Table 3 Univariate and multivariate analyses of contributing factors associated with SVR

Parameter	Univariate analysis, OR (95% CI), *P*-value	Multivariate analysis, OR (95% CI), *P*-value
Age (years)	0.989 (0.968–1.011), 0.619	
Male/female	1.314 (0.886–1.949), 0.488	
Albumin (g/dL)	2.547 (1.399–4.637), 0.119	1.702 (0.728–3.979), 0.531
AST (U/L)	0.999 (0.995–1.005), 0.982	
ALT (U/L)	1.004 (1.001–1.007), 0.250	1.015 (1.009–1.020), 0.005
GGT (U/L)	0.989 (0.983–0.996), 0.101	0.981 (0.973–0.989), 0.022
Alkaline phosphatase (U/L)	0.995 (0.996–0.998), 0.101	0.998 (0.995–1.002), 0.698
Total bilirubin (mg/dL)	0.335 (0.166–0.679), 0.121	0.475 (0.257–0.877), 0.225
Prothrombin time activity (%)	1.002 (0.988–1.016), 0.895	
AFP (ng/mL)	0.984 (0.972–0.995), 0.154	0.999 (0.979–1.020), 0.986
Liver stiffness (kPa)	0.917 (0.884–0.952), 0.019	0.866 (0.810–0.927), 0.033
Platelet ($\times 10^4/\mu L$)	1.030 (0.997–1.065), 0.366	0.958 (0.913–1.006), 0.381
HCV RNA viral load (KIU/mL)	0.850 (0.561–1.287), 0.695	
PLNE	0.318 (0.176–0.576), 0.053	0.179 (0.090–0.354), 0.012

AFP, α-fetoprotein; ALT, alanine aminotransferase; AST, aspartate aminotransferase; CI, confidence interval; GGT, γ-glutamyltransferase; HCV, hepatitis C virus; IL, interleukin; OR, odds ratio; PLNE, perihepatic lymph node enlargement; SVR, sustained virological response.

Association between PLNE and the mutations at position 70 of HCV core protein and at ISDR or IL-28B polymorphism

Recently, as a human factor, *IL-28B* polymorphism has been shown to be a strong predictor for the response to PEG IFN and RBV therapy for chronic hepatitis C.[21–23] On the other hand, as a viral factor, the mutations at position 70 of HCV core protein[24] and those at ISDR of NS5A protein[25] have been revealed to be associated with the treatment outcome by IFN. Thus, the potential associations between PLNE and these factors were evaluated. To this end, we first sought to analyze them in the original cohort, however, the stored samples were not enough for these determinations. Thus, we newly enrolled 45 chronic hepatitis C patients to only assess the potential associations between PLNE and *IL-28B* polymorphism, the mutations at position 70 of HCV core protein or those at ISDR of NS5A protein.

The clinical features of these 45 patients with chronic hepatitis C with HCV genotype 1 are summarized in Table 4. Among these patients, PLNE was observed in 18 patients (40%). Regarding *IL-28B* genotypes, the frequency of the rs8099917 risk allele (G), namely, minor allele, was not different between the patients with PLNE and those without: 22.2% in the former and 23.1% in the latter ($P = 0.76$ by χ^2-test; Fig. 2a). As a viral factor, the mutations at position 70 of HCV core protein were observed in 44.4% patients with PLNE, and in 42.3% patients without ($P = 0.87$ by χ^2-test; Fig. 2b), and less than two substitutions at ISDR were observed in 52.9% patients with PLNE, and in 64.0% patients without ($P = 0.69$ by χ^2-test; Fig. 2c), none of which were statistically different. These results suggest that PLNE is not associated with *IL-28B* polymorphism, or the mutations

Table 4 Characteristics of patients in whom the mutations at position 70 of HCV core protein and at ISDR or IL-28B polymorphism were measured

Parameter	$n = 45$
Age (years)†	68.1 ± 10.7
Male/female	19/26
Albumin (g/dL)†	3.93 ± 0.39
AST (U/L)†	43.4 ± 18.6
ALT (U/L)†	38.8 ± 20.3
GGT (U/L)†	38.1 ± 28.9
Alkaline phosphatase (U/L)†	276.3 ± 101.5
Total bilirubin (mg/dL)†	0.83 ± 0.29
Prothrombin time activity (%)	99.1 ± 2.86
AFP (ng/mL)†	16.0 ± 36.3
Platelet count ($\times 10^4/\mu L$)†	15.4 ± 7.0
HCV viral load (log IU/mL)‡	5.94 (5.54–6.60)
PLNE positive/negative	18/27

†Data are presented as mean ± standard deviation.
‡Data are presented as median (25–75% range).
AFP, α-fetoprotein; ALT, alanine aminotransferase; AST, aspartate aminotransferase; GGT, γ-glutamyltransferase; HCV, hepatitis C virus; IL, interleukin; ISDR, interferon sensitivity-determining region; PLNE, perihepatic lymph node enlargement;.

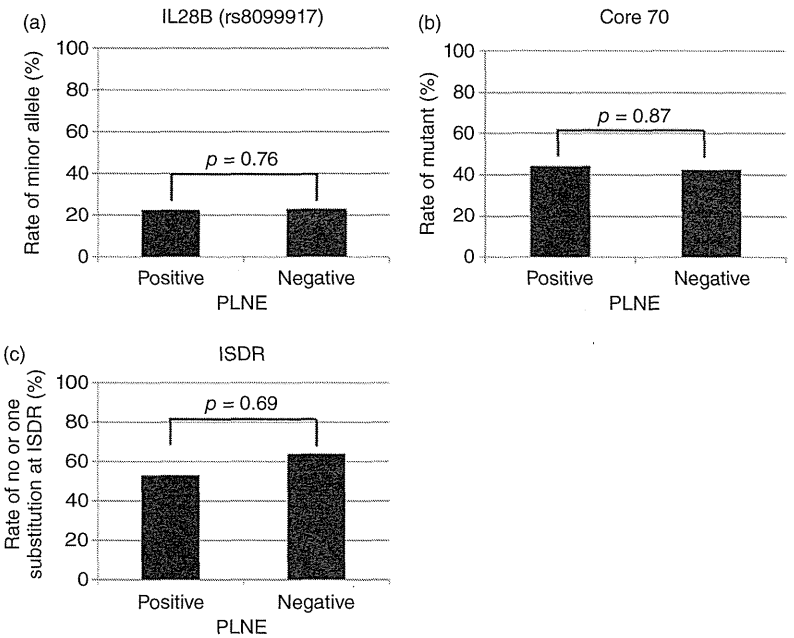

Figure 2 Rate of IL-28B minor allele (a), mutant at position 70 of HCV core protein (Core 70, b), and no or one substitution at ISDR (c) in patients with PLNE (positive) or without PLNE (negative). The rate of IL-28B minor allele (a), mutant at position 70 of HCV core protein (Core 70, b), and no or one substitutions at ISDR (c) was analyzed in patients with PLNE (positive, $n = 18$) or without PLNE (negative, $n = 27$). HCV, hepatitis C virus; IL, interleukin; ISDR, interferon sensitivity-determining region; PLNE, perihepatic lymph node enlargement.

at position 70 of HCV core protein and those at ISDR of NS5A protein.

DISCUSSION

THE FACT THAT chronic hepatitis C patients with PLNE are prone to treatment failure to IFN therapy has been previously reported.[10–12] However, there were limitations because these analyses were performed in patients with various HCV genotypes, viral loads and treatment regimens. It is well known that efficacy of IFN therapy for chronic hepatitis C is poorest in patients with HCV genotype 1 and higher HCV viral load.[13] On the other hand, IFN therapy has been refined with PEG and combination with RBV and protease inhibitors.[26] Thus, to more precisely examine the potential association between PLNE and efficacy of IFN therapy for chronic hepatitis C patients, we sought to analyze the efficacy of PEG IFN and RBV therapy on patients with HCV genotype 1 and HCV RNA of more than 100 KIU/mL in the presence or absence of PLNE. As a result, the SVR rate was significantly lower and the viral decline during the first 4 weeks of treatment was significantly smaller in patients with PLNE than in those without, and PLNE was negatively associated with SVR by multivariate analysis. It is noteworthy that PLNE was not associated with liver stiffness values, a marker of liver fibrosis,[27] although controversy exists whether PLNE may be associated with liver fibrosis,[2,4] and more importantly that PLNE was retained as a negative predictor for SVR by multivariate analysis, independent of liver fibrosis, in the current study.

To examine the underlying mechanism in the association between PLNE and efficacy of PEG IFN and RBV for chronic hepatitis C, we analyzed the potential links between PLNE and well-established host or viral factors to predict outcome by IFN therapy. We especially considered the possibility that PLNE may be associated with *IL-28B* polymorphism, which could be importantly involved in the host immune system.[21–23] However, PLNE was not significantly associated with *IL-28B* polymorphism as a host factor, in addition to no association of PLNE with the mutations at position 70 of HCV core protein[24] and those at ISDR[25] as a viral factor. Thus, we speculate that PLNE may reflect an unknown host factor to play a role in the reaction to HCV infection.

Perihepatic lymph node enlargement in chronic hepatitis C has long been assumed to reflect the immunological response of the host.[7] Indeed, higher $CD8^+$ lymphocyte levels in the blood were observed in chronic hepatitis C patients with PLNE,[7] and HCV-specific IFN-γ production and proliferative response of T cells were found commonly in perihepatic lymph node,[28] suggesting that PLNE indicates an active host immune response

in chronic hepatitis C. Of interest is the finding that biochemical responders to IFN-α therapy for chronic hepatitis C had significantly lower pretreatment levels of $CD8^+$ cells.[29] Thus, increased $CD8^+$ cell levels in chronic hepatitis C patients with PLNE[7] may explain the mechanism, at least in part, of the association between PLNE and the poorer outcome by PEG IFN and RBV therapy. Furthermore, increased oligoclonality of circulating $CD8^+$ cells in chronic HCV infection was also identified as an indicator for poor clinical response to IFN-α therapy.[30] It should be further clarified whether the altered clonality of $CD8^+$ cells may be also involved in the mechanism of the association between PLNE and the efficacy of PEG IFN and RBV therapy.

We previously demonstrated that PLNE is a negative risk for hepatocarcinogenesis in chronic hepatitis C.[10] Currently, we have found that PLNE is a negative predictor for a successful outcome of IFN treatment for chronic hepatitis C, both of which could be paradoxical. However, these findings may be consistently explained by an active host immune system as described above. We previously speculated that the reduced hepatocarcinogenesis in chronic hepatitis C patients with PLNE may be explained by the enhanced host immune system. On the other hand, there are accumulating lines of evidence suggesting that the originally enhanced host immune response may be associated with a negative outcome of IFN treatment for chronic hepatitis C; the higher expression of IFN-stimulated genes in the liver was observed in patients with no response to IFN treatment for chronic hepatitis C.[31–35] Thus, we speculate that PLNE, as an indicator of active host immune responses,[7,28] may be associated negatively with the efficacy of IFN therapy for chronic hepatitis C in line with these previous studies.

One of the limitations of the current study is that the association between PLNE and efficacy of IFN therapy in chronic hepatitis C patients was determined in the retrospective cohort. However, the cohort analyzed was originally set up to prospectively examine the risk of liver stiffness values for HCC development, where patients were consecutively enrolled among those who previously visited our Department of Gastroenterology, The University of Tokyo Hospital,[15] and were followed up for 4.8 years.[10] Thus, we believe that our current evidence could be the same as that from the prospective cohort. As another limitation, the association between PLNE and *IL-28B* polymorphism, the mutations at position 70 of HCV core protein or those at ISDR could not be assessed in the original cohort, because the stored samples of the original cohort were not enough for these determinations.

The combination of PEG IFN and RBV has been the standard of care for chronic hepatitis C,[26] and the further combination of telaprevir[36] or boceprevir[37] with PEG IFN and RBV has been recently employed to improve the treatment efficacy. Nonetheless, it is expected that IFN is still going to play a role in the treatment for chronic hepatitis C. Thus, our evidence of PLNE as a significant predictor for SVR by PEG IFN and RBV therapy may be useful when deciding upon the regimen or timing treatment for chronic hepatitis C patients. Furthermore, because PLNE is originally one of the common clinical signs of chronic liver disease,[1–3] the knowledge regarding the clinical significance of PLNE may shed a light on not only the prediction of treatment outcome of IFN therapy for chronic hepatitis C but also the general pathophysiology of chronic liver disease.

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Short Communication

Perihepatic lymph node enlargement observed at a general health examination: A cross-sectional study

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Aim: Although perihepatic lymph node enlargement (PLNE) is known as a common finding in chronic liver disease, it can be found occasionally at a general health examination. We aimed to clarify the clinical significance of PLNE in general.

Methods: Between January 2008 and December 2011, 4234 subjects were enrolled, who underwent a general health examination at the University of Tokyo Hospital.

Results: PLNE was observed in 69 (1.6%) subjects, among whom 17 (0.4%) had liver disorders and 13 (0.3%) had malignancy, one of whom had both. No disorders were determined in the remaining 40 subjects (0.9%). Among 17 subjects with liver disorder-associated PLNE, anti-hepatitis C virus (HCV) antibody was determined in 11 and serum alanine aminotransferase levels were less than 40 U/L in eight. Among 13 subjects with malignancy-associated PLNE, para-aortic lymph nodes were also enlarged in eight. Among 40 subjects with PLNE of unknown etiology, 27 could be followed up for the mean period of 2.08 years, where no underlying disorders were newly determined with largely unaltered size of PLNE.

Conclusion: The incidence of PLNE in the general population may vary with the prevalence of chronic liver disease, especially HCV infection. When PLNE is observed, liver disorders should be first surveyed including HCV infection even with normal serum alanine aminotransferase levels. PLNE with para-aortic lymph node enlargement may be suggestive of a malignant lesion. The incidence of PLNE of unknown etiology may be approximately 1% in the general population, which may be just followed up without further change.

Key words: chronic liver disease, general health examination, hepatitis C virus, perihepatic lymph node enlargement

INTRODUCTION

PERIHEPATIC LYMPH NODE enlargement (PLNE), usually detected by abdominal ultrasonography, is known as a common finding in patients with chronic liver disease,[1] especially in those with hepatitis C.[2,3] Although PLNE in chronic hepatitis C is reportedly associated with inflammatory activity, stage of liver fibrosis[3–7] or hepatitis C viral load,[8] inconsistent findings have been reported,[2,4,9] suggesting that the clinical significance of PLNE in chronic hepatitis C has not been fully understood yet. PLNE in chronic hepatitis C has long been assumed to reflect an immunological response of the host.[7] We have recently reported that PLNE is a negative predictor for hepatocellular carcinoma development in chronic hepatitis C patients, which may be in line with this previous speculation.[10] Furthermore, PLNE can be also one of the clinical findings in patients with malignancy or lymphoproliferative disorder.

Because PLNE is not usually associated with specific symptoms such as pain, it can be found occasionally at abdominal ultrasonography during a general health examination. In this study, we aimed to clarify the clinical significance of PLNE in general.

METHODS

Subjects

PARTICIPANTS WERE RECRUITED at the time of their health examination at the University of Tokyo Hospital between January 2008 and December 2011.

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Abdominal ultrasonography and venous blood sampling after an overnight fast were carried out as part of a general health examination. Ultrasonographic examination was performed using Xario SSA-660A (Toshiba, Tokyo, Japan) with seven examiners.

Perihepatic lymph node was recognized as one or more masses with an ovoid shape and less echogenic than liver parenchyma, separated from adjacent organs and vessels by a clear-cut cleavage on repeated transverse, sagittal and oblique scans. Lymph nodes were searched for near the trunk of the portal vein, hepatic artery, celiac axis, superior mesenteric vein and pancreas head. Furthermore, Doppler ultrasonography was used to differentiate lymph nodes from vessels.[10] Although some subjects' characteristics such as severe obesity or history of abdominal surgery may affect the visualization of the lymph nodes in the perihepatic area, even subjects with those characteristics were enrolled and analyzed in this study.

Spleen size was also examined by abdominal ultrasonography and expressed as splenic index, calculated with half the maximum craniocaudal length multiplied by the maximum width of the spleen obtained on longitudinal sections.[11]

This study was carried out in accordance with the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the Institutional Research Ethics Committee of the Faculty of Medicine of the University of Tokyo.

Statistical analysis

Data are expressed as mean ± standard deviation (SD) unless otherwise indicated. The categorical variables were compared by Fischer's exact test. For comparing group means, ANOVA was used. Data processing and analysis were performed using StatView ver. 5.0 (SAS Institute, Cary, NC, USA) and SPSS ver. 14.0 (SPSS, Chicago, IL, USA) software.

RESULTS AND DISCUSSION

AMONG THE TOTAL 7259 subjects who underwent abdominal ultrasonography as part of a general health examination between January 2008 and December 2011, 4234 subjects were enrolled to analyze the presence of PLNE after excluding duplicates, because many subjects took a general health examination multiple times over 4 years, such as every year or every other year. Among lymph nodes in the perihepatic area recognized at abdominal ultrasonography, the smallest was 8.5 mm in the longest axis, and the largest was 32.2 mm, while lymph nodes recognized were mostly (96%) more than 10.0 mm, as shown in Figure 1. These recognized lymph nodes in the perihepatic area were

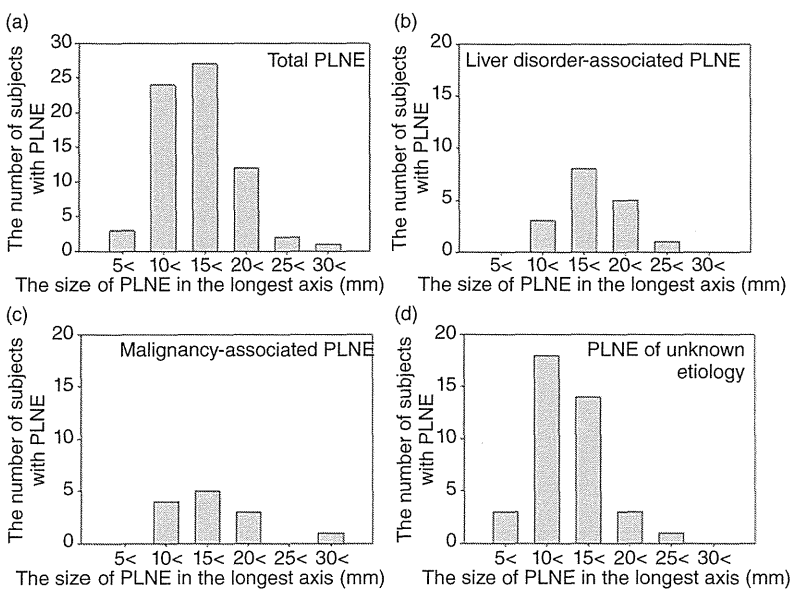

Figure 1 Size of perihepatic lymph node enlargement (PLNE). (a) The size in the longest axis of total PLNE, (b) liver disorder-associated PLNE, (c) malignancy-associated PLNE and (d) PLNE of unknown etiology are shown.

defined as PLNE in the current study. Based on the criteria, PLNE was observed in 69 of 4234 (1.6%) subjects. Among them, PLNE was associated with liver disorders in 17 (0.4%) subjects and with malignancy in 13 (0.3%) subjects, one of which had both liver damage of alcoholic liver disease and malignancy of hepatocellular carcinoma. Finally, no disorders were determined in the remaining 40 (0.9%) subjects.

Characteristics of the subjects with PLNE according to the underlying disorders are shown in Table 1. Female sex was most predominant ($P = 0.003$), the age was youngest ($P = 0.029$) and the size of PLNE was smallest ($P = 0.013$; Fig. 1) in the subjects with PLNE with unknown underlying disorders. Spleen size was not different among the three groups. As a matter of course, serum aspartate aminotransferase and alanine aminotransferase (ALT) levels were highest in the subjects with liver disorder-associated PLNE ($P < 0.001$). On the other hand, serum lactate dehydrogenase levels ($P = 0.01$) and blood urea nitrogen levels ($P < 0.001$) were lowest in the subjects with PLNE with unknown underlying disorders. Furthermore, serum iron levels were also highest in the subjects with liver disorder-associated PLNE ($P = 0.013$). Although white blood cell counts and red blood cell counts were not different among the three groups, platelet counts were highest in the subjects with PLNE with unknown underlying disorders ($P < 0.001$).

The underlying disorders in 17 subjects with liver disorder-associated PLNE are shown in Figure 2(a). Of note, anti-hepatitis C virus (HCV) antibody positivity was determined in 11 of 17 (64.7%) subjects in line with the previous reports showing that PLNE is frequently observed in chronic hepatitis C.[2,3] Furthermore, serum ALT levels were less than 40 U/L in 8 of 11 (72.7%) subjects with positive anti-HCV antibody in line with the previous evidence postulating that the searching for perihepatic lymph nodes could be recommended in routine diagnostic screening for HCV infection even with normal serum ALT levels.[12,13] Among 11 subjects with anti-HCV antibody positivity, HCV RNA was detected in eight, under detection level in one and not measured in two. In the remaining subjects with liver disorder-associated PLNE, hepatitis B virus surface antigen positivity was determined in two, autoimmune hepatitis was diagnosed with positivity of autoantibod-

Table 1 Characteristics of the subjects with PLNE according to the underlying disorders

	Liver disorders	Malignancy	Unknown	*P*-value
Male/female	8/9	7/6	14/26	**0.003**
Age	61.8 ± 11.7	66.0 ± 12.3	56.2 ± 12.0	**0.029**
Size of PLNE in the longest axis (mm)	18.6 ± 4.4	18.7 ± 5.6	15.3 ± 4.2	**0.013**
Spleen index (cm^2)	14.8 ± 3.9	12.7 ± 6.4	13.3 ± 4.2	**0.41**
CRP (mg/dL)	0.12 ± 0.23	0.22 ± 0.29	0.10 ± 0.28	**0.42**
HbA1C (%)	5.1 ± 0.4	5.5 ± 0.4	5.3 ± 0.6	**0.23**
AST (U/L)	34.6 ± 17.2	24.2 ± 8.4	21.6 ± 7.5	**<0.001**
ALT (U/L)	37.1 ± 27.9	19.9 ± 7.1	18.8 ± 7.8	**<0.001**
Total cholesterol (mg/dL)	197.5 ± 29.5	207.0 ± 79.5	201.4 ± 38.6	**0.86**
Total protein (g/dL)	7.4 ± 0.5	7.0 ± 0.5	7.2 ± 0.5	**0.049**
Albumin (g/dL)	4.2 ± 0.3	4.2 ± 0.3	4.2 ± 0.3	**0.92**
LDH (U/L)	200.5 ± 38.3	204.5 ± 40.4	177.2 ± 28.6	**0.01**
γ-GTP (U/L)	52.6 ± 71.1	51.0 ± 78.6	42.8 ± 70.9	**0.87**
ALP (U/L)	233.7 ± 71.3	331.4 ± 406.2	209.0 ± 77.8	**0.12**
Total bilirubin (mg/dL)	1.0 ± 0.4	0.9 ± 0.2	0.9 ± 0.3	**0.14**
BUN (mg/dL)	15.2 ± 3.0	15.8 ± 4.6	12.9 ± 2.9	**<0.001**
Creatinine (mg/dL)	0.71 ± 0.15	0.71 ± 0.15	0.69 ± 0.13	**0.91**
Fe (μg/dL)	122.1 ± 26.8	96.4 ± 21.3	96.9 ± 33.4	**0.013**
White blood cells ($\times 10^3/\mu L$)	4.66 ± 1.10	5.60 ± 2.50	5.20 ± 1.80	**0.37**
Red blood cells ($\times 10^4/\mu L$)	450.1 ± 44.1	439.0 ± 51.5	443.5 ± 40.7	**0.78**
Hemoglobin (g/dL)	13.4 ± 1.0	14.2 ± 1.1	13.5 ± 1.4	**0.14**
Platelet ($\times 10^4/\mu L$)	18.2 ± 4.3	19.7 ± 6.6	24.4 ± 4.7	**<0.001**

ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aminotransferase; BUN, blood urea nitrogen; γ-GTP, γ-glutamyltransferase; LDH, lactate dehydrogenase; PLNE, perihepatic lymph node enlargement.

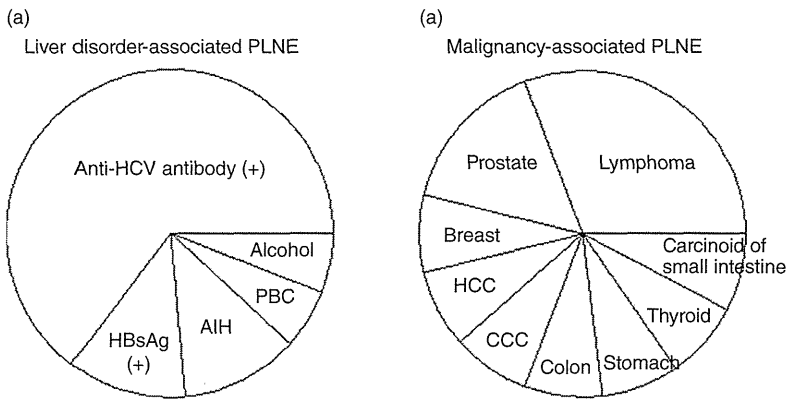

Figure 2 (a) Liver disorder-associated with PLNE. AIH, autoimmune hepatitis; PBC, primary biliary cirrhosis. (b) Malignancy-associated with PLNE. HCC, hepatocellular carcinoma; CCC, cholangiocellular carcinoma.

ies, more than 2 g/dL of serum γ-globulin levels and liver histology in two, primary biliary cirrhosis with positivity of antimitochondrial antibody and elevated serum levels of immunoglobulin M, alkaline phosphatase and γ-glutamyltransferase in one, and alcoholic liver damage with heavy alcohol consumption and exclusion of other liver diseases such as viral hepatitis in one (Fig. 2a).

The underlying diseases in 13 subjects with malignancy-associated PLNE are depicted in Figure 2(b). It is noteworthy that para-aortic lymph nodes were also enlarged in eight of 13 (61.5%) subjects with malignancy-associated PLNE. Thus, when lymph nodes in the perihepatic area are determined at the same time with para-aortic lymph node enlargement, a malignant lesion should be intensively screened.

In the remaining 40 subjects other than those with liver disorder-associated and malignancy-associated PLNE, no underlying disorders were determined even after general blood test, chest X-ray examination, and upper and lower endoscopy. None of these subjects had past history of liver diseases but only one had gall stone. Among the 40 subjects with PLNE with unknown underlying disorders, 27 subjects (67.5%) could be followed up at the next visit for a general health examination. During the mean follow-up period of 2.08 years, no underlying disorders were newly determined with largely unaltered size of PLNE.

As described, PLNE has been evaluated as one of the findings in chronic liver disease, especially in chronic hepatitis C.[1-3] Because we aimed to explore the clinical significance of PLNE in general, we enrolled the subjects, who underwent a general health examination, in the current study, where the vast majority of the enrolled subjects were not outpatients. Although the evidence regarding PLNE in a large cohort has been scarce, Ierna *et al.* analyzed PLNE in 1222 outpatients in Sicily, Italy, who underwent abdominal ultrasonography for various symptoms (e.g. dyspepsia, abdominal pain).[12] Furthermore, Neri *et al.* evaluated PLNE in 7974 subjects who took similarly abdominal ultrasonography in the same area.[13] In the former report, PLNE was determined in 184 patients (15.1%), among whom 171 patients were associated with liver disorders and 142 patients were anti-HCV positive.[12] In the latter reports, PLNE was determined in 684 subjects (8.6%), where all the subjects were associated with liver disorders and 528 subjects were anti-HCV antibody positive.[13] Collectively, the strong association between PLNE and HCV was postulated in these reports.[12,13] The incidence of PLNE in the current study was less (1.6%) than that in the previous reports. This difference may be explained by the fact that HCV was less prevalent in our cohort, although our current evidence still suggests the important contribution of HCV to PLNE. Regarding PLNE of unknown etiology, its incidence was exactly same (0.9%) between the previous report (11/1222)[12] and the current one (40/4234), suggesting that the incidence of PLNE of unknown etiology may be approximately 1% in the general population.

In conclusion, the incidence of PLNE in the general population may vary with the prevalence of chronic liver disease, especially HCV infection. When PLNE is observed at a general health examination, liver disorders should be first surveyed and anti-HCV antibody should be examined even with the subjects with normal serum ALT levels. When PLNE is found at the same time with para-aortic lymph node enlargement, a malignant lesion should be intensively screened. The incidence of PLNE of unknown etiology may be approximately 1%

in the general population, which may be just followed up without further change.

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Original Article

Fibrosis score consisting of four serum markers successfully predicts pathological fibrotic stages of chronic hepatitis B

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Aim: In order to evaluate and judge a fibrotic stage of patients with chronic hepatitis B, multivariate regression analysis was performed using multiple fibrosis markers.

Method: A total of 227 patients from seven hepatology units and institutes were diagnosed by needle biopsy as having chronic liver disease caused by hepatitis B virus. Twenty-three variables and their natural logarithmic transformation were employed in the multivariate analysis. Multiple regression function was generated from data of 158 patients in one hospital, and validation was performed using the other data of 69 patients from six other hospitals.

Results: After stepwise variable selection, multivariate regression analysis finally obtained the following function: $z = 1.40 \times ln$ (type IV collagen 7S) (ng/mL) $- 0.017 \times$ (platelet count) ($\times 1000^3/mm^3$) $+ 1.24 \times ln$ (tissue inhibitor of matrix metalloproteinase-2) (ng/mL) $+ 1.19 \times ln$ (α-2-macroglobulin) (mg/dL) $- 9.15$. Median values of fibrosis scores of F1 ($n = 73$), F2 ($n = 42$), F3 ($n = 31$) and F4 stages ($n = 12$) were calculated as 0.95, 2.07, 2.98 and 3.63, respectively. Multiple regression coefficient and coefficient of determination were 0.646 and 0.418, respectively. Validation with patient data from other institutions demonstrated good reproducibility of fibrosis score for hepatitis B (FSB), showing 1.33 in F1 ($n = 27$), 2.20 in F2 ($n = 20$), 3.11 in F3 ($n = 20$) and 5.30 in F4 ($n = 2$), respectively.

Conclusion: A concise multiple regression function using four laboratory parameters successfully predicted pathological fibrosis stage of patients with hepatitis B virus infection.

Key words: chronic hepatitis, hepatitis B virus, liver cirrhosis, liver fibrosis, multiple regression analysis, stage

INTRODUCTION

WHEN HEPATITIS B virus (HBV)-related chronic liver disease is found by biochemical and virological examination, liver biopsy can establish the definitive diagnosis of chronic hepatitis and its fibrotic staging. Although these pathological procedures are reliable and informative both in diagnosis and treatment, they sometimes require medical invasion and financial costs, including the risk of bleeding from needle puncture, some pain experienced during the procedure and hospital stays of a few days. The pathological examination is, therefore, rarely performed repeatedly in a short period of time, unless disease activity is severe or progression of liver disease is highly suspected. Recently, many authors described the usefulness of ultrasonographic elastography and multiple resonance imaging technology in the estimation of staging of chronic hepatitis and cirrhosis.[1-5] These ways of estimation using the imaging apparatuses seem truly useful for current patients, but they cannot evaluate and compare with past fibrotic states of patients retrospectively. Moreover,

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the same apparatus for elastometry will not be available for repeated measurement for a follow-up examination, for example, several years later.

In spite of the accuracy of biopsy and convenience of elastography in chronic liver disease, clinical diagnosis based on biochemistry and hematology is still indispensable for the daily practice of many patients with HBV-related liver disease. Recently, several studies were published about estimation of hepatitis stages, using one or more serum biomarkers. Discriminant functions or multivariate analyses demonstrated that approximately 60–90% of patients with chronic hepatitis B were correctly classified as having mild hepatitis and severe hepatitis with advanced fibrosis.[2,6–13] Up to the present time, however, the usefulness of the discriminant functions are less valuable for a few reasons. First, these functions were made for the purpose of discrimination of severe hepatic fibrosis from mild fibrosis, and four histological classifications (F1–F4) were neglected in almost of the studies. Second, some studies analyzed both hepatitis B and hepatitis C virus infection, although the significance and actual values of each liver function test in the evaluation of the severity of liver disease were not similar among each viral hepatitis and alcoholic liver disease. Third, biochemical markers for liver fibrosis (e.g. hyaluronic acid, type IV collagen, procollagen III peptide)[14–16] were not always included in those previous studies.

We tried to generate a function estimating fibrotic stages of HBV-related chronic hepatitis, which were objectively diagnosed by liver biopsy. The purpose of this study is, therefore, to make a reliable multiple regression function and to obtain practical coefficients for significant variables also using fibrosis markers.

METHODS

Patients

A TOTAL OF 273 Japanese patients with chronic hepatitis B were recruited for the study from seven hospitals in Japan: Toranomon Hospital, Hiroshima University Hospital (K. Chayama, M.D.), Ehime University Hospital (M. Onji, M.D.), Musashino Red Cross Hospital (N. Izumi, MD), Shishu University Hospital (E. Tanaka, M.D.), Showa University Hospital (M. Imawari, M.D.) and Osaka University Hospital (T. Takehara, M.D.). Inclusion criteria for this study were: (i) positive hepatitis B surface antigen for more than 6 months; (ii) persistent or intermittent elevation in aspartate aminotransferase (AST)/alanine aminotransferase (ALT) levels; and (iii) liver biopsy showing chronic hepatitis (F1–F4). We excluded those patients with overt alcoholic liver disease or fatty liver, association of other types of liver disease (e.g. hepatitis C, primary biliary cirrhosis, autoimmune hepatitis), or those associated with hepatocellular carcinoma or other malignancy. Among the patients, 244 patients fulfilled the conditions for the study: complete demographic data, basic laboratory data of hematology and biochemistry, required liver biopsy specimens, and sufficient amount of frozen sera. Also, we excluded additional 17 patients with eventual histological diagnosis as F0 stage.

Finally, a total of 227 patients who were diagnosed as having chronic hepatitis or cirrhosis (F1–F4) were analyzed for the following hematological, biochemical and histopathological examination. There were 172 males and 55 females aged 16–70 years (median, 39 years).

All the patients presented written informed consent in individual hospitals and medical centers, and the study was approved in each ethical committee.

Hematological and biochemical examination

Hematological and standard biochemical evaluation had been performed in each medical institution: white blood cells, red blood cells, hemoglobin, platelets, total bilirubin, AST, ALT, AST/ALT ratio (AAR), γ-glutamyl transpeptidase (γ-GTP), total protein, albumin and γ-globulin.

Special biochemical examinations including "fibrosis markers" were carried out using stored frozen sera at −20°C or lower: α-2-macroglobulin, haptoglobin concentration, haptoglobin typing, apolipoprotein A1, hyaluronic acid, tissue inhibitor of matrix metalloproteinase (TIMP)-1, TIMP-2, procollagen III peptide and type IV collagen 7S.

Histological diagnosis of chronic hepatitis and cirrhosis

All the 227 cases fulfilled required standards of histological evaluation: sufficient length of specimen, hematoxylin–eosin staining, and at least one specimen with fiber staining. Four independent pathologists (Y. T., J. F., F. K. and T. F.), who were not informed of patients' background and laboratory features except for age and sex, evaluated the 227 specimens regarding the stages of fibrosis and activity. Pathological classification of chronic hepatitis staging was based on Desmet *et al.*[17]

Before judgment of histological staging of individual specimens, the pathologists discussed the objective and reproducible judgment of pathological diagnosis of

hepatitis. They made a panel about obvious criteria using typical microscopic pictures for each stage, and it was always referred to during the procedure of pathological judgment. When inconsistent results were found in the diagnosis of hepatitis stage among the pathologists, the final judgment accepted majority rule among them.

Statistical analysis

Non-parametric procedures were employed for the analysis of background characteristics and laboratory data among patients in each stage, including Mann–Whitney *U*-test, Kruskal–Wallis test and χ^2-test.

The normality of the distribution of the data was evaluated by a Kolmogorov–Smirnov one-sample test. Because certain variables partly did not conform to a normal distribution, natural logarithmic transformation of bilirubin, AST, ALT, γ-GTP, α-2-macroglobulin, hyaluronic acid, type IV collagen 7S and TIMP-2 were also analyzed in the following calculation. The natural logarithmic transformation of the results yielded a normal distribution or symmetrical distribution for all the analyzed factors. After the procedures, the following multiple regression analysis became rationally robust against deviations from normal distribution. In order to avoid introducing into the model any variables that were mutually correlated, we checked the interaction between all pairs of the variables by calculating variance inflation factors. Of the highly correlated variables, less significant factors were removed from the viewpoint of multicollinearity.

Multivariate regression analysis was performed using 158 patient data from Toranomon Hospital (training dataset) to generate a training data of predicting function. We used a stepwise method for selection of informative subsets of explanatory variables in the model. Multiple regression coefficient and coefficient of determination were also taken into account in the selection of variables. Next, we validated the obtained predictive function using the remaining 69 patient data from the other six liver institutions (validation dataset).

A *P*-value of less than 0.05 with two-tailed test was considered to be significant. Data analysis was performed using the computer program SPSS ver. 19.[18]

For evaluation of the efficiency and usefulness of obtained function for fibrosis estimation, we compared various fibrosis scores for hepatitis B and C, including AAR,[19] AST-to-platelet ratio index (APRI),[20] FIB-4,[21] FibroTest[22] and discrimination function of cirrhosis from hepatitis in Japanese patients.[23]

RESULTS

Pathological diagnosis

FOUR PATHOLOGISTS INDEPENDENTLY judged the fibrotic stages and inflammatory activity for 227 specimens of chronic hepatitis/cirrhosis caused by HBV. One hundred patients (44.1%) had a fibrosis stage of F1, 62 (27.3%) F2, 51 (22.5%) F3 and 14 (6.2%) F4. In the subgroup of the 158 patients in the training group, judgment as F1 was made in 73 cases, F2 in 42, F3 in 31 and F4 in 12. Of the 69 patients in the validation group, judgment as F1 was made in 27, F2 in 20, F3 in 20 and F4 in two.

According to hepatitis activity classification, A0 was found in five (2.2%), A1 in 100 (44.1%), A2 in 107 (47.1%) and A3 in 15 (6.6%).

Laboratory data of each hepatitis stage in the training group

There were 124 men and 34 women with a median age of 39 years ranged 16–70 years. Laboratory data of 158 patients in the training group are shown in Table 1. Although several individual items were well correlated with the severity of hepatic fibrosis, significant overlap values were noted among F1–F4 stages: platelet count, γ-globulin, α-2-macroglobulin, haptoglobin, hyaluronic acid, TIMP-2 and type IV collagen 7S.

Significant variables serving staging of hepatitis

Univariate analyses using trend analysis with the Cochran–Armitage method showed that the fibrotic stage of chronic hepatitis B (FSB) was significantly correlated with platelet count (Spearman: $r = -0.45$, $P < 0.001$), γ-GTP ($r = 0.19$, $P = 0.017$), γ-globulin ($r = 0.29$, $P < 0.001$), α-2-macroglobulin ($r = 0.32$, $P < 0.001$), hyaluronic acid ($r = 0.36$, $P < 0.001$), TIMP-2 ($r = 0.16$, $P = 0.043$), procollagen III peptide ($r = 0.30$, $P < 0.001$) and type IV collagen 7S ($r = 0.55$, $P < 0.001$).

Regression function generated from training patient group

After stepwise variable selection, multivariate regression analysis finally obtained the following function: $z = 1.40 \times ln$ (type IV collagen 7S) (ng/mL) $- 0.017 \times$ (platelet count) ($\times 1000^3/mm^3$) $+ 1.24 \times ln$ (TIMP-2) (ng/mL) $+ 1.19 \times ln$ (α-2-macroglobulin) (mg/dL) $-$ 9.15. Median values of the fibrosis score of F1 ($n = 73$), F2 ($n = 42$), F3 ($n = 31$) and F4 stages ($n = 12$) were calculated as 0.95, 2.07, 2.98 and 3.63, respectively

Table 1 Demography and laboratory data of 158 patients in training group

	F1 ($n = 73$)	F2 ($n = 42$)	F3 ($n = 31$)	F4 ($n = 12$)
Demographics				
Men : women	58:15	33:9	23:8	10:2
Age (median, range)	36 (16–70)	39.5 (18–66)	39 (25–64)	43 (32–59)
Laboratory data (median, range)				
WBC (×1000/mm^3)	5.4 (2.5–10.6)	5.1 (2.4–8.7)	4.9 (3.0–8.7)	4.1 (3.7–6.6)
Hemoglobin (g/dL)	15.3 (10.3–18.8)	15.4 (12.5–17.9)	15.2 (11.5–17.2)	14.45 (12.1–18.2)
Platelet (×1000/mm^3)	204 (124–341)	173 (82–308)	155 (96–220)	130 (86–230)
Albumin (g/dL)	4.1 (3.2–4.9)	4.0 (3.2–5.1)	4.0 (3.3–4.9)	3.95 (3.4–4.6)
Bilirubin (mg/dL)	0.8 (0.2–1.7)	0.8 (0.3–2.3)	0.9 (0.4–5.4)	0.85 (0.6–2.3)
AST (IU/L)	48 (16–450)	55 (17–588)	54 (17–1446)	76.5 (27–396)
ALT (IU/L)	102 (10–839)	90 (12–886)	85 (19–2148)	89 (18–809)
γ-GTP (IU/L)	37 (7–247)	55 (8–687)	44 (14–564)	69 (33–262)
γ-Globulin (g/dL)	1.29 (0.78–2.11)	1.495 (0.62–3.20)	1.43 (0.90–2.30)	1.735 (0.92–2.47)
γ-Globulin (%)	17.3 (10.8–26.1)	19.3 (8.5–35.6)	19.9 (12.9–28.6)	22.55 (13.9–30.2)
α-2-Macroglobulin (mg/dL)	226 (116–446)	276 (148–495)	261 (202–565)	286.5 (166–425)
Haptoglobin (mg/dL)	77 (<5–318)	59 (<5–238)	61 (<5–151)	48.5 (<5–145)
Apolipoprotein A-I (mg/dL)	134 (89–212)	143 (78–250)	133 (87–189)	125 (73–169)
Hyaluronic acid (μg/L)	16 (<5–130)	32.5 (<5–204)	38 (<5–418)	49 (24–335)
TIMP-1 (ng/mL)	168 (93–271)	172 (116–314)	157 (119–365)	192 (145–365)
TIMP-2 (ng/mL)	80 (41–135)	80.5 (35–121)	92 (38–251)	85.5 (70–123)
Procollagen III peptide (U/mL)	0.75 (0.53–1.90)	0.835 (0.45–1.20)	0.89 (0.58–2.50)	1.05 (0.71–2.20)
Type IV collagen 7S (ng/ml)	4.0 (2.7–7.7)	4.6 (2.6–9.6)	5.6 (2.3–15.0)	7.2 (4.2–14.0)

ALT, alanine aminotransferase; AST, aspartate aminotransferase; γ-GTP, γ-glutamyl transpeptidase; TIMP, tissue inhibitor of matrix metalloproteinase; WBC, white blood cells.

(Fig. 1). The multiple regression coefficient and coefficient of determination were 0.646 ($P < 0.001$) and 0.418 ($P < 0.001$), respectively.

Because the generated regression function was obtained by multivariate analysis with stepwise variable selection, several variables were removed from the function due to multicollinearity among them. Mutual correlation among the fibrosis predictors are shown in Table 2.

A 28-year-old man of F1 fibrotic stage (Fig. 2a) had a serum type IV collagen concentration of 4.4 ng/mL, platelet 221 × 10^3 count/mm^3, TIMP-2 75 ng/mL and α-2-macroglobulin 226 mg/dL. The regression function provided a fibrosis score of 0.99. Another man aged 46 years had F3 fibrosis on histological examination (Fig. 2b). His type IV collagen was 5.3 ng/mL, platelet 137 × 10^3 count/mm^3, TIMP-2 92 ng/mL and α-2-macroglobulin 255, and the regression function calculated his fibrosis score as 3.10.

Validation of discriminant function

Validation data of 69 patients (Table 3) were collected from the other six institutions in Japan. When applying

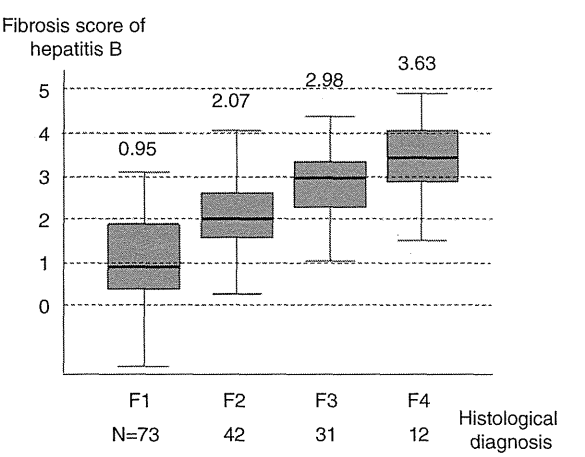

Figure 1 Box and whisker plots of fibrotic score of each histological fibrosis group in the training dataset. The fibrosis score of hepatitis B was generated by the function, $z = 1.40 \times ln$ (type IV collagen 7S) (ng/mL) − $0.017 \times$ (platelet count) (×1000^3/mm^3) + $1.24 \times ln$ (tissue inhibitor of matrix metalloproteinase-2) (ng/mL) + $1.19 \times ln$ (α-2-macroglobulin) (mg/dL) − 9.15.

Table 2 Correlation coefficients (Spearman's ρ) among fibrosis predictors used in multivariate analysis

	Platelet	gamma-globulin	*ln* (α-2-macroglobulin)	*ln* (hyaluronate)	*ln* (P-III-P)	*ln* (IV collagen)	*ln* (TIMP-2)
Platelet ($\times 10^3/mm^3$)	1.000	−0.214 ($P = 0.008$)	−0.260 ($P = 0.001$)	−0.384 ($P < 0.001$)	−0.045 ($P = 0.58$)	−0.297 ($P < 0.001$)	0.094 ($P = 0.24$)
γ-Globulin (g/dL)		1.000	0.276 ($P = 0.001$)	0.349 ($P < 0.001$)	0.342 ($P < 0.001$)	0.414 ($P < 0.001$)	0.268 ($P = 0.001$)
ln (α-2-macroglobulin) (mg/dL)			1.000	0.281 ($P < 0.001$)	0.141 ($P = 0.078$)	0.171 ($P = 0.032$)	−0.079 ($P = 0.32$)
ln (hyaluronic acid) (mg/L)				1.000	0.373 ($P < 0.001$)	0.493 ($P < 0.001$)	0.089 ($P = 0.27$)
ln (procollagen III peptide) (U/mL)					1.000	0.600 ($P < 0.001$)	0.145 ($P = 0.071$)
ln (type IV collagen) (mg/L)						1.000	0.358 ($P < 0.001$)
ln (TIMP-2) (mg/L)							1.000

TIMP, tissue inhibitor of matrix metalloproteinase.

the regression function for the validation set, the fibrosis score demonstrated good reproducibility, showing 1.33 in patients with chronic hepatitis of F1 ($n = 27$), 2.20 of F2 ($n = 20$), 3.11 of F3 ($n = 20$) and 5.30 of F4 ($n = 2$), respectively (Fig. 3). Although F4 fibrosis stage consisted of only two patients and the score 5.30 was regarded as of rather higher value, the scores of other stages of fibrosis were concordant with histological fibrosis.

Comparisons of efficacy with various fibrosis scores (Fig. 4)

In order to evaluate the efficacy and usefulness of the obtained FSB, we compared it with previously reported fibrosis scores using training data. AAR, APRI and FibroTest showed only slight correlation with actual histological stage. FIB-4 demonstrated an increasing trend of the score associated with histological fibrosis, but significant overlapping scores were found in F1–F4. Spearman's correlation coefficients of AAR, APRI, FIB-4 and FibroTest were 0.199 ($P = 0.012$), 0.265 ($P = 0.001$), 0.412 ($P < 0.001$) and 0.330 ($P < 0.001$), respectively. Our FSB showed a Spearman's correlation coefficient of 0.625 ($P < 0.001$), and was a much higher value than the others. The dichotomous discrimination function for cirrhosis and hepatitis C in Japanese patients[23] showed good differentiation also in patients with hepatitis B virus.

DISCUSSION

RECOGNITION OF SEVERITY of chronic hepatitis is essential in managing patients with chronic HBV infection: estimation of length of infection, existence of any previous hepatitis activity, presumption of current fibrotic stage, and prediction of future fibrosis progression and hepatocarcinogenesis. Differential diagnosis of cirrhosis from chronic hepatitis is especially important in the evaluation of chronic HBV infection. Identification of liver cirrhosis often leads to an important change in management of the patient: need for fiberscopic examination for esophageal varices, ultrasonographic exploration for the association of liver cancer, and prediction of hepatic decompensation. Guidelines published by the American Association of Study of Liver Disease[24] recommend liver biopsy for HBV carriers with aminotransferase elevation or for any candidates of antiviral therapy, because hepatic fibrosis sometimes shows unexpectedly far advancement to cirrhosis, and because it is very difficult to evaluate and translate the liver function tests or ultrasonographic findings compared to chronic hepatitis type C.

Table 3 Demography and laboratory data of 69 patients in training group

	F1 ($n = 27$)	F2 ($n = 20$)	F3 ($n = 20$)	F4 ($n = 2$)
Demographics				
Men : women	18:9	15:5	13:7	2:0
Age (median, range)	36 (13–64)	45 (14–64)	36.5 (24–59)	32 (25–39)
Laboratory data (median, range)				
WBC (×1000/mm^3)	5.0 (2.8–8.7)	5.8 (2.8–11.6)	5.3 (3.2–8.1)	3.85 (2.7–5.0)
Hemoglobin (g/dL)	14.8 (12.4–17.4)	15.0 (12.4–16.9)	14.4 (11.1–16.4)	14.4 (12.5–16.3)
Platelet (×1000/mm^3)	204 (86–322)	180 (90–275)	147 (90–276)	130 (67–183)
Albumin (g/dL)	4.4 (2.8–5.2)	4.2 (3.5–5.1)	4.3 (3.4–4.9)	4.45 (4.0–4.9)
Bilirubin (mg/dL)	0.9 (0.4–6.4)	0.8 (0.2–1.6)	0.75 (0.4–1.7)	1.15 (1.1–1.2)
AST (IU/L)	52 (17–575)	50.5 (21–272)	65 (22–284)	248.5 (51–446)
ALT (IU/L)	84 (16–1101)	101.5 (19–554)	86.5 (16–1113)	453.5 (74–833)
γ-GTP (IU/L)	42 (14–332)	54 (16–205)	52.5 (13–191)	193 (57–329)
γ-Globulin (g/dL)	1.30 (1.04–1.59)	1.35 (1.18–2.53)	1.62 (1.16–1.97)	1.545 (1.51–1.58)
γ-Globulin (%)	17.9 (14.3–22.1)	19.6 (15.5–30.8)	22.0 (16.5–24.6)	20.15 (19.3–21.0)
α-2-Macroglobulin (mg/dL)	287 (160–687)	270 (89–452)	272.5 (211–463)	389 (313–465)
Haptoglobin (mg/dL)	58 (<5–229)	74 (<5–154)	56.5 (<5–198)	<5 (<5–<5)
Apolipoprotein A-I (mg/dL)	146 (95–216)	137 (87–162)	120 (88–170)	100.5 (74–127)
Hyaluronic acid (μg/L)	27 (<5–113)	36 (10–1050)	59 (14–439)	331 (225–437)
TIMP-1 (ng/mL)	168.5 (83–302)	176 (127–408)	182 (104–303)	390.5 (283–498)
TIMP-2 (ng/mL)	76 (25–143)	86.5 (28–154)	77.5 (32–141)	100.5 (91–110)
Procollagen III peptide (U/mL)	0.71 (0.27–2.20)	0.88 (0.63–2.80)	0.995 (0.60–2.10)	1.75 (1.50–2.00)
Type IV collagen 7S (ng/ml)	3.6 (2.7–17.0)	5.25 (3.3–13.0)	5.7 (3.0–16.0)	15.5 (15.0–16.0)

ALT, alanine aminotransferase; AST, aspartate aminotransferase; γ-GTP, γ-glutamyl transpeptidase; TIMP, tissue inhibitor of matrix metalloproteinase; WBC, white blood cells.

Recently, non-invasive estimation of severity of liver fibrosis has been reported in patients with HBV-related chronic hepatitis.[2,6–13] However, these studies were principally aimed at differentiation of advanced fibrotic stages of F3 or F4 from mild fibrotic stages of F1 or F2. Those discrimination functions were insufficient to recognize the stepwise progression of viral hepatitis from F1–F4. This dichotomy (mild or severe) of chronic hepatitis B seemed less valuable in the study of disease progression, disease control abilities of antiviral drugs and estimation of histological improvement after anti-inflammatory drugs. A histology-oriented, practical and reliable formula is therefore required for the diagnosis and investigation of chronic hepatitis B.

This study aimed to establish non-invasive evaluation and calculation of liver fibrosis for patients with chronic hepatitis B virus infection. Although it was retrospectively performed as a multicenter study of eight institutions, judgment of histological diagnosis was independently performed by four pathologists in another hospital, who were informed only of the patient's age, sex and positive HBV infection. Objective judgment of the histological staging and grading in sufficient biopsy specimens could be obtained.

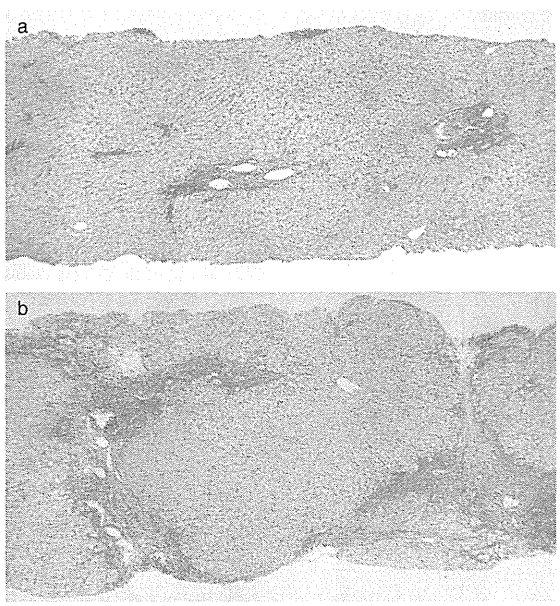

Figure 2 Case presentations of the training set. (a) A 28-year-old man with F1 fibrosis. Final regression function provided his fibrosis score as 0.99. (b) A 45-year-old man with F3 fibrosis. His regression coefficient was calculated as 3.10. Silver stain, ×40.

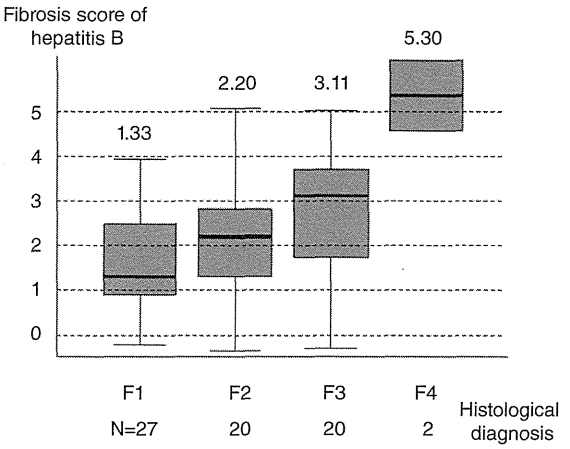

Figure 3 Box and whisker plots of fibrotic score of each group of histological fibrosis in the validation dataset. The fibrosis score of hepatitis B was generated by the function, $z = 1.40 \times ln$ (type IV collagen 7S) (ng/mL) − 0.017 × (platelet count) ($\times 1000^3/mm^3$) + 1.24 × *ln* (tissue inhibitor of matrix metalloproteinase-2) (ng/mL) + 1.19 × *ln* (α-2-macroglobulin) (mg/dL) − 9.15.

As many as 227 patients with chronic hepatitis B were analyzed in this study, who had been diagnosed as having chronic hepatitis or cirrhosis by liver biopsy performed in experienced liver units in Japan. To obtain the most suitable equation approximating histological fibrotic stage, multivariate analysis was performed using two demographic parameters (age and sex) and 21 hematological and biochemical markers with or without logarithmic transformation. They included many kinds of fibrosis markers: α-2-macroglobulin, haptoglobin concentration, haptoglobin typing, apolipoprotein A1, hyaluronic acid, TIMP-1, TIMP-2, procollagen III peptide and type IV collagen 7S Multiple regression analysis finally generated a first-degree polynomial function consisting of four variables: type IV collagen 7S, platelet count, TIMP-2 and α2-macroglobulin. A constant numeral (−9.15) was finally adjusted in the regression equation in order to obtain fitted figures for a fibrotic stage of F1–F4. From the magnitude of the standardized partial regression coefficient of individual variable in the function, platelet count demonstrated the most potent contribution toward the prediction of liver fibrosis. Type IV collagen 7S and *ln* (TIMP-2) proved to be the second and third distinctive power in the model, respectively.

The FSB was sufficiently fitted to actual fibrotic stages with certain overlapping as is usually found in histological ambiguity judged by pathologists. Because judgment of fibrosis in chronic hepatitis often shows a transitional histological staging, pathological examination cannot always make a clear-cut diagnosis discriminating F1–F4. Considering the limitation of the pathological difficulty in differentiating the four continuous disease entities, the obtained regression function showed satisfactory high accuracy rates in the prediction of liver disease severity. The FSB can provide one or two decimal places (e.g. 3.2 or 3.24) and the utility of the score is possibly higher than the mere histological stage of F1–F4. The reproducibility was confirmed by the remaining 67 patients' data obtained from the other six hospitals. Although the validation data were collected from a different geographic area and different chronological situation, the FSB showed similar results in prediction of histological staging.

The FSB seemed a very useful quantitative marker in evaluating fibrotic severity of hepatitis B patients without invasive procedures and without any specialized ultrasonography or magnetic resonance imaging. The FSB also has an advantage of measurement, in which old blood samples are available for retrospective assessment of varied clinical settings: for example, old sera from 20 years prior to the time of initial liver biopsy, or paired sera before and after long-term antiviral therapy. These kinds of retrospective assessments of fibrotic staging will be valuable in estimating a long-term progression of liver disease, in evaluating efficacy of long-term medication or other medical intervention, or in making a political judgment from the viewpoints of socioeconomic efficacy.

The score can be calculated for any patients with chronic HBV infection. Although this multiple regression model dealt with appropriate logarithmic transformation for non-normal distribution parameters, the regression analysis was based on a linear regression model. Very slight fibrosis can be calculated as less than 1.00, which is commonly found to a slight degree in chronic hepatitis with tiny fibrotic change as F0. Very severe fibrosis might be calculated as more than 4.00, which is an imaginary and nonsense number in the scoring system of fibrosis. The FSB is, however, very useful and valuable in a real clinical setting: estimation of severity of liver fibrosis in an outpatient clinic, evaluation of the natural progression of a patient's fibrosis over 10 years and assessment of a long-term administration of interferon in patients with chronic hepatitis B from the viewpoint of fibrotic change. Recent development of new nucleoside/nucleotide analogs requires evaluation for long-term histological advantage, for aggravation of hepatitis stage during viral and biochemical breakthrough caused by HBV mutation, and even for

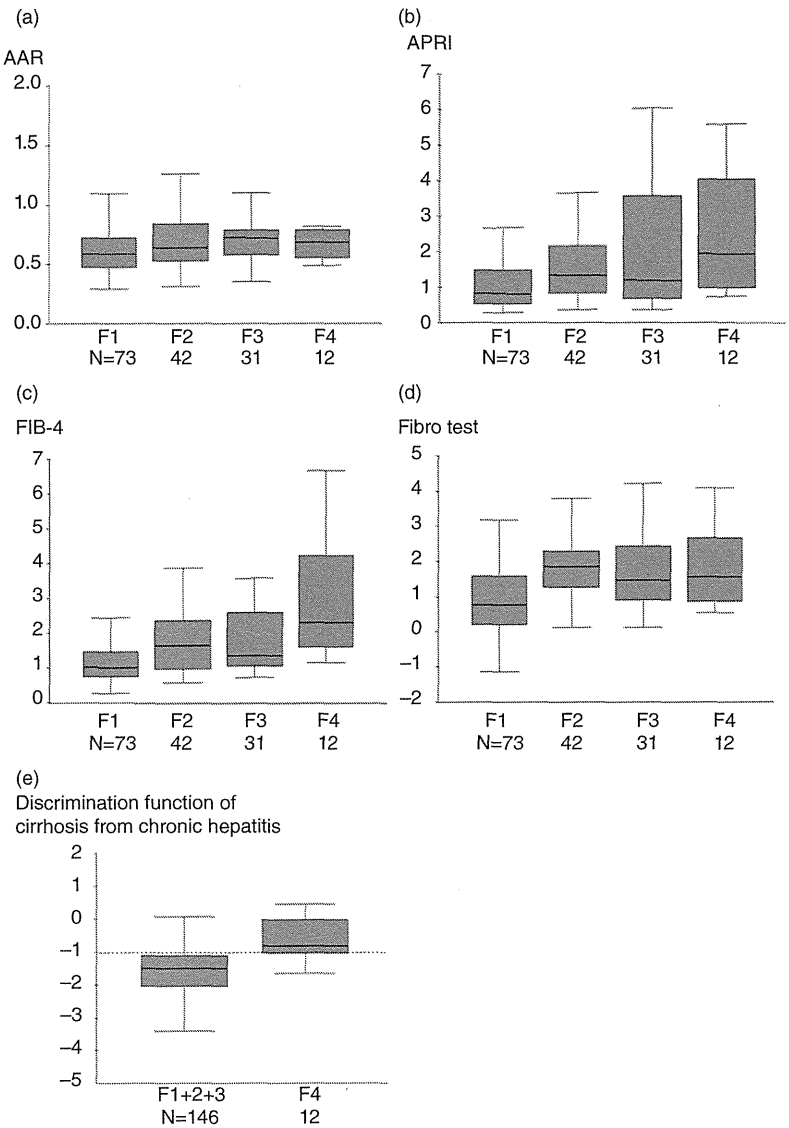

Figure 4 Previously published fibrosis scores. (a) Aspartate aminotransferase/alanine aminotransferase ratio (AAR),[19] (b) aspartate aminotransferase-to-platelet ratio index (APRI),[20] (c) FIB-4,[21] (d) FibroTest[22] and (e) discrimination function of cirrhosis from hepatitis in Japanese patients.[23]

the best management of patients with chronic hepatitis B. The FSB seems one of the ideal methods of approximating the fibrotic stage of chronic hepatitis B. Repeated measurement is quite suitable for patients with an unestablished treatment or trial, every 1 or 2 years, for example. Because the current regression function was generated from the data of HBV-related chronic liver disease, this equation would not be suitable for the recognition of hepatitis C virus-related chronic liver disease, alcoholic liver disease, and other congenital or autoimmune liver diseases. To recognize the latter diseases, other studies of individual diseases must be performed.

We compared the usefulness of the FSB with that of other fibrosis scores.[19–23] The more simple and less expensive AAR or APRI could not estimate fibrotic stages with poor correlation coefficients of 0.199 and 0.265, which are much lower than the coefficient of the FSB of 0.625. FibroTest, which contained three costly fibrosis markers (α-2-macroglobulin, haptoglobin and apolipo-

protein A1), also showed a low correlation coefficient of 0.330, suggesting that its usefulness was limited in HBV positive oriental patients. Although FIB-4 demonstrated the best coefficient of 0.412 among the fibrosis scores, significant overlaps were found between neighboring stages and obtained scores were not coordinated for real histological classification.

In conclusion, the FSB was a useful and reliable biomarker for prediction of liver fibrosis in patients with chronic HBV infection. The FSB is expected to be introduced and utilized in varied kinds of studies and trials. Its accuracy and reproducibility require further validation using higher numbers of patients in several countries other than Japan.

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Clinical Study

The Effectiveness of Liraglutide in Nonalcoholic Fatty Liver Disease Patients with Type 2 Diabetes Mellitus Compared to Sitagliptin and Pioglitazone

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Background. Liraglutide leading to improve not only glycaemic control but also liver inflammation in non-alcoholic fatty liver disease (NAFLD) patients. *Aims.* The aim of this study is to elucidate the effectiveness of liraglutide in NAFLD patients with type 2 diabetes mellitus (T2DM) compared to sitagliptin and pioglitazone. *Methods.* We retrospectively enrolled 82 Japanese NAFLD patients with T2DM and divided into three groups (liraglutide: $N = 26$, sitagliptin; $N = 36$, pioglitazone; $N = 20$). We compared the baseline characteristics, changes of laboratory data and body weight. *Results.* At the end of follow-up, ALT, fast blood glucose, and HbA1c level significantly improved among the three groups. AST to platelet ratio significantly decreased in liraglutide group and pioglitazone group. The body weight significantly decreased in liraglutide group (81.8 kg to 78.0 kg, $P < 0.01$). On the other hands, the body weight significantly increased in pioglitazone group and did not change in sitagliptin group. Multivariate regression analysis indicated that administration of liraglutide as an independent factor of body weight reduction for more than 5% (OR 9.04; 95% CI 1.12–73.1, $P = 0.04$). *Conclusions.* Administration of liraglutide improved T2DM but also improvement of liver inflammation, alteration of liver fibrosis, and reduction of body weight.

1. Introduction

Nonalcoholic fatty liver disease (NAFLD) is reported to be the most common liver disease, increasing in prevalence in Western countries as well as in Japan because of the raising prevalence of obesity [1, 2]. NAFLD shows a wide disease spectrum ranging from simple steatosis to steatohepatitis and finally to cirrhosis. Approximately 3% of the patients who have NAFLD will develop cirrhosis [3]. The main pathophysiological problem in NAFLD patients is insulin resistance. Thus, there is a clear association between NAFLD and metabolic syndrome which induces type 2 diabetes mellitus (DM), obesity, hypertension, and dyslipidemia [4]. Improvement of insulin resistance and sensitivity has therapeutic effect in preventing the progression of NAFLD because the accumulation of triglycerides in hepatocytes is considered to be the first step in the current two-hit theory of the pathophysiological development of NAFLD [5]. Several studies indicated that improving insulin resistance and sensitivity would reduce fatty liver change and might prevent the second step of hepatocytes injury due to oxidative stress [6–8].

Glucagon like peptide-1 (GLP-1) is a naturally existing incretin hormone with a potent blood-glucose reducing action only during hyperglycemia because it induces insulin secretion and reduces glucagon secretion in a glucose-dependent mechanism [9]. In addition, GLP-1 prolongs gastric emptying and induces satiety, leading to decreased energy intake and body weight [10, 11]. Therefore, GLP-1 has a great potential among type 2 DM patients. However,

its half-life is extremely short because GLP-1 is rapidly degraded by the enzyme dipeptidyl peptidase-4 (DPP-4) [9]. Liraglutide, one of the GLP-1 analogues, has 97% aminoacid sequence identity to native human GLP-1 and an acyl side-chain attachment, which makes it bind to albumin. These small structural differences prolong the half-life of GLP-1 to 13 hours, making it possible for once daily administration [12]. Several studies showed that liraglutide was well tolerated, improved glycaemic control with a low risk of hypoglycemia, improved functions of beta-cell, and was associated with body weight reduction [13]. The receptors of GLP-1 analogue also exist in human hepatocytes and administration of GLP-1 analogue reported to directly reduce liver steatosis and fibrosis in *in vivo* study [14, 15].

DPP-4 inhibitors (DPP-4I) are also novel drugs as GLP-1 analogue which affect incretin hormone. DPP-4 is one of the serine proteases enzymes that lead inactivation of incretin hormone such as GLP-1. DPP-4I is a class of oral hypoglycemics that block the activity of DPP-4. The mechanism of DPP-4I is to increase GLP-1 levels, which inhibit glucagon release, which in turn increases insulin secretion, decreases gastric emptying, and decreases blood glucose levels [16]. Serum DPP-4 activity is reported to be significantly higher in NAFLD patients [17]. Thus, administration of DPP-4I might have possibility to improve fatty liver change as same mechanism as GLP-1 analogue. However, the effectiveness of DPP-4 inhibitors on NAFLD patients is still unknown. Future use of GLP-1 analogue and DPP-4I for NAFLD may be significant advance in treatment of this common form of disease.

On the other hand, pioglitazone has already several clinical evidences on treatment of NAFLD [18]. Pioglitazone, a thiazolidinedione derivative (TZD), is a peroxisome proliferator-activated receptor γ (PPARγ) agonist that ameliorates insulin resistance and improves glucose and lipid metabolism in type 2 DM [19]. Insulin resistance in NAFLD is frequently associated with chronic hyperinsulinemia, hyperglycemia, and an excessive supply of plasma free fatty acids to the liver. Pioglitazone reverses these abnormalities by improving insulin resistance in adipose tissues, the liver, and muscles [20]. However, there is a disadvantage of increasing body weight [21] which may affect on long-term outcomes because weight reduction is one of the important treatment of NAFLD [8]. According to these backgrounds, we conducted this retrospective cohort study to compare the efficacy and effectiveness among liraglutide, one of the GLP-1 analogues compared to sitagliptin, one of the DDP-4 inhibitors and pioglitazone.

2. Patients and Methods

2.1. Patients. Between April 1, 2003 and March 31, 2011, a total of 126 patients who were clinically diagnosed NAFLD with type 2 DM visited the out patient clinic of Department of Diabetes and Metabolism or Department of Gastroenterology, Mitsui Memorial Hospital. We retrospectively analyzed 82 of them, excluding 44 patients only treated with insulin injection or exercise and diet therapy. We divided the rest 82 patients into three groups: liraglutide-treated group (N = 26), sitagliptin-treated group (N = 36), and pioglitazone-treated group (N = 20) (Figure 1). All of these patients were negative for hepatitis B and C virus infection, anti-mitochondrial antibody, and antinuclear antibody. Hemochromatosis and Wilson's disease were diagnosed in none of them. Clinical diagnosis of NAFLD was based on the following criteria: existence of fatty liver change in ultrasonography, alcohol consumption less than 20 g ethanol per day, and continuous elevation of alanine aminotransferase (ALT) equal or over 40 IU/L for more than 6 months. Diagnosis of DM was based on medical history or 75 g oral glucose tolerance test. Dyslipidemia was defined as blood total cholesterol concentration over 220 mg/dL or triglyceride over 150 mg/dL, or history of taking oral drugs for dyslipidemia. Hypertension was defined as systolic blood pressure over 140 mmHg or diastolic blood pressure over 90 mmHg, or taking oral drugs for hypertension. Body mass index (BMI) was calculated as body weight in kilogram (kg) divided twice by body height in meter (m), which was also routinely measured at the beginning of the treatment. The evaluation of liver fibrosis depended on calculation of aspartate aminotransferase (AST) to platelet counts ratio (APRI) index [22]. APRI index was calculated as AST level (IU/L) divided by upper limit of AST (37 IU/L) and platelet counts ($\times 10^9$/L), and finally multiplied by 10^2. APRI over 1.5 was considered as bridging fibrosis and over 2.0 as liver cirrhosis. This study was conducted according to STROBE statement [23].

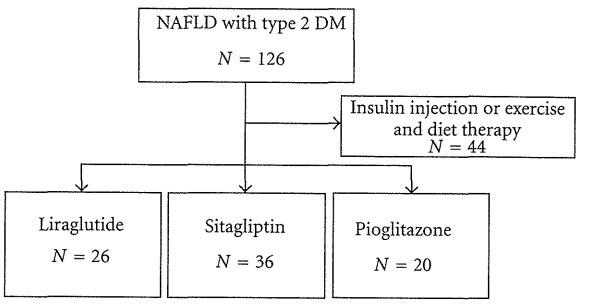

FIGURE 1: A total of 126 patients who were clinically diagnosed NAFLD with type 2 DM visited the outpatient clinic of Department of Diabetes and Metabolism or Department of Gastroenterology, Mitsui Memorial Hospital. We retrospectively analyzed 82 of them, excluding 44 patients only treated with insulin injection or exercise and diet therapy. We divided the rest 82 patients into three groups: liraglutide-treated group (N = 26), sitagliptin-treated group (N = 36), and pioglitazone-treated group (N = 20).

2.2. Treatment and Followup. All patients were treated in our out patient clinic and had uncontrollable type 2 diabetes (HbA1c over 6.5%) with exercise and diet therapy. The administration of each medicine, liraglutide or sitagliptin or pioglitazone, was determined by our out patient clinic doctors. Liraglutide was subcutaneously injected once daily 0.3 mg for the first week, 0.6 mg for the next week, and