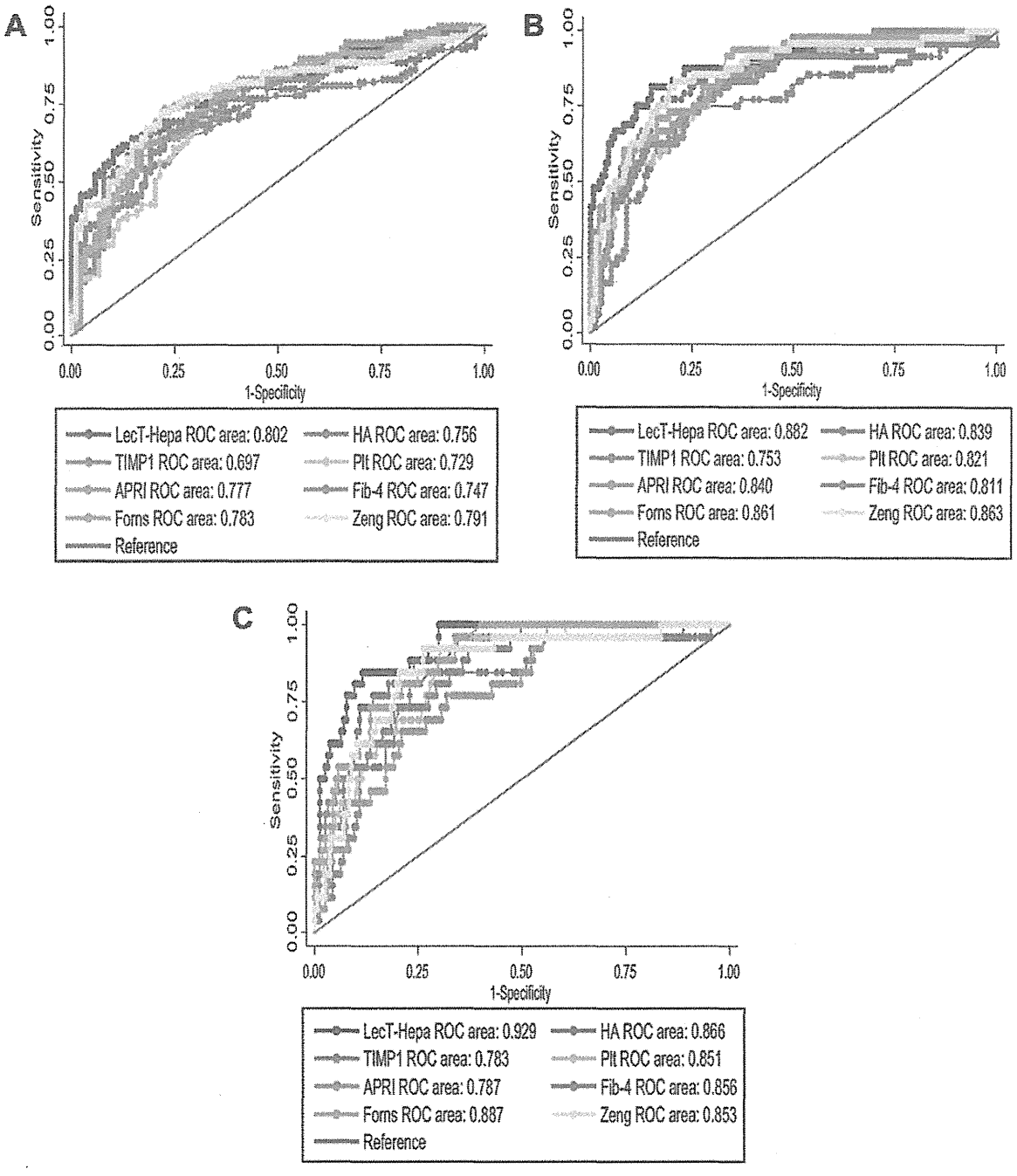


Fig. 3. Comparison of ROC curves in the performance of LecT-Hepa, HA, TIMP1, Plt, APRI, Fib-4 Index, Forns index, Zeng's score for the diagnosis of significant fibrosis (A), severe fibrosis (B), and cirrhosis (C). ROC: receiver operating characteristic curve; TIMP1: tissue inhibitors of metalloproteinases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

Increase in platelet count based on inosine triphosphatase genotype during interferon beta plus ribavirin combination therapy

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Key words
chronic hepatitis C, inosine triphosphatase, natural interferon β, platelet count, ribavirin.

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Abstract

Background and Aim: The inosine triphosphatase (*ITPA*) genotype is associated with ribavirin-induced anemia and pegylated interferon α (PEG IFN-α)-induced platelet reduction during PEG IFN-α plus ribavirin combination therapy. Natural IFN-β plus ribavirin therapy is associated with increases in platelet counts during treatment. We investigated decreases in platelet counts according to *ITPA* genotype during natural IFN-β/ribavirin therapy to determine if patients with low platelet counts were eligible for this combination therapy.

Methods: A total of 187 patients with chronic hepatitis C received PEG IFN-α/ribavirin or natural IFN-β/ribavirin therapy. Decreases in platelet counts based on *ITPA* genotype were investigated during treatment through 24 weeks.

Results: Platelet counts decreased during week 1 of PEG IFN-α/ribavirin therapy, but increased during week 2, after which platelet counts decreased gradually. Platelet counts decreased until week 4 of natural IFN-β/ribavirin therapy, after which platelet counts increased. Platelet counts after week 8 were higher relative to pretreatment platelet counts. Patients with the *ITPA*-CC genotype showed a smaller decrease in platelet counts during natural IFN-β/ribavirin therapy than those with the *ITPA*-CA/AA genotype; platelet counts after week 8 of this therapy were higher than pretreatment platelet counts, regardless of pretreatment platelet counts. Multivariate logistic regression analyses showed that natural INF-β/ribavirin therapy was the only significant independent predictor for an increase in platelets through week 8.

Conclusion: Natural IFN-β/ribavirin therapy is safe for patients with the *ITPA*-CC genotype, even if their pretreatment platelet counts are low.

Introduction

The introduction of pegylated interferon-α (PEG IFN-α) plus ribavirin (PEG-IFN/RBV) combination therapy has led to an improved sustained virological response (SVR) rate in patients with chronic hepatitis C receiving IFN therapy.[1–6] However, cytopenia has been observed during PEG-IFN/RBV therapy. Specifically, cases of RBV-induced anemia and PEG-IFN-induced thrombocytopenia or neutropenia have been reported, and we have previously described cases of RBV-induced anemia.[7] A genome-wide association study (GWAS) identified the inosine triphosphatase gene (*ITPA*) single nucleotide polymorphism (SNP) as being strongly associated with RBV-induced anemia.[8–10] This *ITPA* SNP was also reported to play a role in the decreases in platelet counts that occur during PEG-IFN/RBV therapy.[11,12] In Japan, natural INF-β plus ribavirin (IFN-β/RBV) therapy has been indicated for the treatment of chronic hepatitis C. This therapy is associated with greater increases in platelet counts than seen with PEG-IFN/RBV therapy.[13] Therefore, we investigated the association between the *ITPA* genotype and decreases in platelet count during IFN-β/RBV therapy to determine if patients with a low platelet count were eligible for IFN-β/RBV therapy.

Methods

Patients. A total of 187 patients with chronic hepatitis C who received IFN therapy for at least 24 weeks at the Shinkokura Hospital between January 2009 and April 2011 were included in the study. The study protocol was in compliance with the Good Clinical Practice Guidelines and the 1975 Declaration of Helsinki, and was approved by the Institutional Review Board. Each

patient provided informed consent before participating in this trial.

Criteria for exclusion were as follows: (i) clinical or biochemical evidence of hepatic decomposition or advanced cirrhosis identified by ascites, encephalopathy, or hepatocellular carcinoma; (ii) IFN-β/RBV: a white blood cell count of less than 3×10^9/L and a platelet count of less than 50×10^9/L, PEG-IFN/RBV: a white blood cell count of less than 4×10^9/L and a platelet count of less than 80×10^9/L; (iii) concomitant liver disease other than hepatitis C (hepatitis B surface antigen- or human immunodeficiency virus-positive); (iv) excessive active alcohol consumption exceeding 60 g/day or drug abuse; (v) severe psychiatric disease; and (vi) antiviral or corticosteroid therapy in the 12 months prior to enrollment.

IFN-β/RBV combination therapy. Interferon-β (Feron; Toray Industries, Tokyo, Japan) was given intravenously at a dose of 6 million units (MU) daily for 4 weeks, followed by three times a week for 20–44 weeks. The ribavirin (Rebetol; MSD, Tokyo, Japan) dose was adjusted according to body weight (600 mg for ≤ 60 kg; 800 mg for > 60 to ≤ 80 kg; and 1000 mg for > 80 kg), based on the guidelines of the Ministry of Health, Labor and Welfare of Japan.[5] The drug was administered orally after breakfast and dinner.

PEG-IFN/RBV combination therapy. Pegylated interferon-α-2B (PEG-Intron; MSD) was injected subcutaneously at a median dose of 1.5 μg/kg (range: 1.3–1.5 μg/kg) once a week. Ribavirin was administered twice a day according to body weight, as described for IFN-β/RBV combination therapy.

This study was a prospective, nonrandomized open trial. Platelet counts and hemoglobin levels were measured at baseline and at weeks 1, 2, 4, 8, 12, and 24.

We genotyped each patient for two SNPs: rs8099917, an *IL28B* SNP previously reported to be associated with therapy outcome, and rs1127354 (14), an *ITPA* SNP reported to be associated with ribavirin-induced anemia[14] and decreases in platelet counts.[11] Samples were genotyped using the Illumina HumanHap610-Quad Genotyping BeadChip or with the Invader or TaqMan assay, as described elsewhere.[15–17]

Statistical analysis. Statistical analysis was performed using PASW Statistics, version 18 (SPSS, Chicago, IL, USA) and R, version 2.11. Categorical data were analyzed using the χ^2 test and Fisher's exact tests, and continuous data were analyzed using the nonparametric Mann–Whitney *U*-test. Univariate and multivariate logistic regression analyses were used to determine the factors that significantly contributed to the increase in platelets $> 0 \times 10^9$/L from week 0 through week 8. The odds ratios (OR) and 95% confidence intervals (95% CI) were also calculated. All *P*-values found to be less than 0.05 by the two-tailed test were considered significant. Variables that achieved statistical significance ($P < 0.1$) on univariate analysis were entered into a multiple logistic regression analysis to identify significant independent predictive factors. The potential pretreatment factors associated with increases in platelets $> 0 \times 10^9$/L from week 0 to week 8 included the following variables: age, sex, method of IFN treatment, hepatitis C virus (HCV) genotype, *ITPA* genotype, *IL28B* genotype, hemoglobin, platelet count, alanine aminotransferase (ALT), γ-glutamyl transpeptidase (γ-GTP), and HCV RNA level.

Results

The clinical backgrounds of chronic hepatitis C patients before combination therapy with IFN-β/RBV or PEG-IFN/RBV are shown in Table 1. The mean age of patients receiving IFN-β/RBV therapy was 59.3 years and that of patients receiving PEG-IFN/RBV therapy was 57.9 years, with no difference between the two patient groups. The PEG-IFN/RBV group had more men, although the number was not significantly higher. All baseline laboratory parameters, including hemoglobin levels, platelet counts, ALT levels, γ-GTP levels, and HCV loads, showed no differences

Table 1 Clinical background before combination therapy with interferon β plus ribavirin (IFN-β/RBV) or pegylated interferon plus ribavirin (PEG-IFN/RBV) in chronic hepatitis C patients

		IFN-β/RBV	PEG-IFN/RBV	*P*-value
		n = 45	*n* = 137	
Age	Year (SD)	59.3 (14.3)	57.9 (10.4)	ns
Sex	M/F	22/23	73/64	ns
Hb	g/dL (SD)	14 (1.5)	14.2 (1.4)	ns
Platelet	10^9/L (SD)	178 (59)	183 (59)	ns
ALT	IU/L (SD)	84.1 (63.3)	76.5 (64)	ns
γ-GTP	IU/L (SD)	79.1 (56.29)	69.5 (58.5)	ns
HCV	logIU/mL (SD)	6.7 (1.1)	6.4 (0.9)	ns
HCV genotype	1/2	21/24	102/35	< 0.001
ITPA (rs1127354)	CC/CA or AA	36/9	99/38	ns
IL28B (rs8099917)	TT/TG or GG	35/10	96/41	ns
Decrease in platelet count at week 1	10^9/L (SD)	−47 (32)	−47 (43)	ns
Decrease in platelet count at week 4	10^9/L (SD)	−42 (33)	−28 (33)	< 0.05
Decrease in platelet count at week 8	10^9/L (SD)	19 (36)	−35 (43)	< 0.0001

ALT, alanine aminotransferase; γ-GTP, γ-glutamyl transpeptidase; HCV, hepatitis C virus; ITPA, inosine triphosphate pyrophosphatase; ns, not significant; SD, standard deviation.

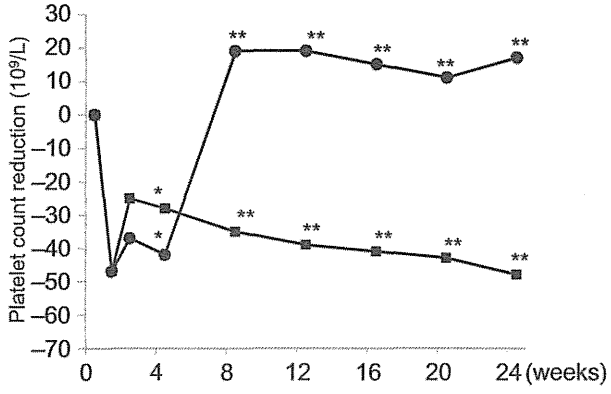

Figure 1 Decreases in platelet count during combination therapy with IFN-β/RBV or PEG-IFN/RBV (closed circle, IFN-β/RBV; closed square, PEG-IFN/RBV; $*P < 0.05$, IFN-β/RBV *versus* PEG-IFN/RBV at week 2; $**P < 0.0001$, IFN-β/RBV *versus* PEG-IFN/RBV at weeks 8, 12, 16, 20, and 24). IFN-β, interferon β; RBV, ribavirin; PEG-IFN, pegylated interferon.

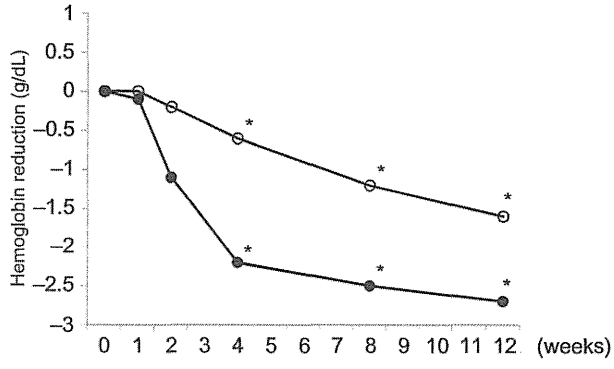

Figure 2 Decreases in hemoglobin levels according to inosine triphosphate pyrophosphatase *(ITPA)* genotype during combination therapy with IFN-β/RBV (closed circle, *ITPA*-CC; open circle, *ITPA*-CA/AA; $*P < 0.01$, *ITPA*-CC *versus ITPA*-CA/AA (rs1127354) at weeks 4, 8, and 12). IFN-β, interferon β; RBV, ribavirin.

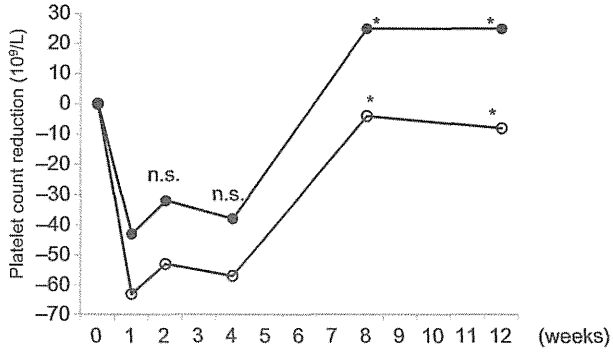

Figure 3 Decreases in platelet count according to inosine triphosphate pyrophosphatase *(ITPA)* genotype during combination therapy with IFN-β/RBV (closed circle, *ITPA*-CC; open circle, *ITPA*-CA/AA; $*P < 0.05$, *ITPA*-CC *versus ITPA*-CA/AA (rs1127354) at weeks 8 and 12). IFN-β, interferon β; RBV, ribavirin.

between the two patient groups. Significantly more patients with HCV genotype 1 were in the PEG-IFN/RBV group ($P < 0.001$). A total of 74% (135/182) patients had the *ITPA*-CC genotype, while 72% of patients had the *IL28B* TT genotype. The frequencies of the *ITPA*-CC genotype and the *IL28B* TT genotype were comparable between the two patient groups. There was no difference in the decreases in platelet counts at week 1; however, at weeks 4 and 8, decreases in platelet counts differed significantly between the two patient groups ($P < 0.05$, $P < 0.0001$).

Platelet count decreases that occurred during combination therapy with IFN-β/RBV or PEG-IFN/RBV are depicted in Figure 1. A decrease in platelet counts of 47×10^9/L was observed at week 1 during IFN-β/RBV therapy. Subsequently, platelet counts transiently increased at week 2, but reduced again at week 4. Platelet counts reduced for 4 weeks after the start of treatment, as IFN-β/RBV therapy involved continuous, daily dosing with IFN-β for 4 weeks after the start of treatment. As per the treatment protocol, IFN-β administration was subsequently reduced to thrice-weekly dosing. At week 8, platelet counts increased and were significantly higher than the pretreatment platelet counts ($P < 0.001$). Platelet counts remained unchanged after week 8. A reduction of 47×10^9/L was observed at week 1 during PEG-IFN/RBV therapy, similar to the reduction that was observed during IFN-β/RBV therapy. Subsequently, platelet counts increased at week 2, decreased at week 4, and gradually decreased further after week 8. The decrease in platelet counts at week 4 during IFN-β/RBV therapy was significantly larger than the decrease observed during PEG-IFN/RBV therapy ($P < 0.05$). However, platelet counts after week 8 of IFN-β/RBV treatment were significantly higher than those during PEG-IFN/RBV therapy ($P < 0.0001$), due to a rapid increase in platelet counts after week 4 of the IFN-β/RBV regimen.

Decreases in hemoglobin levels in relation to the *ITPA* genotype (rs1127354: CC, CA/AA) are shown in Figure 2. At week 2, a large decrease in hemoglobin levels was observed in patients with the *ITPA*-CC genotype. There was no difference in hemoglobin levels based on *ITPA* genotype up to week 2 in patients receiving IFN-β/RBV therapy. Patients with the *ITPA*-CC genotype showed a significantly larger decrease in hemoglobin levels at weeks 4, 8, and 12 than those with the *ITPA*-CA/AA genotype ($P < 0.01$).

Platelet counts during combination therapy with IFN-β/RBV according to the *ITPA* genotype is shown in Figure 3. Similar changes in platelet count decreases were observed in patients with the *ITPA*-CC and *ITPA*-CA/AA genotypes. Patients with the *ITPA*-CC genotype showed a smaller decrease in platelet counts at weeks 1, 2, 4, 8, 12, 24 during therapy compared to those with the *ITPA*-CA/AA genotype. Specifically, patients with the *ITPA*-CC genotype showed a statistically lower degree of platelet decrease at weeks 8, 12, and 24 than those with the *ITPA*-CA/AA genotype ($P < 0.05$). Patients with the *ITPA*-CC genotype had significantly increased platelet counts at week 8 compared with the pretreatment platelet counts ($P < 0.0001$).

Decreases in platelet counts during combination therapy with PEG-IFN/RBV in relation to the *ITPA* genotype are shown in

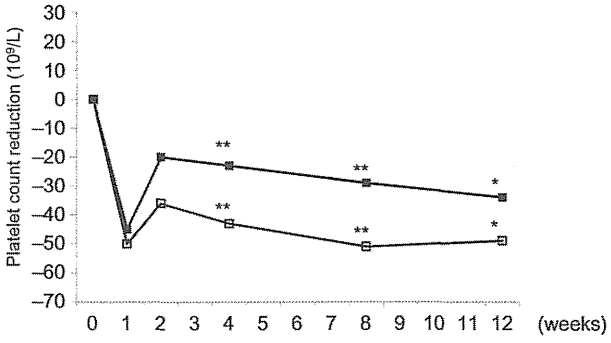

Figure 4 Decreases in platelet count according to inosine triphosphate pyrophosphatase *(ITPA)* genotype during combination therapy with PEG-IFN/RBV (closed square, *ITPA*-CC; open square, *ITPA*-CA/AA; $*P < 0.05$, *ITPA*-CC *versus* *ITPA*-CA/AA (rs1127354) at week 12; $**P < 0.01$, *ITPA*-CC *versus* *ITPA*-CA/AA (rs1127354) at weeks 4 and 8). PEG-IFN, pegylated interferon; RBV, ribavirin.

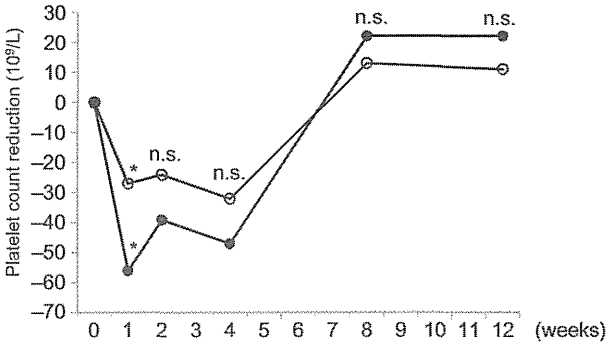

Figure 5 Decreases in platelet count relative to pretreatment platelet counts during combination therapy with IFN-β/RBV (closed circle, $\geq 150 \times 10^9$/L; open circle, $< 150 \times 10^9$/L; $*P < 0.05$, $\geq 150 \times 10^9$/L *versus* $< 150 \times 10^9$/L at week 1). IFN-β, interferon β; RBV, ribavirin.

Figure 4. Similar changes in platelet count decreases were observed in patients with the *ITPA*-CC and *ITPA*-CA/AA genotypes. Patients with the *ITPA*-CC genotype showed a lower degree of platelet reduction at weeks 1, 2, 4, 8, 12, 24 during therapy compared to those with the CA/AA genotype. Specifically, patients with the *ITPA*-CC genotype had a significantly smaller decrease in platelet counts at weeks 4, 8, and 12 than those with the *ITPA*-CA/AA genotype ($P < 0.01$, $P < 0.05$).

Platelet reduction during combination therapy with IFN-β/RBV compared with pretreatment platelet counts is shown in Figure 5. At week 1, patients with a low pretreatment platelet count ($< 150 \times 10^9$/L) showed a significantly smaller decrease in platelet counts than those with a high pretreatment platelet count ($\geq 150 \times 10^9$/L; $P < 0.01$). Five patients had pretreatment platelet counts of $\leq 100 \times 10^9$/L, and a decrease in platelet counts of $\leq 40 \times 10^9$/L was observed in these patients at week 1. Patients with low pretreatment platelet counts showed a small decrease in platelet counts at week 1, after which there was no difference in platelet counts between the groups of patients with high and low

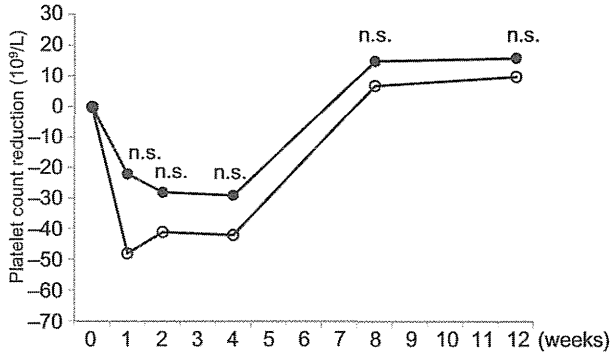

Figure 6 Decreases in platelet count according to inosine triphosphate pyrophosphatase *(ITPA)* genotype during combination therapy with IFN-β/RBV in patients with pretreatment platelet counts ($< 150 \times 10^9$/L) (closed circle, *ITPA*-CC; open circle, *ITPA*-CA/AA). IFN-β, interferon β; RBV, ribavirin.

pretreatment platelet counts. Among patients with both high and low pretreatment platelet counts, platelet counts at week 8 were significantly increased compared with pretreatment platelet counts ($P < 0.01$, $P < 0.05$).

Decreases in platelet counts according to *ITPA* genotype during combination therapy with IFN-β/RBV for patients with pretreatment platelet counts ($< 150 \times 10^9$/L) are shown in Figure 6. For patients with pretreatment platelet counts of $< 150 \times 10^9$/L, patients with the *ITPA*-CC genotype showed a smaller decrease in platelet counts than those with the *ITPA*-CA/AA genotype.

The results of univariate and multivariate logistic regression analyses of factors associated with the increase in platelets $> 0 \times 10^9$/L from week 0 to 8 are shown in Table 2. Univariate and multivariate logistic regression analyses revealed that IFN-β/RBV therapy was the only significant independent predictor for the increase in platelets $> 0 \times 10^9$/L from week 0 to week 8.

Only one patient in the IFN-β/RBV group was withdrawn from the study by week 24. The reason for discontinuation was proteinuria. The dose of IFN was reduced only in the one patient. The dose of ribavirin was reduced in four of 45 patients, all of whom had the *ITPA*-CC genotype.

Discussion

This study showed that the platelet counts of patients undergoing IFN-β/RBV combination therapy for chronic hepatitis C infection after week 8 are higher than those before treatment. Moreover, patients with the *ITPA*-CC genotype showed a smaller decrease in their platelet counts not only during IFN-β/RBV, but also with PEG-IFN/RBV therapy, compared to those with the *ITPA*-CA/AA genotype. In particular, the results demonstrated that platelet counts after week 8 during IFN-β/RBV therapy were higher than pretreatment platelet counts, regardless of pretreatment platelet counts. Compared with pretreatment platelet counts, patients with the *ITPA*-CC genotype had markedly increased platelet counts after week 8 of IFN-β/RBV therapy. Multivariate logistic regression analyses showed that IFN-β/RBV therapy was the factor that contributed to increased platelet counts at week 8 relative to pretreatment platelet counts.

Table 2 Results of univariate and multivariate logistic regression analyses of factors associated with the increase in platelets > 0 (10^9/L) from week 0 to week 8

			Simple regression		Multiple logistic regression	
Factor	Range		Odds ratio	*P*-value	Odds ratio	*P*-value
Age (years)	≥ 60/< 60		1.219	0.389	–	–
Sex	Male/Female		1.219	0.554	–	–
Genotype	1/2		1.303	0.451	–	–
Method of IFN therapy	IFN-β/RBV/PEG-IFN/RBV		20.797	< 0.0001	23.596	< 0.0001
ITPA	CC/CA or AA		0.468	0.073	–	–
IL28B	TT/TG or GG		0.569	0.153	–	–
Baseline hemoglobin	< 14/≥ 14	g/dL	0.569	0.153	–	–
Baseline platelet count	< 150/≥ 150	10^9/L	0.737	0.399	–	–
Baseline ALT	≥ 50/< 50	IU/L	1.646	0.140	–	–
Baseline γ-GTP	≥ 45/< 45	IU/L	1.603	0.166	–	–
Baseline viral load	≥ 6.0/< 6.0	LogIU/mL	1.833	0.091	–	–

ALT, alanine aminotransferase; γ-GTP, γ-glutamyl transpeptidase; IFN-β, interferon-β; ITPA, inosine triphosphate pyrophosphatase; RBV, ribavirin; PEG-IFN, pegylated interferon.

A GWAS identified several new host genetic variants that may be important for PEG-IFN/RBV therapy in chronic hepatitis C. One of these was the SNP in the *IL28B* gene that was strongly associated with therapy outcome,[18–21] and another was the *ITPA* gene that was associated with RBV-induced anemia during PEG-IFN/RBV therapy in chronic hepatitis C.[8–10]

Tanaka *et al.* reported that one SNP (rs11697186) located on the *DDRGK1* gene on chromosome 20 showed strong associations with a decrease in platelet counts in response to PEG-IFN/RBV therapy, and fine mapping with 22 SNPs around the *DDRGK1* and *ITPA* genes showed that rs11697186 had strong linkage disequilibrium with rs1127354, known as a functional variant of the *ITPA* gene.[11] We investigated the changes in platelet count decreases during IFN-β/RBV or PEG-IFN/RBV therapy relative to the *ITPA* genotype (CC, CA/AA). PEG-IFN/RBV therapy was associated with a larger decrease in hemoglobin levels among patients with the *ITPA*-CC genotype than those with the *ITPA*-CA/AA genotype.[8–10] A reactive increase in platelet counts was observed from week 1 through week 4 of treatment, with patients with the *ITPA*-CC genotype showing a higher degree of a reactive increase in platelet counts. This trend was similar to findings reported by Tanaka *et al.*, who reported that a reactive increase in platelet counts occurred secondary to RBV-induced anemia through week 4.[11]

In this investigation, decreases in hemoglobin levels were also observed from weeks 2 through 4 during IFN-β/RBV therapy. Secondarily, a temporary reactive increase in platelet counts occurred. IFN-β/RBV therapy involves continuous daily dosing of IFN-β for 4 weeks, and therefore, platelet counts typically decrease up until week 4, after which platelet counts rapidly increase following a reduction in the dosing frequency of IFN-β to thrice-weekly dosing. However, patients receiving IFN-β/RBV therapy had higher platelet counts at week 8 than pretreatment platelet counts. Arase *et al.* reported that platelet counts increased following a reduction in the dosing frequency of IFN-β from continuous daily dosing to thrice-weekly dosing.[13] We could demonstrate evidence of a relationship between the reduction of the dosing frequency of IFN-β and increases in platelet counts because we developed a treatment protocol using a 4-week continuous daily dosing of IFN-β and complied strictly with the protocol-defined duration of continuous daily dosing of 4 weeks. A higher degree of these recurrent increases in platelet counts was observed in patients with the *ITPA*-CC genotype than in those with the *ITPA*-CA/AA genotype. As with PEG-IFN/RBV therapy, patients with the *ITPA*-CC genotype showed a smaller decrease in platelet counts during IFN-β/RBV therapy. In the present study, our results demonstrated that the *ITPA* genotype was strongly involved in platelet reduction during IFN therapy, in both PEG-IFN RBV and IFN-β/RBV therapy.

The *ITPA* genotype is strongly associated with ribavirin-induced anemia and IFN-induced platelet reduction, although the reasons for these associations are not clear. Erythropoietin (EPO) is produced when hemoglobin reduction occurs as a result of ribavirin-induced anemia. The sequence homology of thrombopoietin (TPO) and EPO may explain the synergy of the physiological roles of TPO and EPO in platelet production. When EPO is elevated, as in iron deficiency anemia, an amino acid sequence similar to TPO may increase the platelet count.[22]

In Japan, the IFN-β/RBV regimen used in the present study has been indicated for chronic hepatitis C patients receiving IFN-based therapy. The SVR rate among patients with HCV genotype 1 who were treated with IFN-β/RBV was lower (approximately 40%) than that among those treated with PEG-IFN/RBV.[13] We reported that IFN-β/RBV therapy was associated with a lower incidence of depressive symptoms or sleep disorders than PEG-IFN/RBV therapy.[23] Therefore, we have also used IFN-β/RBV therapy in elderly patients or patients with concurrent depression. Patients with HCV genotype 2 who were treated with IFN-β/RBV had an SVR rate of approximately 87%, which was similar to that observed in those treated with PEG-IFN/RBV.[24] This study is a prospective, nonrandomized open trial. Thus, the SVR rate among patients with HCV genotype 1 who were treated with PEG-IFN/RBV was higher than the SVR rate of those treated with IFN-β/RBV. IFN-β/RBV therapy was performed only in patients with depression or sleep disorder, thus the number of enrolled patients with HCV genotype 1 who were treated with IFN-β/RBV was small. As for patients with HCV genotype 2, since there was no difference in the SVR rate between IFN-β/RBV and PEG-IFN/RBV therapies, the number of enrolled patients was not different.

Therefore, more patients with HCV genotype 1 were included in the PEG-IFN/RBV group.

In this investigation, there were few discontinuations, dose reductions of IFN, and dose reductions of ribavirin in the IFN-β/RBV group. This is likely due to the fact that few patients developed anorexia, no patients showed weight loss, and dietary intake was adequate during the IFN-β/RBV therapy.

In the present study, patients with the *ITPA*-CC genotype showed a higher increase in platelet counts after week 8 during IFN-β/RBV therapy than those with the *ITPA*-CA/AA genotype. Platelet counts after week 8 were increased compared with pretreatment platelet counts, regardless of pretreatment platelet counts. In patients with low pretreatment platelet counts, patients with the *ITPA*-CC genotype showed a smaller decrease in platelet count than those with the CA/AA genotype, and the platelet counts were increased after week 8. The IFN-β/RBV regimen appears to be a safe strategy for IFN therapy for patients with the *ITPA*-CC genotype, even if they have low pretreatment platelet counts.

The present study demonstrated that as with PEG-IFN/RBV therapy, patients with the *ITPA*-CC genotype showed a smaller decrease in platelet counts during IFN-β/RBV therapy. Platelet counts after week 8 of IFN-β/RBV therapy were increased compared with pretreatment platelet counts, regardless of pretreatment platelet counts. Therefore, we concluded that IFN-β/RBV therapy is safe for patients with the *ITPA*-CC genotype, even if their pretreatment platelet counts are low.

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原　著

当院におけるHIV，HCV重複感染症例に対するペグインターフェロン，リバビリン併用療法の治療成績

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要旨：名古屋医療センターにてHIV，HCV重複感染10症例に対しペグインターフェロン，リバビリン併用療法が行われた．HCV genotypeは，1bが3例，3bが2例，2b，2c，3a，4a，6nがそれぞれ1例ずつであった．9例に抗HIV療法が併用され，そのうち5例で抗HIV剤の変更が行われた．予定治療完遂例は7例であった．全例で治療中に重篤な有害事象は認めなかった．HCVの持続的ウイルス陰性化は，genotype　1または4で4例中1例（25%），1または4以外で6例中5例（83%）に認められた．HIV，HCV重複感染症例に対する本治療法は，安全で有用な治療と考えられた．

索引用語：C型慢性肝炎，HIV，ペグインターフェロン，リバビリン，HCV genotype

背　景

近年，本邦におけるヒト免疫不全ウイルス（human immunodeficiency virus；HIV）感染者は増加の傾向にある．一般に血液媒介感染であるHIV感染はC型肝炎ウイルス（hepatitis C virus；HCV）重複感染を合併する頻度が高く，本邦のHIV感染者の約2割弱がHCV感染者と推測されており[1)]，中でもHIV陽性の血友病患者におけるHCV感染は約98%と報告されている[2)]．また，近年の抗HIV療法の進歩にともない日和見感染は減少し，HCVにより予後が決定される頻度が増加している．わが国での調査では，1997年から2006年におけるHIV，HCV重複感染のある血液凝固疾患症例の死因は，43%がHCVによるものと報告されており，HIV感染者の診療におけるC型慢性肝炎の治療は大きな課題となっている[2)]．

I　目　的

HIV，HCV重複感染例におけるペグインターフェロン（PEG-IFN），リバビリン（RBV）併用療法は，HCV単独感染に対する治療の場合とは異なった問題点がいくつか存在する．まず，HIV

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感染症に対する多剤併用抗ウイルス療法（highly active anti-retroviral therapy；HAART）は，CD4陽性細胞数を維持することでより安全にPEG-IFN，RBV併用療法を可能とする反面，薬物相互作用による有害事象により治療薬の減量につながることもあり，治療成績の低下の一因ともなり得る[3)4)]．また，C型慢性肝炎のウイルス側の因子としてHCV genotypeはPEG-IFN，RBV併用療法の治療成績に大きく関わる因子と考えられているが，その感染経路が単独感染とは若干異なるHIV重複感染者において，1b，2a，2b以外の国内におけるHCV単独症例ではまれなgenotypeもある程度の頻度で存在しており，それらの症例の治療成績に関するわが国での報告は少ない．以上のことをふまえて，厚生労働省指定エイズ対策東海ブロック拠点病院である名古屋医療センターにおける同治療の現状を検討し，その問題点を明らかにすることを目的とした．

II 対象と方法

2004年12月から2011年4月までに当院でPEG-IFN，RBV併用療法を行ったHIV，HCV重複感染例全症例を対象に，患者背景，HCV genotype，治療成績，副作用を検討した．投与薬剤のadherenceは，投与期間中の予定投与量に対する総投与量の割合と定義し，症例ごとに検討した．HCV genotypeは，C/E1およびNS5B領域の塩基配列に基づいて分類した．リファレンスの配列は，Los Alamos研究所のHCV sequence databaseから取得し，系統樹作成は，maximum likelihood法にてreplication 1000回の条件で行った[5)6)]．

治療における薬剤選択と投与量は，HCV単独感染に対する治療に準じて行われたが，全例PEG-IFNα2bが用いられた．投与期間は，genotype 1または4では48～72週，genotype 1または4以外では24～48週を目標とし，genotype，副作用および治療反応性に応じて決定した．

また，対象患者の母集団である，同時期に当院で診療したHIV陽性患者でHCV-RNA陽性が判明している29例のうち，PEG-IFN，RBV併用療法の対象と考えられた27例のHCV genotypeについても検討した．

III 結 果

対象症例の背景因子と治療結果をTable 1に示す．対象症例は10例，男性7例，女性3例，平均年齢は40.5±12.7歳で，女性は3例とも40歳未満であった．人種は，黄色人種が8例，白色人種が2例（症例3，7）であった．推測感染地域は，国内が5例と半数にとどまっており，そのうちの2例（症例1，6）は血友病に対する輸入非加熱血液製剤が感染源と考えられた．表には示さなかったが，感染経路は先述の2例が輸入非加熱血液製剤，4例が男性同性愛，2例が経静脈麻薬常習，2例が不明であった．HCV genotypeは，1bが3例，3bが2例，2b，2c，3a，4a，6nがそれぞれ1例ずつであった．ウイルス量は全例5 logIU/m*l*以上であった．C型慢性肝炎の推測罹病期間は平均9.9±2.3年で，6例において10年以内と比較的短かった．習慣飲酒者は3例で，飲酒量は20～40g/日であった．なお表には示さなかったが，全例においてHBs抗原は陰性であった．治療前検査結果の平均値はそれぞれ，ALT 83±55IU/*l*，白血球6700±1600/μ*l*，ヘモグロビン14.3±1.8g/d*l*，血小板20.2±5.9×10^4/μ*l*であった．肝生検については2例で施行されており，いずれもF2以下であった．

治療期間は，治療反応性不良や副作用にて治療中断となった3症例を含め，最短で12週，最長で48週であった．genotype 1または4の症例では4例中2例（50%）において48週投与が行われ，1例では治療反応性不良にて32週で，1例ではインフルエンザ様症状の副作用にて24週で中止となった．genotype 1または4以外の6例においては，3例（50%）で48週，2例（33%）で24週の投与が行われ，1例で治療反応性不良にて12週で中止となった．治療成績は，全体でみると持続的ウイルス陰性化（SVR）を6例（60%）に認めた．genotype 1または4高ウイルス症例においてSVRは4例中1例（25%），無効1例，治療中の再燃（breakthrough）1例，治療後の再燃1例であった．genotype 1または4以外の症例では，SVRを6例のうち5例（83%）に認め，

Table 1. 患者背景と PEG-IFN/RBV 併用療法の効果

症例	性	年齢	人種	HCV genotype	HCV-RNA (logIU/ml)	HCV 推測感染地域	HCV の罹病期間 (年)	飲酒量 (g/日)	ALT (IU/l)	T-Bil (mg/dl)	Alb (g/dl)	WBC (/μl)	Hb (g/dl)	Plt (×10⁴/μl)	肝生検	adherence (%) PEG-IFN	adherence (%) RBV	副作用	HCV-RNA 陰性化時期 (週)	治療期間 (週)	治療効果
1	M	30 歳代	黄色	1b	6.0	国内*	25	20	38	0.91	4.4	8100	16.4	24.2	未検	100	100		陰性化せず	48	NR
2	M	40 歳代	黄色	1b	6.0	国内	2	0	78	1.03	4.5	6700	15.1	23.9	未検	100	100		4	48	SVR
3	F	20 歳代	白色	1b	6.1	ヨーロッパ	7	0	136	0.76	3.3	4600	12.4	11.8	未検	50	100	抑鬱	8	32	BT
4	M	60 歳代	黄色	2b	6.4	国内	20	0	31	1.35	4.4	8800	12.4	12.5	未検	70	65	WBC, Hb 減少	8	48	SVR
5	M	30 歳代	黄色	2c	7.0	国内	10	30	69	0.40	5.0	6000	14.9	28.3	A2F1	100	100		8	24	SVR
6	M	40 歳代	黄色	3a	5.9	国内*	30	0	78	1.58	3.8	6800	13.1	16.3	未検	70	70	WBC, Plt, Hb 減少	8	48	SVR
7	M	30 歳代	白色	3b	6.3	南アジア	4	40	94	0.68	4.7	6300	16.1	18.6	A2F2	100	100		4	24	SVR
8	F	30 歳代	黄色	3b	6.8	東アジア	3	0	28	0.51	4.4	4900	12.9	18.0	未検	50	100	WBC 減少	陰性化せず	12	NR
9	F	30 歳代	黄色	4a	5.8	東南アジア	9	0	66	0.55	4.2	5200	12.8	19.6	未検	100	100	インフルエンザ様症状	16	24	Rel
10	M	50 歳代	黄色	6n	6.7	東南アジア	13	0	212	0.65	5.0	9400	17.3	28.7	未検	100	100		8	48	SVR

*：国内での輸入非加熱血液製剤による感染，PEG-IFN：ペグインターフェロン，RBV：リバビリン，adherence：総投与量/投与期間中の予定投与量，NR：non-response，SVR：sustained virological response，BT：virological breakthrough，Rel：relapse.

Table 2. HIV 治療の状況

症例	IFN 前 CD4 陽性細胞数 (/μl)	IFN 投与中の CD4 陽性細胞数最少値 (/μl)	IFN 前 HIV-1 定量 (copies/ml)	IFN 投与中の HIV-1 定量最高値 (copies/ml)	HIV 治療 (HAART)	HIV 治療の変更	HIV 治療の変更時期
1	631	455	1.0×10^5	1.0×10^5	なし		
2	599	344	検出せず	検出せず	AZT, 3TC, NVP	ABC, 3TC, NVP	IFN 導入 12 週前
3	599	393	検出せず	2.0×10^4	TDF, FTC, EFV		
4	488	111	検出せず	検出せず	ABC, 3TC, LPV/r		
5	532	416	検出せず	検出せず	AZT, 3TC, EFV	TDF, FTC, RAL	IFN 導入時
6	461	116	検出せず	検出せず	AZT, 3TC, LPV/r	TDF, 3TC, LPV/r	IFN 導入 2 週後
7	590	344	検出せず	検出せず	TDF, FTC, EFV	TDF, FTC, RAL	IFN 導入 24 週前
8	711	533	検出せず	検出せず	TDF, FTC, FPV		
9	323	119	検出せず	検出せず	AZT, 3TC, SQV-HGC, RTV	ABC, 3TC, SQV-HGC, RTV	IFN 導入 6 週前
10	935	490	検出せず	検出せず	d4T, 3TC, NVP		

AZT；ジドブジン，3TC；ラミブジン，NVP；ネビラピン，ABC；アバカビル，TDF；テノホビル，FTC；エムトリシタビン，EFV；エファビレンツ，LPV/r；ロピナビル（少量リトナビル含有），RAL；ラルテグラビル，FPV；ホスアンプレナビル，SQV-HGC；サキナビル，RTV；リトナビル，d4T；サニルブジン．

無効1例であった．

PEG-IFN，RBV 併用療法中の血球減少に対して4例で PEG-IFN の減量を，2例で RBV の減量が必要であった．PEG-IFN の adherence は最低で50%，RBV の adherence は最低で65% であった．治療経過全般を通じて全例において重篤な副作用は認めなかった．

Table 2 に HIV に対する治療について示した．PEG-IFN，RBV 併用療法開始前の CD4 陽性細胞数は323～935/μl であったが，併用療法中の白血球の減少にともない全例でその減少を認めた．日和見感染症を発症しやすいとされる CD4 陽性細胞数200/μl 以下への減少は3例に認めたが，全症例において治療中に日和見感染症の合併は認めていない．PEG-IFN，RBV 併用療法開始前の HIV-1 定量値は，1例（症例1）で 1.0×10^5copies/ml であったが，この症例は経過観察のみで CD4 陽性細胞数が500/μl 以上を維持していた．HAART については，この1例を除いて9例に PEG-IFN，RBV 併用療法開始前から行われていた．そのうち，1例（症例4）は IFN 治療を開始することを前提に開始24週前に HAART が開始されており，その他の症例は以前より HAART が行われていた．PEG-IFN，RBV 併用療法の開始に当たって，4例がジドブジン（AZT）から他の核酸系逆転写酵素阻害剤に変更が行われた．変更時期については1例（症例6）で PEG-IFN，RBV 併用療法開始2週後，1例（症例5）で同併用療法開始とほぼ同時期に，他の3例（症例2，7，9）は同併用療法の開始6～24週前に変更されていた．PEG-IFN，RBV 併用療法開始2週後に AZT を変更した症例は消化器科と感染症科の連絡不行き届きで，AZT を変更する前に PEG-IFN，RBV 併用療法が開始されたことにより，急速に貧血を呈し，一時 PEG-IFN，RBV 併用療法が中止となった．その後 AZT をテノホビル（TDF）に変更し，速やかに貧血は改善し PEG-IFN，RBV 併用療法の再開が可能となり，その後も貧血，好中球減少，CD4 陽性細胞数減少を認めたが48週間の投与を行うことができた（Figure 1）．また，非核酸系逆転写酵素阻害剤のエファビレンツ（EFV）は抑鬱などの精神症状をきたしやすいことを理由に，PEG-IFN，RBV 併用療法導入に当たって2症例（症例5，7）において他の薬剤に変更が行

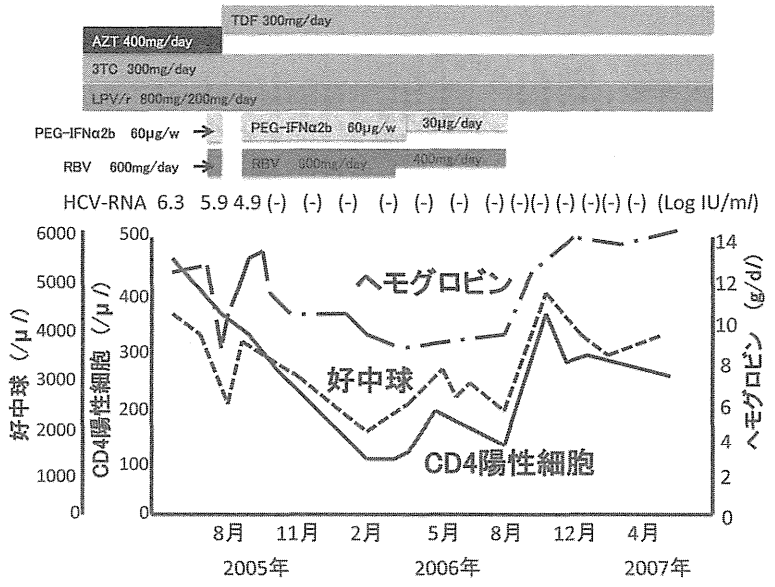

Figure 1. 症例6 治療経過：HIVに対するAZT，3TC，LPV/rによるHAART治療中にペグインターフェロン，リバビリン併用療法を開始したが，急激なヘモグロビン値，好中球数の減少を認め，著しい倦怠感を訴えた．ペグインターフェロン，リバビリン併用療法を中止とし，AZTをTDFに変更し，血球数の回復を待ってからペグインターフェロン，リバビリン併用療法を再開した．その後は，血球減少を認めるも48週間の治療を完遂できSVRが得られた．AZT：ジドブジン，TDF：テノホビル，3TC：ラミブジン，LPV/r：ロピナビル（少量リトナビル含有），PEG-IFNα2b：ペグインターフェロンα2b，RBV：リバビリン．

われた．なお，症例3はHAARTに対するadherenceが不良なため，治療中にHIVの再活性化が認められたが，CD4陽性細胞数は200/μl以上を維持していた．

今回の検討対象の母集団である当院におけるHIV，HCV重複感染例のうち27例でHCV genotypeを検討した．非加熱血液製剤による感染症例12症例では，genotype 1aが8例（66.7%）と最も多く，3aが2例（16.7%），1bおよび2aがそれぞれ1例（8.3%）であった．一方，非加熱血液製剤以外での感染症例15症例では，genotype 1bが6例（40.0%）と最も多く，1aおよび3bがそれぞれ2例（13.3%），2b，2c，3a，4a，6nがそれぞれ1例（6.7%）であった（Table 3）．

IV 考 察

今までのところ，HIV，HCV重複感染におけるC型慢性肝炎治療は，HCV単独感染と同様にPEG-IFN，RBV併用療法を中心に行われてきている．一般にHIV，HCV重複感染症例でのPEG-IFN，RBV併用療法は，HCV単独感染肝炎と比較して著効率が低いとされているが，その理由は十分には明らかにされていない．実際の治療に当たっては，治療導入時に十分なCD4陽性細胞数を有することが抗HCV治療の成績向上につながるとの報告もあり[7]，必要に応じてHAARTを併用することが推奨されている[8]．その際にHAARTとの薬物相互作用による有害事象に留意しつつ，十分な量の抗HCV薬を十分な期間投与することが重要と考えられている[9]．

今回，当院でのHIV，HCV重複感染症例におけるC型慢性肝炎に対するPEG-IFN，RBV併用療法の現状を検討したところ，忍容性にはおおむね問題がなかった．一部症例でHIVに対する治療内容の変更を必要としたが，重篤な合併症は経験しなかった．なお，一般にCD4陽性細胞数が200/μl以下の場合は，日和見感染の危険が高く

Table 3. 当院におけるHIV/HCV重複感染者のHCV genotype

HCV genotype	感染経路 非加熱血液製剤 (n=12)	非加熱血液製剤以外 (n=15)	全体 (n=27)
1a	8 (66.7%)	2 (13.3%)	10 (37.0%)
1b	1 (8.3%)	6 (40.0%)	7 (25.9%)
2a	1 (8.3%)	0 (0%)	1 (3.7%)
2b	0 (0%)	1 (6.7%)	1 (3.7%)
2c	0 (0%)	1 (6.7%)	1 (3.7%)
3a	2 (16.7%)	1 (6.7%)	3 (11.1%)
3b	0 (0%)	2 (13.3%)	2 (7.4%)
4a	0 (0%)	1 (6.7%)	1 (3.7%)
6n	0 (0%)	1 (6.7%)	1 (3.7%)

なるといわれており，HCVに対するIFNを用いた治療の導入は推奨されず，HIVの治療が優先されている[10]．今回の症例は，治療開始時においては全例その基準を満たしていた．一方，経過中にはすべての症例で白血球減少を認め，それにともなってCD4陽性細胞数も減少を認めた．ただし，PEG-IFNの投与量は通常のC型慢性肝炎治療に準じて好中球数に応じて適宜増減を行い，結果的にほぼCD4陽性細胞数に合わせたIFN投与量の増減が行われていた．3例でCD4陽性細胞数200/μlを下回ったが，治療中の日和見感染の合併症は認めなかった．一般に，IFN投与時にCD4陽性細胞数の減少を認めてもTリンパ球におけるその割合は低下せず，易感染症をきたすことはないとされており，IFNの減量基準も通常の基準に従えば問題がないと考えられた[11][12]．

治療を受けた症例のHCV genotypeは1が3例，4が1例でそれら以外が6例であった．PEG-IFN，RBV併用療法導入に当たって，genotype 1および4の症例には難治が予測されることを説明してはいるが，なるべく積極的に導入する方向で対応している．しかし，当院におけるHIV, HCV重複感染症例でHCV genotypeの検討を行った27例のうち，genotype 1または4の症例は，今回HCVの治療を受けた症例では10例中4例（40.0%）で，受けなかった症例では17例中14例（82.4%）であった．今回治療を受けた症例のうち母集団の中では最も多いgenotype 1の症例が少なかったのは，難治が予測され治療開始を躊躇する症例が多かった結果と考えられる．広く知られるように世界的にHCV genotypeは，1aおよび1bが最も多く，次に2a，2b，3a，3bが多いといわれている[13]．わが国ではgenotype 1bが最も多く，2a，2bが次に多い[14]．ただし，わが国における血友病症例などでの輸入非加熱血液製剤による感染では，1a，1bがそれぞれ約30%，3aが約20%と推測されている[15]．当院で治療経過観察中の輸入非加熱血液製剤による感染症例では，12例のうちgenotype 1aが8例（66.7%）と最も多かった．一方，血液製剤以外の感染症例15症例ではgenotype 1bが6例（40.0%）と最も多く，それ以外では1a，3bがそれぞれ2例（13.3%），2b，2c，3a，4a，6nがそれぞれ1例（6.7%）と多様であった．HIV，HCV重複感染症例の中でも血液製剤以外での感染症例におけるgenotypeの多様性は，国外での感染や，国内においても経静脈麻薬常習者や，男性同性愛者などの外国人を含む狭いコミュニティでの感染であることに関連していると思われる．今回，治療を受けた患者のうち5症例は海外での感染と推測され，うち3症例（症例3，7，8）は海外出身者であった．推定感染地域から考えても，今回の症例群は日本でのHCV単独感染例における感染経路とは異なり，その結果genotypeの分布も異なる結果となっている．今回当院でのHIV，HCV重複感染でPEG-IFN，RBV併用療法を施行した症