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Original Article

Disease Progression-Related Markers for Aged Non-Alcoholic Fatty Liver Disease Patients

Kosaku Morimoto, Yasuto Takeuchi, Akinobu Takaki*, Nozomu Wada, Atsushi Oyama, Takuya Adachi, Hideki Onishi, Hidenori Shiraha, and Hiroyuki Okada

Department of Gastroenterology and Hepatology, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama 700-8558, Japan

Liver fibrosis is an important phenomenon in non-alcoholic fatty liver disease (NAFLD) progression. Standard markers reflecting liver fibrosis, including the FIB-4 index, increase with age. This study aimed to identify fibrosis progression-related markers that are diagnostically beneficial even in aged individuals. Serum levels of pro- and anti-inflammatory cytokines were measured by multiple enzyme-linked immunosorbent assay. Two standard NAFLD or fibrosis progression-related markers — the FIB-4 index and APRI score — were analyzed along with cytokine levels to define the best approach to discriminate advanced fibrosis. Ninety-eight NAFLD patients were enrolled: 59 and 39 patients with fibrosis stages 1-2 and 3-4 respectively. In addition to the FIB-4 index and APRI score, the following factors showed significant differences between stages 1-2 and stages 3-4 in a multivariate analysis: platelet counts, IP-10, and RANTES. The fibrosis stage, FIB-4, APRI, PDGF-BB, and RANTES were related to the prognosis. In aged patients, IP-10, GM-CSF, and RANTES differed between stages 1-2 and stages 3-4. FIB-4 and APRI were beneficial for their correlation with fibrosis. However, to stratify either young or elderly advanced fibrosis patients, and to identify patients likely to have a bad outcome, RANTES was the best marker.

Key words: NAFLD, NASH, liver fibrosis, chemokine, FIB-4

Non-alcoholic fatty liver disease (NAFLD) is a chronic progressive liver disease that may lead to liver cirrhosis and hepatocellular carcinoma (HCC), and its incidence is increasing worldwide [1]. The advanced stage of NAFLD is known as non-alcoholic steatohepatitis (NASH), while simple fatty liver is known as non-alcoholic fatty liver (NAFL). In recent years, studies on the pathogenesis and progression of NAFLD have revealed that it is associated with multiple parallel hits from factors such as lipids, inflammatory cytokines, oxidative stress, ER stress, and insulin resistance [2]. However, the heterogeneity of the NAFLD patient population often makes it difficult to define the main disease-progression-related pathway in individual patients. In addition, risk stratification is necessary because NAFLD is a progressive disease. The disease condition of NAFLD patients is defined by two approaches: the diagnosis of NAFL or NASH, and the diagnosis of the activity grade and fibrosis stage. Diagnosis of NASH and diagnosis of the activity grade and fibrosis stage require pathological examination of a liver biopsy specimen. However, liver biopsy is time-consuming, is associated with a risk of mortality and has the potential for sampling errors [3]. Advanced liver fibrosis has been shown to predict worse survival,

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*Corresponding author. Phone: +81-86-235-7219; Fax: +81-86-225-5991
E-mail: akitaka@md.okayama-u.ac.jp (A. Takaki)

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and thus should be precisely and quickly evaluated [4]. Recently, non-invasive clinical biological markers or radiological examinations have been employed to predict advanced stages of NAFLD. As clinical biological markers, formulas with standard liver fibrosis-related factors are widely used. The FIB-4 index ((AST × age) / (platelet count × √ALT)), NAFLD Fibrosis Score (NFS) ($-1.675 + 0.037 \times$ age $+ 0.094 \times$ BMI $+ 1.13 \times$ impaired fasting glycemia / diabetes [yes = 1, non = 0]), and APRI (AST / upper limit of normal range of AST / platelet $\times 100$) are markers that can be simply calculated from standard laboratory data and which show acceptable correlations with histological liver fibrosis and the prognosis [5]. The EASL-EASD-EASO Clinical Practice Guidelines recommend use of the FIB-4 index and NFS for ruling out advanced fibrosis [6]. However, because both of these scores include age in their formulas, care is required in the evaluation. In addition, BMI is included in the NFS, but the distribution of BMI differs substantially in different geographic areas: obesity (BMI > 30) is relatively prevalent in Western countries, while it is relatively rare in East Asian countries such as Japan. To overcome these disadvantages, other markers that are independent of factors that reflect the clinical status (*e.g.*, age or BMI) are desired.

Immune responses and inflammation are known to be involved in metabolic diseases, including NASH [7]. Liver and adipose tissue-derived cytokines are known to promote the progression of metabolic disease. Even in simple fatty liver, macrophage infiltration and macrophage attractant chemokine CCL2 expression are significantly increased [8]. Neutrophils are also accepted to be involved in the progression of NAFLD. The neutrophil-to-lymphocyte ratio (NLR) is a significant marker for the diagnosis of advanced NAFLD [9]. Dendritic cells have also been shown to be involved in NAFLD progression, although their effect is complex, as pro-inflammatory data and anti-inflammatory data have both demonstrated [7]. T cells are also involved in the progression of NAFLD. In advanced NAFLD, CD4 (+) and CD8 (+) T cell infiltration increases, and inflammatory cytokines, such as IL-6 or IL-8, are also increased [8]. Serum cytokine levels do not directly reflect the liver inflammatory status, but can indicate the final balance of these immune responses. Moreover, several serum cytokines are recognized as important markers for the differentiation of NAFLD stages [10].

The objective of the present study was to investigate the effectiveness of standard predictive markers of the NAFLD stage and the profiles of multiple cytokines for differentiating between progressive and non-progressive NAFLD patients, including aged patients.

Materials and Methods

Patients. Ninety-eight patients with NAFLD (NAFL, n = 25; NASH, n = 73) who were diagnosed by liver biopsy at Okayama University Hospital from 2005 to 2016 were enrolled. The last observation period was December 2022. The diagnostic system reported by Matteoni *et al.* was adopted to diagnose NASH [11]. The METAVIR scoring system was used to analyze the activity grade and stage of liver fibrosis in patients. Their fibrosis stages were as follows: stage 1-2 (n = 59), stage 3-4 (n = 39). Serum assays were also conducted for 20 healthy donors. The patients were diagnosed as not having cancer and were negative for hepatitis B and hepatitis C viral markers and autoantibodies. The baseline characteristics of the patients are summarized in Table 1A. Aged patients were defined as ≥ 65 years old according to the WHO definition. The prognosis of the patients was defined based on additional events after a liver biopsy (*e.g.*, cirrhosis to death [n = 2], cirrhosis to hepatocarcinogenesis [n = 4], non-cirrhotic condition to symptomatic cirrhosis diagnosed by emergence of varix or ascites [n = 4]). Of the 4 hepatocarcinogenesis patients, 3 were in the advanced fibrosis stage, and 1 was in the mild fibrosis stage.

Informed consent was obtained from each patient included in the study, and the study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki, and was approved by the Ethics Committee of Okayama University Hospital.

Blood sample collection and preparation. Fasting blood samples were collected from the patients on the day after admission or at the outpatient clinic, and the serum was collected within 48 h, meaning that no intervention was performed before sample collection. The serum aliquots were stored at −30°C until analysis.

Evaluation of standard noninvasive NASH diagnostic formulas. FIB-4 and APRI were evaluated for their effectiveness in the differential diagnosis of NAFL and NASH, and in the differential diagnosis of fibrosis stages 1-2 and 3-4. The FIB-4 index and APRI score were calculated using the original formulas [12, 13].

Table 1A Baseline clinical characteristics

	Control	All NAFLD	NAFL	NASH	*P*-value
Age	46.5 (40.3–49.0)	54.5 (17–78)	42 (19–66)	56 (17–78)	0.018*
Male sex (%)	55	53	56	44	0.293
BMI	n.t.	27 (14.3–40.7)	26.1 (14.3–36.1)	27.6 (16.7–40.7)	0.347
Albumin (g/dL)	n.t.	4.4 (3.1–5.1)	4.6 (3.2–5.0)	4.3 (3.1–5.1)	0.021*
Platelet ($\times 10^4/\mu L$)	n.t.	22.6 (6.7–284)	25.6 (10.1–284)	21.8 (6.7–79.1)	0.004*
T. bil (mg/dL)	n.t.	0.65 (0.2–6.04)	0.67 (0.31–1.23)	0.65 (0.2–6.04)	0.383
PT-INR	n.t.	0.97 (0.83–1.47)	0.92 (0.85–1.15)	0.98 (0.83–1.47)	0.002*
AST (U/L)	n.t.	51.5 (0.14–201)	33 (0.14–81)	61 (21–201)	<0.001*
ALT (U/L)	n.t.	66.5 (14–452)	50 (16–128)	75 (14–452)	0.024*
Triglyceride (mg/dL)	n.t.	159.5 (32–687)	169 (70–687)	154 (32–392)	0.310
HOMA-IR	n.t.	3.44 (0.55–47.56)	1.80 (0.55–47.56)	3.95 (1.32–30.88)	<0.001*
FIB-4 index	n.t.	1.69 (0.007–13.4)	0.7 (0.007–5.2)	2.1 (0.31–13.4)	<0.001*
liver biopsy finding					
activity grade (1 ; 2–3)	n.t.	70 ; 28	25 ; 0	45 ; 28	<0.001*
fibrosis stage (1–2 ; 3–4)	n.t.	59 ; 39	25 ; 0	34 ; 39	<0.001*
Matteoni (1–2 ; 3–4)	n.t.	25 ; 73	25 ; 0	0 ; 73	<0.001*

NAFLD, non-alcoholic fatty liver disease; NAFL, non-alcoholic fatty liver; NASH, non-alcoholic steatohepatitis.

Multiple cytokine assays. Measurement of multiple cytokines was performed using a BioPlex 200 System (Bio-Rad Laboratories, Hercules, CA, USA) in accordance with the manufacturer's protocols. The assay was a Bio-Plex Pro Human Cytokine Grp 1 Panel 27-Plex, which targets IL-1β, IL-1ra, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12 (p70), IL-13, IL-15, IL-17, basic fibroblast growth factor (FGF), eotaxin, granulocyte colony stimulating factor (G-CSF), granulocyte-macrophage CSF (GM-CSF), interferon (IFN)-γ, IP-10, monocyte chemoattractant protein (MCP)-1, macrophage inflammatory protein (MIP)-1α, MIP-1β, platelet-derived growth factor subunit B (PDGF-BB), regulated upon activation normal T-cell expressed and secreted (RANTES), tumor necrosis factor (TNF)-α, and vascular endothelial growth factor (VEGF). Samples were tested in duplicate, and the median values were used for further analyses.

Statistical analyses. Statistical comparisons were performed using JMP version 14.0.0 (SAS Institute, Cary, NC, USA). The Wilcoxon rank-sum test was used to compare continuous data, and the chi-squared test was used to compare categorical data. The log-rank test was used for the additional event analysis. *P* values of < 0.05 were considered statistically significant.

Results

Clinical characteristics of the NALF vs. NASH patients. NASH patients were older and had a higher prevalence of diabetes mellitus, lower serum albumin, lower hemoglobin and platelet counts, higher prothrombin time international ratio (PT-INR), higher AST/ALT, and higher homeostasis model assessment as an index of insulin resistance (HOMA-IR) values than NAFL patients. Known NAFLD improvement effect-related agents, such as vitamin E, angiotensin II receptor blocker (ARB), peroxisome proliferator-activated receptor (PPAR)-activator bezafibrate, dipeptidyl-peptidase 4 (DPP-IV) inhibitors, and insulin, were administered in 31% of NAFLD patients, and this percentage was not markedly different between the NAFL and NASH patients. All NAFL patients showed lower histological activity and fibrosis scores. NASH patients were grouped according to their histological activity (Table 1A).

Clinical characteristics of the patients according to fibrosis stages. Patients with advanced fibrosis were older and had a higher prevalence of diabetes mellitus, with lower hemoglobin and platelet counts, lower serum albumin, higher PT-INR, higher AST, and lower triglyceride levels than patients with mild fibrosis (Table 1B). In addition, advanced fibrosis patients showed higher histological activity in comparison to

Table 1B Clinical characteristics according to fibrosis stages

	Stage 1–2	Stage 3–4	*P*-value
Age	47 (17–78)	60 (30–75)	0.002
Male sex (%)	54.2	35.9	0.073
BMI	27.5 (14.3–40.7)	26.9 (19.3–38.4)	0.360
Albumin (g/dL)	4.5 (3.2–5.1)	4.2 (3.1–4.8)	<0.001
Platelet ($\times 10^4/\mu L$)	25.2 (10.1–284)	15.7 (6.7–30.5)	<0.001
T. bil (mg/dL)	0.65 (0.2–1.58)	0.71 (0.34–6.04)	0.083
PT-INR	0.95 (0.83–1.15)	1.01 (0.9–1.47)	<0.001
AST (U/L)	47 (0.14–201)	61 (21–194)	0.034
ALT (U/L)	69 (16–452)	58 (14–159)	0.250
Triglyceride (mg/dL)	174 (32–687)	126 (43–392)	0.011
HOMA-IR	3.09 (0.55–47.6)	3.58 (1.32–22.0)	0.052
FIB-4 index	0.89 (0.0072–5.2)	2.57 (0.94–13.4)	<0.001
liver biopsy finding			
activity grade (1 ; 2–3)	48 ; 11	22 ; 17	0.008
fibrosis stage (1–2 ; 3–4)	59 ; 0	0 ; 39	<0.001
Matteoni (1–2 ; 3–4)	25 ; 34	0 ; 39	<0.001

patients with less advanced fibrosis.

Because several markers were shown to be significantly different, a multivariate logistic regression analysis was carried out to clarify the most important factors. Of the factors shown to be significant on a univariate analysis, diabetes mellitus, low platelet counts, low albumin, high AST, and high TG were shown to be significant on a multivariate analysis.

Different patterns of serum cytokine concentrations in NAFL and NASH. As shown in Figure 1A, FIB-4 and APRI showed strong power to discriminate NAFL and NASH. Of the 27 cytokines that were measured, the levels of two cytokines, *i.e.*, IP-10 and IL-15, were significantly higher in the samples from NASH than those from NAFL patients ($p < 0.05$). In contrast, VEGF was significantly lower in the NASH serum samples ($p < 0.05$). The levels of other cytokines, such as the representative cytokines shown in the figure (IL-17, PDGF-BB, and RANTES), were roughly the same between NAFL and NASH samples.

Different patterns of serum cytokine concentrations in mild and advanced fibrosis. FIB-4 and APRI showed good correlation with the progression of the fibrosis stage (Fig. 1B). Of the measured cytokines, the level of IP-10 in patients with advanced stage fibrosis was higher than that in patients with mild fibrosis, while the levels of VEGF, IL-17, PDGF-BB, and RANTES were lower in advanced stage patients. IL-15, which differed between NAFL and NASH, did not differ to a statistically significant extent between stages 1-2 and stages 3-4. We next conducted a multivariate analysis using the cytokines and clinical factors shown to be significant in Table 1B. Low platelet count, high IP-10, and low RANTES were found to be significant in the multivariate analysis (Table 2).

Because aging is correlated with fibrosis, we next conducted a Spearman's correlation test to investigate whether or not the cytokine levels were correlated with age. The results showed that two of the five significant markers, PDGF-BB and RANTES, were significantly negatively correlated with age (data not shown).

Clinical course of patients after liver biopsy. Patients with NASH or advanced fibrosis showed no worsening of mortality. Patients with NASH showed no increase of additional events; however, patients with advanced fibrosis showed more frequent additional events than the others (Fig. 2A). These data suggested that advanced fibrosis was more important for prognosis than a diagnosis of NASH in our cohort.

Cytokine patterns and the clinical course of patients after liver biopsy. The clinical course of patients was compared according to the titers of FIB-4, APRI, and selected cytokines (Fig. 2B). Patients with high FIB-4 and APRI showed a worse clinical course. Patients with low PDGF-BB and RANTES showed a worse clinical course, while other cytokines (*e.g.*, IP-10, VEGF, IL-15, and IL-17) showed no such correlation with the clinical course. The cytokines useful for dividing NASH and

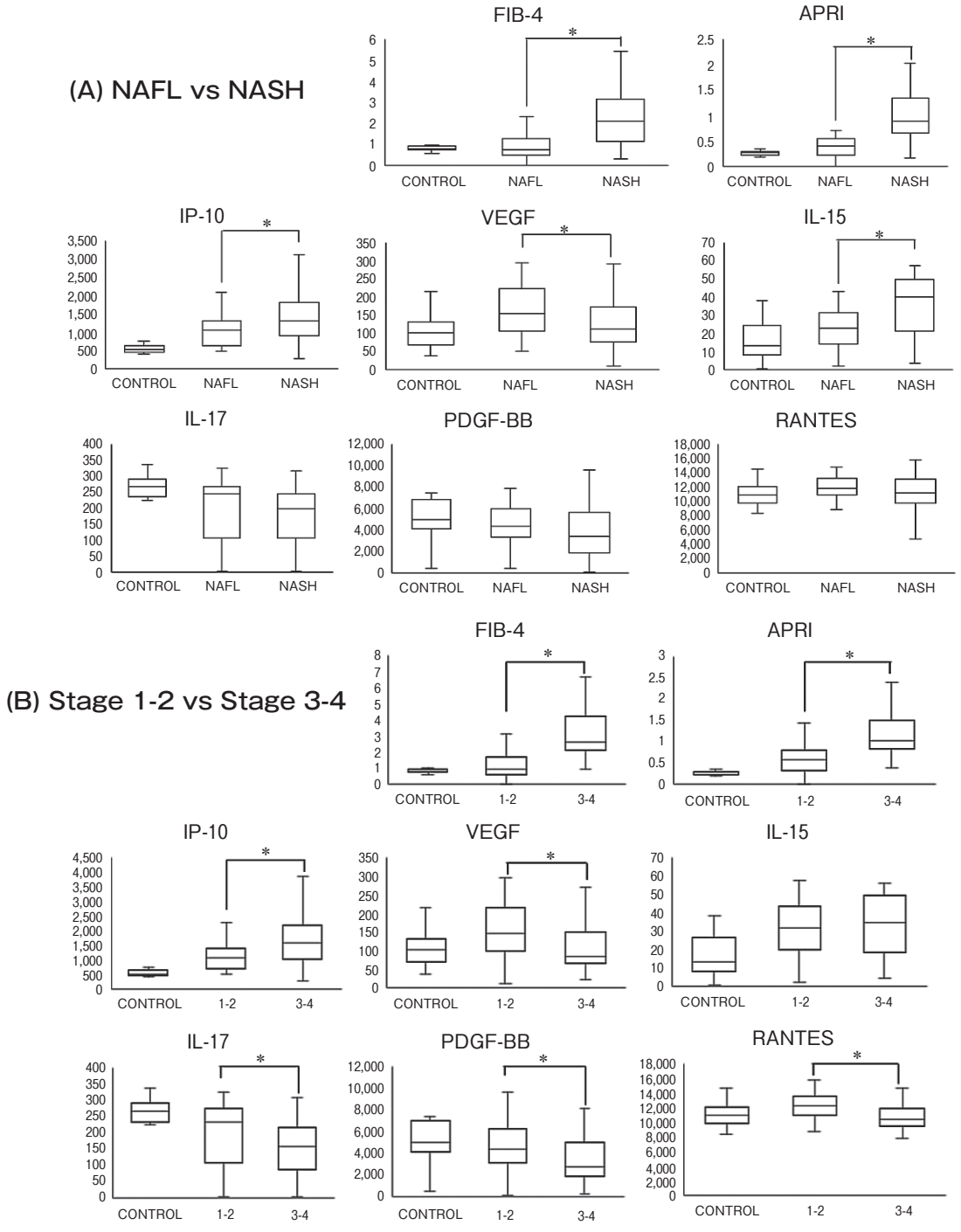

Fig. 1 Candidate markers for discrimination between NAFL and NASH or fibrosis stages 1-2 and 3-4. Titers of two simple clinical markers of the progression of liver fibrosis progression (FIB-4 and APRI) and concentrations of cytokines were investigated to discriminate between (**A**) NAFL and NASH or (**B**) fibrosis stages 1-2 and 3-4. (**A**) Both FIB-4 and APRI were effective for discriminating NAFL and NASH. Of the measured cytokines, IP-10 and IL-15 were higher in NASH than in NAFL patients. In contrast, VEGF was lower in NASH than in NAFL patients. (**B**) FIB-4 and APRI were also effective for discriminating stages 1-2 and stages 3-4. IP-10 was higher in stages 3-4 than in stages 1-2, while VEGF, IL-17, PDGF-BB, and RANTES were lower in stages 3-4 than in stages 1-2. Other cytokines showed no significant difference between these clinical stages. $^{*}p<0.05$.

Table 2A Standard laboratory data and cytokines in NAFL vs NASH and Stage 1-2 vs Stage 3-4 in <65 years old patients

<65	NAFL	NASH	*P*-value	Stage 1-2	Stage 3-4	*P*-value
FIB-4 index	0.70 (0.42–1.16)	1.70 (0.94–3.08)	<0.001*	0.77 (0.45–1.18)	2.55 (1.98–3.84)	<0.001*
APRI	0.36 (0.22–0.55)	0.88 (0.66–1.36)	<0.001*	0.54 (0.29–0.75)	1.14 (0.81–1.61)	<0.001*
IP-10	1,013 (617–1,222)	1,330 (880–1,712)	0.015*	1,055 (684–1,420)	1,536 (970–1,930)	0.006*
GM-CSF	26.3 (16.3–55.9)	33.4 (16.9–57.9)	0.727	30.8 (16.4–53.7)	36.3 (16.0–59.0)	0.763
PDGF-bb	4,367 (3,344–6,277)	4,045 (2,119–7,489)	0.536	4,906 (3,376–6,347)	2,796 (1,517–5,151)	0.012*
RANTES	12,068 (10,793–13,447)	11,837 (9,689–14,578)	0.440	12,266 (11,000–13,663)	10,425 (9,271–12,303)	0.005*
VEGF	153 (104–218)	120 (80–281)	0.212	147 (116–218)	89 (68–155)	<0.001*

Table 2B Standard laboratory data and cytokines in NAFL vs NASH and Stage 1-2 vs Stage 3-4 in ≥65 years old patients

≥65	NAFL	NASH	*P*-value	Stage 1-2	Stage 3-4	*P*-value
FIB-4 index	2.13 (1.94–2.31)	2.61 (2.11–4.20)	0.256	2.28 (1.91–2.96)	2.90 (2.30–5.18)	0.069
APRI	0.81 (0.49–1.13)	1.00 (0.60–1.34)	0.614	0.74 (0.50–1.18)	1.01 (0.67–1.39)	0.226
IP-10	1,978 (1,957–1,999)	1,196 (943–2,134)	0.614	1,050 (901–1,967)	2,047 (1,146–3,212)	0.041*
GM-CSF	45.2 (29.4–61.0)	40.9 (31.0–102.7)	0.750	78.7 (42.2–126.2)	32.5 (27.2–41.2)	0.020*
PDGF-bb	3,841 (3,065–4,616)	2,750 (466–4,757)	0.449	3,065 (449–4,697)	2,750 (1,924–5,093)	0.705
RANTES	11,549 (11,174–11,924)	10,589 (10,086–12,650)	0.308	11,924 (10,616–14,606)	10,431 (9,870–10,940)	0.027*
VEGF	431 (232–629)	79 (54–112)	0.043*	84 (60–204)	78 (46–223)	0.762

NAFL showed no impact on the clinical course after liver biopsy.

Standard laboratory data and cytokines in NAFL vs. NASH and stages 1-2 vs. 3-4 in young patients. In young patients (<65 years), the FIB-4 index and APRI score in the NASH group were higher than those in the NAFL group, and they were higher in stages 3-4 than in stages 1-2 (Table 2A). IP-10 was increased in NASH and in stages 3-4, while PDGF-BB, RANTES, and VEGF were decreased in stages 3-4.

Standard laboratory data and cytokines in NAFL vs. NASH and stages 1-2 vs. 3-4 in aged patients. In aged patients (≥65 years), the FIB-4 index and APRI values did not differ to a statistically significant extent between NASH and NAFL, or between stages 1-2 and stages 3-4 (Table 2B). On the other hand, in stages 3-4, IP-10 was significantly greater than in stages 1-2, while GM-CSF and RANTES were significantly lower in stages 3-4.

Discussion

In this study, FIB-4 and APRI, standard indexes related to the progression of fibrosis, showed good correlation with NASH, advanced fibrosis, and disease progression. Although several cytokines showed a similar trend, several others showed the opposite pattern, including lower levels in patients with advanced fibrosis. Advanced fibrosis was also correlated with low platelet counts, high IP-10, and low RANTES. The prognosis was correlated with advanced fibrosis but not with NASH. The fibrosis-related cytokines PDGF-BB and RANTES showed good correlation with the prognosis. In aged patients, high IP-10, low GM-CSF and low RANTES predicted the progression of fibrosis. RANTES was the only marker that showed good correlation with the progression of fibrosis in the overall study population and in aged patients.

Recently, advanced fibrosis has been shown to be a strong prognostic factor, even stronger than a diagnosis of NASH [14]. A meta-analysis revealed that NAFLD patients with fibrosis had increased all-cause mortality as the stage progressed, with the mortality rate ratio (MRR) to stage 0 increasing from 1.58 (stage 1) to 2.52 (stage 2), 3.48 (stage 3) and 6.40 (stage 4) [4]. Of course, the same study showed a stronger effect of fibrosis on the risk of liver-related mortality, with MRRs of 1.41 (stage 1) 9.57 (stage 2), 16.69 (stage 3), and 42.30 (stage 4). Our present study also showed that advanced fibrosis is a risk factor for additional events after liver biopsy, while the diagnosis of NASH had no effect. This is consistent with previous reports [4, 14].

Prediction of the fibrosis stage via serological or other non-invasive markers, either alone or in combi-

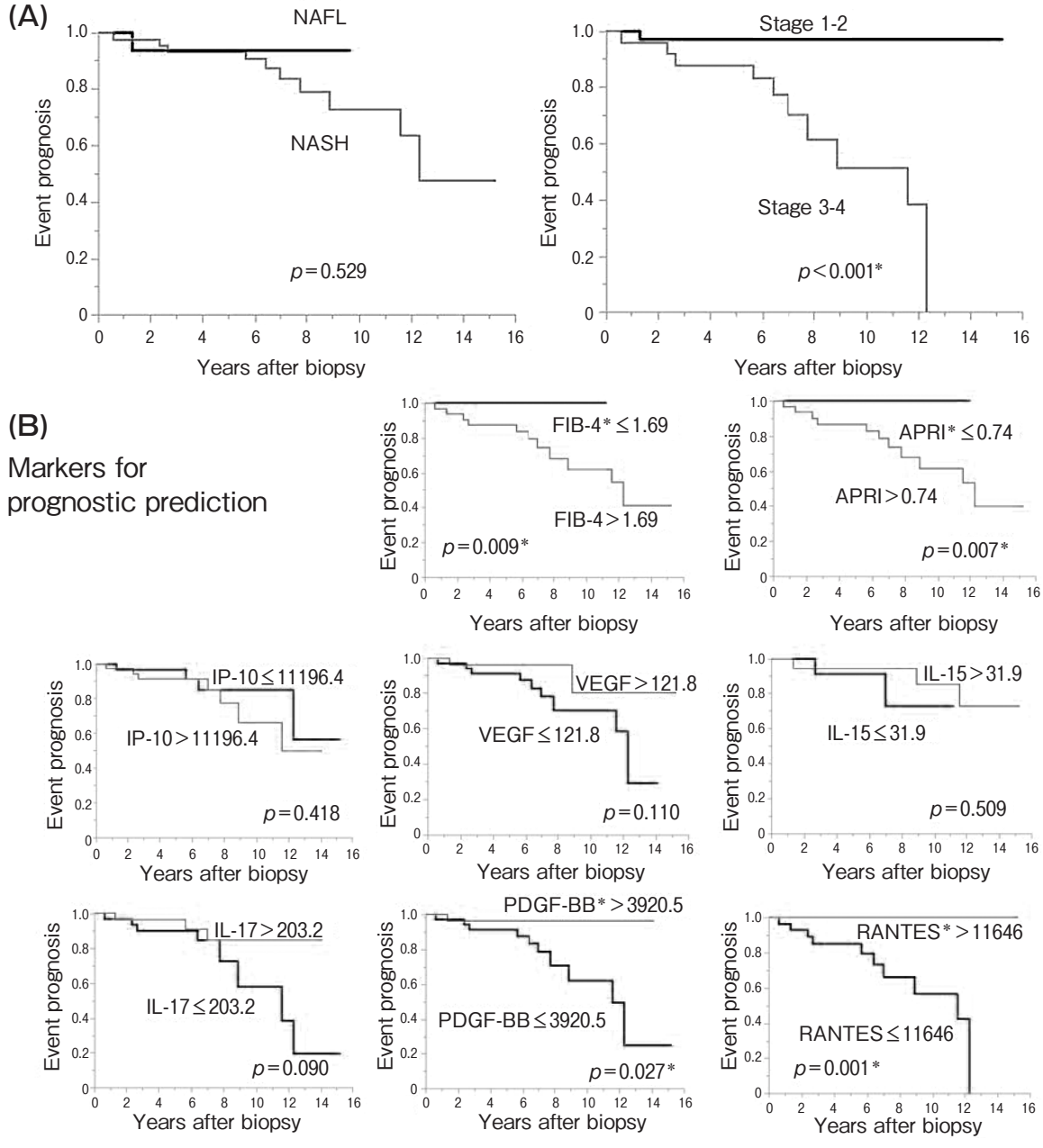

Fig. 2 Candidate markers for the prediction of clinical outcome. Two simple clinical markers of the progression of liver fibrosis (FIB-4 and APRI) and cytokine concentrations were investigated to predict clinical outcomes. The prognosis of the patients was defined as an additional event after liver biopsy (cirrhosis to death, cirrhosis to hepatocarcinogenesis, non-cirrhotic chronic liver disease to emergence of varix or ascites). (**A**) Prognosis of the patients with NAFL vs. NASH and fibrosis stages 1-2 vs. 3-4. (**B**) FIB-4, APRI and cytokines were investigated to determine their prognostic ability. FIB-4, APRI and cytokines PDGF-BB and RANTES were considered useful for predicting the prognosis.

nation with the clinical status, is important, and several markers have been shown to be effective. FIB-4 and NFS are easy to calculate and are widely used in screening for liver fibrosis; however, as age is involved in their calculation, the specificity declines from FIB-4 (35% specificity) and NFS (20%) [15]. Conversely,

Younes *et al*. showed that the inclusion of age in their calculation makes these markers valuable for predicting the prognosis in terms of liver-related mortality or overall mortality [16]. In our present analysis, high FIB-4 and APRI scores were correlated with a higher frequency of additional events. Of the measured cytokines, those related to inflammatory response, such as IP-10, showed no correlation with the prognosis, while those related to fibrosis, *i.e.*, PDGF-BB and RANTES, were predictive of the prognosis.

PDGF plays a major role in the activation of hepatic stellate cells (HSCs). The PDGF-B mRNA expression has been shown to increase in the early stage of HSC activation; this increase is quickly followed by a marked decrease [17]. Immunohistochemical staining of PDGF-BB protein and analysis of PDGF-BB mRNA expression revealed that both the protein and mRNA were expressed in portal areas and perisinusoidal cells, where myofibroblast-like cells were seen in liver specimens of chronic hepatitis patients [18]. Based on the hepatic expression of PDGF in chronic hepatitis with fibrous expansion, serum PDGF-BB should be correlated with the progression of liver fibrosis; however, the results are confusing. One report showed that serum PDGF-BB increased as alcoholic liver disease progressed [19], while in chronic hepatitis B patients, the serum PDGF-BB level was negatively correlated with the fibrosis stage [20] and the level in chronic hepatitis C patients was lower than that in healthy subjects [21]. Multiple explanations have been put forward to account for the decrease in serum PDGF-BB levels with the progression of chronic hepatitis. One explanation is that the activation of the PDGF system is strongest in an early stage of fibrosis and declines afterwards, leading to lower serum levels in advanced-stage liver fibrosis [17]. Another explanation is that the change is due to a decrease of platelets in advanced liver fibrosis, because the platelets produce PDGF-BB [22].

Another fibrosis-related marker, RANTES, also called CC chemokine ligand 5 (CCL5), directly activates proinflammatory M1 macrophage polarization and impedes M2 polarization [23]. In patients with HBV-related chronic hepatitis and cirrhosis, the serum RANTES levels of patients with moderate-to-severe hepatitis were higher than those in patients with mild hepatitis, while the levels in patients with cirrhosis were lower than those in patients with chronic hepatitis [24]. Immunohistological staining of RANTES revealed positivity in the cytoplasm of hepatocytes, while this was diminished in damaged hepatocytes and fibrous bands of cirrhosis tissue, likely in line with the serum quantity data. The results of our present analysis with NAFLD seem to agree with the previous results from patients with chronic hepatitis and cirrhosis type B. RANTES showed a good correlation with the progression of fibrosis, even in aged patients, suggesting the superiority of this marker for defining advanced NAFLD.

Although the above-mentioned fibrosis-related markers showed good correlation with the prognosis and stages of fibrosis in aged patients, markers used to discriminate between NAFL and NASH, such as IP-10, VEGF and IL-15, showed no such correlations. IP-10 is a proinflammatory type 1 helper T (Th1) cell-related marker, which is also referred to as interferon-gamma induced protein of 10 kD or C-X-C motif chemokine ligand 10 (CXCL10), and which has already been shown to be an effective marker for discriminating NASH from NAFL and advanced chronic hepatitis C [10]. However, IP-10 did not show a good correlation with the prognosis in our cohort, although it showed a good correlation in patients with advanced fibrosis, even in aged patients.

Our present results highlight the important finding that RANTES is predictive of advanced fibrosis in young as well as aged NAFLD patients and also predictive for their prognosis. However, there are several limitations associated with this study. The number of patients included was small, and the background characteristics, such as the age-related prevalence of diabetes mellitus, differed between the mild and advanced fibrosis groups. In addition, we investigated only serum cytokine concentrations, which of course reflect only conditions in the sera, and we were unable to detect increases in the status of immune-related markers reflecting pathology in the liver. As a next step, we must investigate the cytokine expression pattern in the liver in many more patients.

In conclusion, RANTES is our most highly recommended non-invasive marker, as it was correlated with advanced fibrosis stages in both young and aged patients and could predict their prognosis. It should be emphasized that FIB-4 and APRI, two easily calculated fibrosis-related markers, were also prognostic factors. However, their power was diminished in the aged patients in our cohort. RANTES showed a good correlation with all of our present results. Further studies

in larger study populations should be performed to confirm the results.

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