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New Discriminant Method for Identifying the Aggressive Disease Phenotype of Non-alcoholic Fatty Liver Disease

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Abstract

Objective To detect the aggressive phenotype (AP) of non-alcoholic fatty liver disease (NAFLD) based on the initial laboratory data and clinical characteristics.

Methods We enrolled 144 patients with histologically proven NAFLD. For the first analysis, 24 NAFLD patients underwent repeat biopsy to establish a discriminant formula for predicting the AP of NAFLD (D-APN). The AP was defined by NAFLD that had been maintained or progressed to a fibrotic stage beyond stage 2. In the second analysis, we analyzed the distribution of the AP in each stage of disease and the incidence of the PNPLA3 rs738409 GG genotype in AP in 120 other patients.

Results After the analysis, the following function was found to discriminate the disease phenotype: z=0.150 ×body mass index (kg/m^2)+0.085×age (years)+1.112×ln (AST) (IU/L)+0.127×ln (m-AST)-12.96. A positive result indicates the AP of NAFLD. The discriminant functions had a positive predictive value of 94% and a negative predictive value of 71%.

The distribution of the AP and the incidence of the PNPLA3 GG genotype in the AP in each stage of the disease among the 120 patients were as follows: non-alcoholic fatty liver, 30%/33%; non-alcoholic steatohepatitis (NASH) stage 1, 53%/26%; stage 2, 71%/70%; stage 3, 92%/57%; and stage 4, 93%/64%; there was a significant increase in the incidence of the AP as the disease progressed ($p<0.001$).

Conclusion The new discriminant formula was useful for predicting disease progression potential in NAFLD patients and the incidence of the PNPLA3 GG genotype was elevated according to the distribution of AP.

Key words: non-alcoholic fatty liver disease, aggressive, phenotype, fibrosis, hepatocellular carcinoma

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Introduction

Non-alcoholic fatty liver disease (NAFLD) is a common cause of chronic liver disease in Western countries (1-4) and - more recently - in many Asian nations (5, 6). In particular, patients with non-alcoholic steatohepatitis (NASH), a subcategory of NAFLD, are at increased risk of developing hepatocellular carcinoma (7). Similar to individuals with viral hepatitis, patients with NAFLD and advanced fibrosis have a higher risk of hepatocarcinogenesis (8-10).

In addition, we often encounter patients who have the same fibrotic stage at diagnosis of NAFLD; however, during follow up, they present with different progression patterns (i.e., the progression of fibrosis will be extremely rapid in one patient and very slow in another - during the same follow-up period).

Thus, there is a need to be able to identify the rapid progressive type, or the "*aggressive phenotype*" and the slow progressive type, or the "*indolent phenotype*" at an earlier fibrotic stage; this would enable the more intensive follow-up of patients with the aggressive phenotype.

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To date, several non-invasive scoring systems (NAFLD fibrosis score, BARD score, APRI and FIB-4 index) (11-14) have been constructed for the purpose of discriminating severe hepatic fibrosis from mild fibrosis; however, these scoring systems only estimate the current fibrotic stage, and cannot predict the speed of disease progression in the early stage of the disease.

Thus, the first aim of the present retrospective study was to define the rapid progressive type, or "*aggressive phenotype*" of NAFLD using a non-invasive discriminant formula that does not depend on the various markers of fibrosis (i.e., hyaluronic acid, type-IV collagen 7S).

Recently the impact of the PNPLA3 genotype on the pathogenesis and genetic risk of NAFLD appears to be straightforward (15-19), and Hotta et al. reported that the G-allele of rs738409 was significantly associated with increases in the fibrotic stage in NAFLD patients, even after adjustment for age, gender, and BMI (17). Thus, another aim of the present study was to elucidate the relationship between the aggressive phenotype and the rs738409 GG genotype.

Materials and Methods

Study population

From January 1980 to July 2015, 220 patients were histopathologically diagnosed with NAFLD at Toranomon Hospital, Tokyo, Japan; 144 of these patients were enrolled in this retrospective study. The criteria for inclusion were as follows: (1) past daily alcohol intake of <20 g; (2) no underlying viral hepatitis, autoimmune hepatitis, drug-induced liver disease, or primary biliary cirrhosis; (3) no underlying systemic autoimmune diseases, such as systemic lupus erythematosus or rheumatoid arthritis; (4) no underlying metabolic diseases, such as hemochromatosis, alpha-1-antitrypsin deficiency, or Wilson disease; and (5) an analysis of the PNPLA3 rs738409 genotype and m-AST (a marker of apoptosis) at the time of the initial biopsy.

We divided these 144 patients into two groups. One group was the construct group, which included 24 patients who received repeat biopsy. In addition to the above-mentioned inclusion criteria, these patients also satisfied the following criteria: (1) all patients underwent examination for mitochondrial-AST (m-AST) (a marker of necroapoptosis) in several biopsies, (2) the fibrotic stage was maintained or progressed in comparison to the initial biopsy, and (3) the biopsies were performed over a period of more than one year.

The other group was the validation group, which included 120 patients with histologically proven NAFLD. The study was approved by the Institutional Review Board of Toranomon Hospital.

The definition of diabetes mellitus

Diabetes mellitus was diagnosed based on the 2010 criteria of the American Diabetes Association (20). These criteria include: (1) casual plasma glucose ≥200 mg/dL; (2) fasting plasma glucose ≥126 mg/dL; and (3) 2-hour post-glucose (oral glucose tolerance test) ≥200 mg/dL.

The determination of the PNPLA3 genotype

PNPLA3 rs738409 was genotyped with the TaqMan SNP genotyping assay (Applied Biosystems, Foster City, CA, USA).

The histopathological examination of the liver

Liver biopsy specimens were obtained using a 14-gauge modified Vim Silverman needle (Tohoku University style, Kakinuma Factory, Tokyo, Japan), a 16-gauge core tissue biopsy needle (Bard Peripheral Vascular, Tempe, AZ, USA) or surgical resection. Tissue was fixed in 10% formalin, and sections were stained with Hematoxylin and Eosin, Masson trichrome, silver impregnation, and periodic acid-Schiff after diastase digestion. Fibrosis was scored using the five-grade scale proposed by Brunt et al. (21): stage 0, normal connective tissue; stage 1, pericellular or perivenular fibrosis in zone 3 (pericentral vein area); stage 2, zone 3 perisinusoidal/pericellular fibrosis with focal or extensive periportal fibrosis; stage 3, bridging or septal fibrosis; and stage 4, cirrhosis.

NAFLD activity was scored using "The NAFLD Activity Score (NAS)," an eight-grade scale proposed by Kleiner et al. (22), which is determined based on the unweighted sum of the scores for steatosis (0-3), lobular inflammation (0-3), and ballooning degeneration (0-2).

The definition of the disease phenotype of NAFLD (aggressive or indolent)

In this study, we defined the aggressive phenotype as one that was maintained or progressed to a fibrotic stage of ≥2; in contrast, in patients with the indolent phenotype, the fibrotic stage was maintained at <2 after several biopsies (Fig. 1a).

For example, a patient with stage 2 fibrosis at the first biopsy (Fig. 1b) and stage 3 fibrosis at the second biopsy (Fig. 1c), would be classified as having the aggressive phenotype.

Statistical analysis

Non-parametric procedures, including the chi-squared test and the Mann-Whitney *U* test, were employed for the analysis of background characteristics and laboratory data among the patients with each phenotype at the time of the first biopsy. The Cochran-Armitage trend test was used to investigate the significance of trends in the incidence of the aggressive phenotype and the rs738409 GG genotype in patients with the aggressive phenotype based on pathological fibrotic stage. The Kolmogorov-Smirnov one-sample test was used to evaluate the normality of the distribution of the data.

Because certain variables did not completely conform to a

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Disease phenotype	Histological staging: First biopsy	Histological staging: Repeated biopsy	Number of patient
Indolent phenotype of NAFLD Maintained or progressed fibrotic stage under - 2	NAFL	Stage 1	1
	Stage 1	Stage 1	5
Aggressive phenotype of NAFLD Maintained or progressed fibrotic stage beyond -2	Stage 1	Stage 2	3
	Stage 1	Stage 3	2
	Stage 2	Stage 2	1
	Stage 2	Stage 3	2
	Stage 3	Stage 3	8
	Stage 3	Stage 4	2

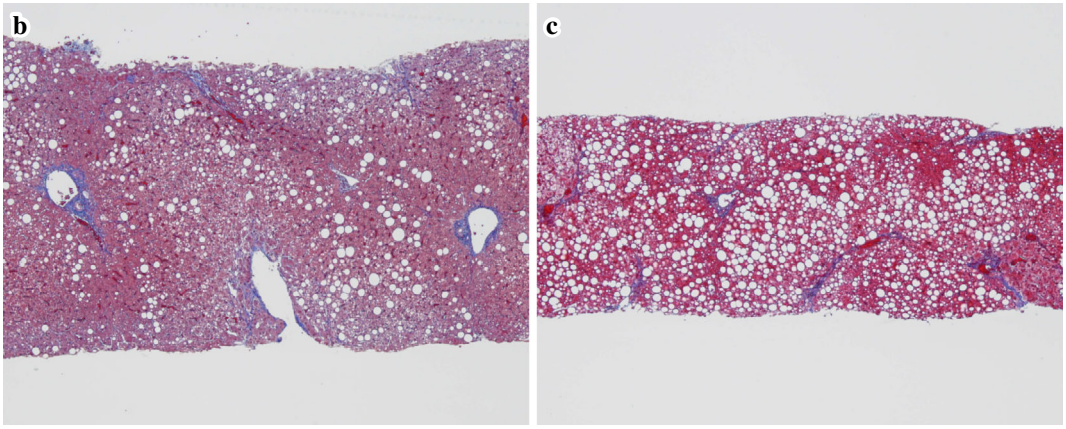

Figure 1. **(a) The histological background and distribution of the aggressive and indolent phenotypes of NAFLD. (b) A 36-year-old man with stage 2 fibrosis at the time of the first biopsy (c); four years later he received a second biopsy that revealed stage 3 fibrosis, which is consistent with the aggressive phenotype. (Masson trichrome staining of liver tissue; Original magnification, 1×40)**

normal distribution, the bilirubin, AST, m-AST, ALT, GGT, triglyceride, and ferritin were subjected to natural logarithmic transformation. Following natural logarithmic transformation, each of the factors was normally or symmetrically distributed. After these procedures, the following discriminant analysis became rationally robust against deviations from normal distribution.

All of the factors that were found to be at least marginally associated with the aggressive phenotype of NAFLD ($p < 0.05$) in a univariate analysis were simultaneously entered into a multivariate discriminant analysis to predict the aggressive phenotype of NAFLD.

The sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) for identifying the aggressive phenotype and the indolent phenotype were determined for this discriminant formula. Next, we validated the obtained predictive function using the data from the remaining 120 patients in the validation dataset.

The data were analyzed using the SPSS software program (version 16.0 for Windows; SPSS, Chicago, IL, USA).

Results

The laboratory data for each phenotype (aggressive or indolent) in the construct group

Table 1 summarizes the profiles and data of 24 NAFLD patients who received repeat biopsy, according to the disease phenotype (aggressive or indolent). Patients with the aggressive phenotype were significantly older and had significantly higher BMI, serum AST and m-AST values than those with the indolent phenotype. In addition, the serum ferritin level tended to be higher in patients with the aggressive phenotype. As for interval of the biopsy period, patients with the indolent phenotype presented with a significantly longer interval than those with the aggressive phenotype.

The discriminant formula generated from the construct group

After the analysis, we obtained the following formula: z= 0.150×body mass index (kg/m^2)+0.085×age (year)+1.112×ln (AST) (IU/L)+0.127×ln (m-AST)-12.96. A positive result indicates the aggressive phenotype of liver disease, while a negative result indicates the indolent phenotype (discrimi-

Table 1. Demographic Characteristics and Laboratory Data of the 24 Patients with Non-alcoholic Fatty Liver Disease according to Disease Phenotype in the Construct Group.

	Indolent phenotype (n=6)	Aggressive phenotype (n=18)	p value
Gender, M:F, n	5:1	10:8	0.465
Age, years*	34 (26-44)	49.5 (33-65)	0.002
Body mass index, kg/m^2*	24.3 (20.5-26.6)	27.9 (23.0-34.0)	0.015
Albumin, g/dL*	4.4 (3.7-4.8)	4.3 (3.7-4.9)	0.770
Total bilirubin, mg/dL*	0.8 (0.6-1.1)	0.9 (0.3-2.2)	0.871
AST, IU/L*	58.5 (29-72)	88.5 (36-150)	0.015
ALT, IU/L*	95.5 (56-211)	136.5 (41-281)	0.177
γ-GTP, IU/L*	59 (17-178)	74.0 (26-505)	0.494
Platelet count, ×10^9/L*	247 (165-389)	200 (130-253)	0.199
Fasting blood sugar, mg/dL*	95.5 (92-112)	96.0 (65-273)	0.770
Diabetes mellitus, yes/no	0/6	4/18	
Total cholesterol, mg/dL*	208.5 (193-253)	213.5 (181-256)	0.871
Triglycerides, mg/dL*	124.5 (95-232)	129 (51-337)	0.770
LDL-cholesterol, mg/dL*	133 (115-189)	134 (99-205)	0.721
HDL-cholesterol, mg/dL*	43 (35-54)	44 (31-84)	0.923
Ferritin, ng/mL*	142 (16-536)	342 (10-1123)	0.104
m-AST (IU/L)*	3.5 (2-5)	4.5 (2-17)	0.047
NA score*	4 (1-6)	5 (2-7)	0.415
Interval of biopsy, years*	10.1 (4.4-13.8)	4.0 (1.0-15.8)	0.047

ALT: alanine aminotransferase, AST: aspartate aminotransferase, γ-GTP: gamma-glutamyl transpeptidase, HDL: high-density lipoprotein, LDH: lactate dehydrogenase, LDL: low-density lipoprotein, m-AST: mitochondrial-aspartate aminotransferase, NA score: NAFLD activity score

*Expressed as median (range).

Table 2. Distribution of Scores of the 120 Patients with Non-alcoholic Fatty Liver Disease in the Validation Group.

	NAFL (n=10)	NASH Stage-1 (n=43)	NASH Stage-2 (n=14)	NASH Stage-3 (n=38)	NASH Stage-4 (n=15)	p value
Age, years*	46.5 (24-78)	46.0 (23-73)	55.5 (35-70)	61.5 (29-83)	67 (36-85)	<0.001
Body mass index, kg/m^2*	24.3 (21.6-27.8)	26.0 (20.1-38.2)	25.8 (23.0-33.3)	27.6 (21.2-37.9)	26.2 (18.1-40.4)	0.158
AST, IU/L*	29.5 (14-55)	51.0 (23-164)	48.5 (25-139)	63 (17-198)	48 (26-152)	0.003
m-AST (IU/L) *	4.5 (≤2-10)	4.0 (≤2-13)	4.0 (≤2-8)	5.0 (≤2-11)	4.0 (≤2-18)	0.929
Score of discriminant formula*†	-1.478 (-3.244-1.403)	0.498 (-2.904-3.540)	0.886 (-1.944-2.600)	1.661 (-1.691-4.752)	2.166 (-0.316-3.215)	<0.001
Number of aggressive phenotype (%)	3/10 (30%)	24/43 (53%)	10/14 (71%)	35/38 (92%)	14/15 (93%)	

AST: aspartate aminotransferase, m-AST: mitochondrial-aspartate aminotransferase, NAFL: non-alcoholic fatty liver, NASH: non-alcoholic steatohepatitis

*Expressed as median (range).

†Calculated with the following formula: z=0.150×body mass index (kg/m^2)+0.085×age (years)+1.112×ln (AST) (IU/L)+0.127×ln (m-AST)–12.96.

nant formula for predicting the aggressive phenotype of NAFLD, named "D-APN"). This discriminant formula has a sensitivity of 89%, a specificity of 83%, a PPV of 94%, and an NPV of 71%.

The validation of the discriminant function

The distribution of the scores of the 120 patients in the validation group is shown in Table 2. The discriminant formula scores were gradually elevated along with the stage of progression, and their distribution was significantly different among several stages of fibrosis. Fig. 2 shows the ratio of the aggressive phenotype and the PNPLA3 rs738409 GG genotype in patients with the aggressive phenotype of NAFLD in each histological stage of NAFLD. The ratio of the aggressive phenotype of NAFLD was significantly elevated along with the stage of progression ($p<0.001$), and more than 70% of the patients with stage ≥2 fibrosis had the aggressive phenotype. Similarly, the incidence of the PNPLA3 rs738409 GG genotype in patients with the aggressive phenotype was significantly elevated along with the stage of progression ($p<0.001$).

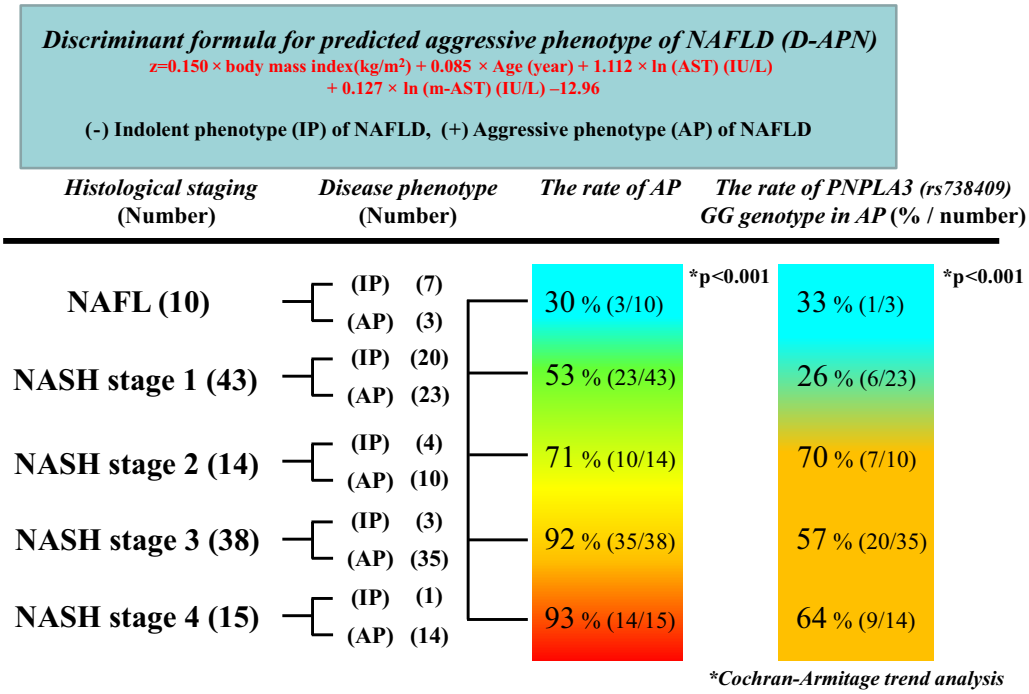

Figure 2. **The distribution of the aggressive phenotype of NAFLD and the ratio of the PNPLA3 rs738409 GG genotype in patients with the aggressive phenotype in the validation group of 120 NAFLD patients.**

Discussion

In Japan, many patients with NAFLD are diagnosed by US alone, because liver biopsies are associated with the risk of major complications, including intraperitoneal bleeding. Although some non-invasive scoring systems (the NAFLD fibrosis score, the BARD score, and the APRI and FIB-4 indexes) have been used to predict fibrosis (11-14), these studies were principally aimed at differentiating the advanced stages of fibrosis (3 to 4) from mild stages of fibrosis (1 to 2). However, in daily clinical practice, we need to detect the rapidly progressive type at an earlier stage of disease, and we should follow these cases more intensively.

In the present study, we created a discriminant formula to predict the aggressive phenotype of NAFLD that does not depend on the various markers of fibrosis. Similarly, some of the non-invasive scoring systems (the NAFLD fibrosis score, the BARD score, and the APRI and FIB-4 indexes) that are used for predicting fibrosis do not depend on the various fibrotic markers (11-14). There were two differences between our discriminant formula and the previously reported non-invasive scoring systems. First, our discriminant formula is can be used to predict the potential for disease progression in each NAFLD patient at the time of the first blood examination. Thus, in this study, we divided NAFLD patients into two disease phenotypes, the "*aggressive phenotype*" and the "*indolent phenotype*", based on the disease stage in several biopsies. In patients with the aggressive phenotype, the stage of fibrosis was maintained at or progressed beyond 2.

In patients with the indolent phenotype, the stage of fibrosis was maintained at <2. NASH stage 2 is defined by "perisinusoidal and portal/periportal fibrosis," and fibrotic progression in this phase has very important clinical implications; the next stage of progression is fibrotic bridging.

Recently, Vilar-Gomez et al. (23, 24) reported that weight loss (especially ≥10% weight loss) was highly associated with the level of improvement in the histological features and the resolution of NASH. However, in our study, in the cohort of the construct group, weight loss was observed between each liver biopsy in 3 of 6 patients (50%) [≥10% weight loss was observed in 1 of 6 patients (17%)] with the indolent phenotype. Similarly, weight loss was observed in 8 of 18 patients (44%) [≥10% weight loss was observed in 3 of 18 patients (17%)] with the aggressive phenotype. From this result, the use of the factor of weight loss alone may not be sufficient for predicting the resolution or progression of disease.

Second, we used a marker of necroapoptosis, "m-AST," to construct a new discriminant formula. CK-18(M30) is a prominent and specific marker of apoptosis in NAFLD that has been used in a clinical study in specialized tertiary care centers. Although several studies have reported the utility of CK-18(M30) as marker of apoptosis in NAFLD (25-29), it is not useful in daily clinical practice because it is not easy for all institutions (including primary and secondary care centers and general clinics) to obtain. Realistically, in daily clinical practice for NAFLD patients, a commercially-available marker of necroapoptosis, such as m-AST, is more useful.

In addition, our formula yielded very interesting results.

Our estimation formula predicted the ratio of the aggressive phenotype in each fibrotic stage of NAFLD, the ratio was significantly elevated in accordance with the progression of fibrosis, and the incidence of the PNPLA3 GG genotype was significantly elevated in accordance with the distribution of the aggressive phenotype (Fig. 2).

Recently, Singh et al. (30) reported that a low baseline AST:ALT ratio was a sensitive discriminant marker of the progression of fibrosis. In addition small proportion of these patients may rapidly progress to advanced fibrosis. Similarly, in our study population, five of the patients with the aggressive phenotype whose fibrotic stage was <2 at baseline presented a low baseline AST:ALT ratio (the median value was 0.47). However, the AST:ALT ratio changes along with the progression of the disease. Generally, the NASH patients with non-advanced fibrosis have a low AST:ALT ratio (<1.0), while those with advanced fibrosis have higher AST:ALT ratios. Progression is also associated with decreased fatty deposition. Stage 4 patients frequently present with ratios of ≥1.0. Similarly, in our study population, the median AST:ALT ratio in the validation group changed with each fibrotic stage as follows: stage 0, 0.67; stage 1, 0.55; stage 2, 0.57; stage 3, 0.77; and stage 4, 1.20. Thus, 13 of the patients with the aggressive phenotype of NAFLD whose baseline stage of fibrosis was ≥2 presented higher baseline AST:ALT ratio values in comparison to the patients with the aggressive phenotype of NAFLD who had earlier stage of fibrosis at baseline (median AST:ALT ratio 0.75 vs. 0.47). Thus, the AST:ALT ratio is a useful marker for the early stages of NASH, but may not be sufficient for all stages of NASH.

These results accurately reflect our impression of NAFLD in daily clinical medicine; the most important point was that almost 30% patients, even those with NAFL, who had the potential for rapidly progressive disease.

However, the present study is associated with some limitations. First, the older age of the aggressive phenotype group influenced the definition of the aggressive phenotype, because older patients generally have advanced fibrosis. Second, we used 120 patients who did not receive a repeated biopsy for our validation group to predict the latent aggressive phenotype in this study; thus, we cannot be certain of the accuracy of these results in predicting the aggressive phenotype. Singh et al. (30) performed a systematic review and meta-analysis of paired-biopsy studies and reported that patients with NAFL progressed by 1 stage over 14.3 years, while those with NASH progressed by 1 stage over 7.1 years; however, the median follow-up period in our validation group was 4.4 years. Thus, the transaminase levels, platelet counts and body weight should be followed over a longer period to evaluate the utility of D-APN in a future study. Third, this was a retrospective single-center cohort study that evaluated a small number of patients. A further large-scale multicenter study is needed to evaluate this discriminant formula in a larger number of patients who receive repeat biopsy. However, we believe that this new discriminant formula will impact the routine clinical care of patients with NAFLD, especially those whose disease shows the potential for rapid progression. We also think that the progression of many high-risk patients to advanced liver disease, including decompensated liver cirrhosis and hepatocellular carcinoma, will be prevented by the early detection of disease progression using this discriminant formula.

In conclusion, the new discriminant formula, named "D-APN," was useful for predicting the NAFLD patients who had a high potential for disease progression, and the distribution of the aggressive phenotype was associated with the distribution of the PNPLA3 rs738409 GG genotype. In addition, this formula is suitable for repeated use in daily clinical medicine. Its accuracy and reproducibility require further validation with larger numbers of patients in several countries besides Japan.

The authors state that they have no Conflict of Interest (COI).

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