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Validation of the FIB4 index in a Japanese nonalcoholic fatty liver disease population

Yoshio Sumida[1], Masato Yoneda[2], Hideyuki Hyogo[3], Yoshito Itoh[4], Masafumi Ono[5*], Hideki Fujii[6], Yuichiro Eguchi[7], Yasuaki Suzuki[8], Noriaki Aoki[9], Kazuyuki Kanemasa[1], Koji Fujita[2], Kazuaki Chayama[3], Toshiji Saibara[5], Norifumi Kawada[6], Kazuma Fujimoto[7], Yutaka Kohgo[8], Toshikazu Yoshikawa[4] and Takeshi Okanoue[10], for Japan Study Group of Nonalcoholic Fatty Liver Disease (JSG-NAFLD)

Abstract

Background: A reliable and inexpensive noninvasive marker of hepatic fibrosis is required in patients with nonalcoholic fatty liver disease (NAFLD). FIB4 index (based on age, aspartate aminotransferase [AST] and alanine aminotransferase [ALT] levels, and platelet counts) is expected to be useful for evaluating hepatic fibrosis. We validated the performance of FIB4 index in a Japanese cohort with NAFLD.

Methods: The areas under the receiver operating characteristic curves (AUROC) for FIB4 and six other markers were compared, based on data from 576 biopsy-proven NAFLD patients. Advanced fibrosis was defined as stage 3-4 fibrosis. FIB4 index was assessed as: age (yr) × AST (IU/L)/(platelet count (10^9/L) × √ALT (IU/L))

Results: Advanced fibrosis was found in 64 (11%) patients. The AUROC for FIB4 index was superior to those for the other scoring systems for differentiating between advanced and mild fibrosis. Only 6 of 308 patients with a FIB4 index below the proposed low cut-off point (< 1.45) were under-staged, giving a high negative predictive value of 98%. Twenty-eight of 59 patients with a FIB4 index above the high cut-off point (> 3.25) were over-staged, giving a low positive predictive value of 53%. Using these cutoffs, 91% of the 395 patients with FIB-4 values outside 1.45-3.25 would be correctly classified. Implementation of the FIB4 index in the Japanese population would avoid 58% of liver biopsies.

Conclusion: The FIB4 index was superior to other tested noninvasive markers of fibrosis in Japanese patients with NAFLD, with a high negative predictive value for excluding advanced fibrosis. The small number of cases of advanced fibrosis in this cohort meant that this study had limited power for validating the high cut-off point.

Background

Type 2 diabetes mellitus is associated with nonalcoholic fatty liver disease (NAFLD) in clinical practice. NAFLD includes a wide spectrum of liver diseases ranging from simple steatosis, which is usually a benign and non-progressive condition, to nonalcoholic steatohepatitis (NASH), which can progress to liver cirrhosis (LC) and hepatocellular carcinoma (HCC) in the absence of significant alcohol consumption [1-4]. Liver biopsy remains a reliable tool for the diagnosis of NASH [1,5,6], and the most sensitive and specific method for providing prognostic information. However, it may not be practical to perform liver biopsies in every patient with NAFLD to ascertain the presence of NASH [6]. Moreover, biopsies are associated with significant limitations such as pain, risk of severe complications, sampling errors [7,8], cost, and patient unwillingness to undergo invasive testing. Since it is not easy to distinguish simple steatosis from NASH in diabetes clinics, simple scoring systems to derive progressive NASH are required. Numerous noninvasive panels of tests have been developed to stage liver disease, including a combination of clinical and routine laboratory parameters, as well as specialized tests involving direct markers of fibrosis and elastography [9-20]. Of these, the BAAT (body mass index [BMI], age, alanine aminotransferase [ALT],

* Correspondence: onom@kochi-u.ac.jp
[5]Department of Gastroenterology and Hepatology, Kochi Medical School, Kochi, Japan
Full list of author information is available at the end of the article

triglycerides) [14], European liver fibrosis (ELF) score [10], Fibrotest (BioPredictive, Paris, France) [9], Fibroscan (Echosens, Paris, France) [12], acoustic radiation force impulse elastography (Mochida Siemens Medical System Co. Ltd., Tokyo, Japan) [15], hyaluronic acid (HA) [16,17], type IV collagen 7S [18], BARD (BMI, aspartate aminotransferase [AST]/ALT ratio [AAR], diabetes mellitus [DM]) [19], N (Nippon) score [20] and the NAFLD fibrosis score (NFS) [21] have been tested in subjects with NAFLD.

The FIB4 index was developed as a noninvasive panel to stage liver disease in subjects with human immunodeficiency virus and hepatitis C virus (HCV) co-infection [22]. It relies on patient age, AST, ALT, and platelet count, which are routinely measured and are thus available for virtually all subjects with liver disease. This index has also been independently validated in subjects with HCV infection alone [23]. It has recently been demonstrated that its performance characteristics for the diagnosis of advanced fibrosis in NAFLD are better than those of other similar panels that do not require additional testing [24]. However, 74% of the subjects enrolled in the study were Caucasian, and validation of the FIB4 index in other ethnic groups is required before it can be applied globally. In this study, we therefore aimed to assess the accuracy of the FIB4 index for predicting advanced liver fibrosis in a cohort of Japanese patients with NAFLD.

Methods

Patients

A total of 576 patients with well-characterized and liver-biopsy-confirmed NAFLD between 2002 and 2008 were enrolled from the Japan Study Group of NAFLD (JSG-NAFLD), which includes nine hepatology centers in Japan: Center for Digestive and Liver Diseases, Nara City Hospital; Division of Gastroenterology, Yokohama City University Graduate School of Medicine; Department of Medicine and Molecular Science, Graduate School of Biomedical Sciences, Hiroshima University; Department of Gastroenterology and Hepatology, Kochi Medical School; Department of Internal Medicine, Saga Medical School, Saga University; Department of Hepatology, Graduate School of Medicine, Osaka City University; Department of Gastroenterology and Hepatology, Kyoto Prefectural University of Medicine; Division of Gastroenterology and Hematology/Oncology, Department of Medicine, Asahikawa Medical College; and Hepatology Center, Saiseikai Suita Hospital. All patients were also involved in the previous JSG-NAFLD study [25].

The diagnosis of NAFLD was based on the following criteria: (1) liver biopsy showing steatosis in at least 5% of hepatocytes [26]; and (2) appropriate exclusion of liver diseases of other etiologies, including viral hepatitis, autoimmune hepatitis, drug-induced liver disease, primary biliary cirrhosis, biliary obstruction, hemochromatosis, Wilson's disease, or α-1-antitrypsin- deficiency-associated liver disease. Patients who consumed > 20 g alcohol per day and patients with evidence of decompensated LC or HCC were excluded. Written informed consent was obtained from all patients at the time of liver biopsy, and the study was conducted in accordance with the Helsinki Declaration [27]. The study protocol was approved by the ethical committee of Nara City Hospital in Nara, Japan.

Anthropometric and laboratory evaluation

Venous blood samples were taken in the morning after a 12-h overnight fast. Laboratory evaluations in all patients included a blood cell count and measurement of AST, ALT, γ-glutamyl transpeptidase (GGT), cholinesterase (ChE), total cholesterol, triglyceride, high-density lipoprotein (HDL) cholesterol, albumin, fasting plasma glucose (FPG), immunoreactive insulin (IRI), and ferritin. These parameters were measured using standard clinical chemistry techniques. BMI was also calculated; obesity was defined as BMI > 25, according to the criteria of the Japan Society for the Study of Obesity [28]. Patients were assigned a diagnosis of DM if they had documented use of oral hypoglycemic medication, a random glucose level > 200 mg/dL, or FPG > 126 mg/dL [29]. Hypertension was defined as a systolic blood pressure ≥ 130 mmHg or a diastolic blood pressure ≥ 85 mmHg or by the use of antihypertensive agents. Dyslipidemia was defined as serum concentrations of triglycerides ≥ 150 mg/dL or HDL cholesterol < 40 mg/dL and < 50 mg/dL for men and women, respectively, or by the use of specific medication [30]. Based on a review of the literature, the following scores were calculated for each patient: FIB4 [22], AAR, AST to platelet ratio index (APRI) [31], age-platelet index (AP index) [32], BARD score [19], N score [20], and NFS [13]. The values for the upper limit of normal were set according to the International Federation of Clinical Chemistry: AST 35 U/L for men, 30 U/L for women, and were comparable to the values used in other analyses. The specific formulae used to determine these scores are shown in Table 1.

Histologic evaluation

All patients enrolled in this study underwent percutaneous liver biopsy under ultrasonic guidance. The liver specimens were embedded in paraffin and stained with hematoxylin and eosin, and Masson's trichrome. The minimum biopsy size was 20 mm and the number of portal areas was 10. The liver biopsy specimens were reviewed by two hepatopathologists (T.O. and Y.S.) who were blinded to the clinical data. Fatty liver was defined

Table 1 Formulae for determining noninvasive marker panels for detection of liver fibrosis.

Formula	Equation	
FIB4 index	(Age [years] × AST [IU/L])/(platelet count [10^9/L] × $\sqrt{\text{ALT [IU/L]}}$)	
AST to ALT ratio (AAR)	AST/ALT	
AST to platelet ratio index (APRI) [a]	([AST/ULN]/platelet count [10^9/L]) × 100	
Age-platelet index (AP index)	Age (years)	platelet count (10^9/L)
	< 30 = 0	< 225 = 0
	30-39 = 1	200-224 = 1
	40-49 = 2	175-199 = 2
	50-59 = 3	150-174 = 3
	60-69 = 4	125-149 = 4
	≥ 70 = 5	< 125 = 5
	Score is the sum of two (0-10)	
NAFLD fibrosis score	-1.675 + 0.037 × age (years) + 0.094 ×BMI (kg/m^2) + 1.13 × IFG/diabetes (yes = 1, no = 0) + 0.99 × AST/ALT - 0.013 × platelet count (× 10^9/L) - 0.66 × albumin (g/dL).	
BARD score	Scale 0-4	
	BMI ≥ 28 kg/m^2 = 1 point	
	AST/ALT ≥ 0.8 = 2 points	
	Diabetes = 1 point	
N (Nippon) score	Scale 0-4	
	female sex = 1 point	
	older age (> 60 years) = 1 point	
	type 2 diabetes = 1 point	
	hypertension = 1 point	

BMI, body mass index; IFG, impaired fasting glucose; INR, international normalized ratio; ULN, upper limit of normal.[a]ULN for AST: 30 in women, 35 in men.

as the presence of steatosis in at least 5% hepatocytes, while steatohepatitis was diagnosed by steatosis, inflammation, and hepatocyte ballooning [2,3,26]. The individual parameters of NASH histology, including fibrosis, were scored independently using the NASH Clinical Research Network (CRN) scoring system developed by the NASH CRN [26]. Advanced fibrosis was classified as stage 3 or 4 disease (bridging fibrosis or cirrhosis).

Statistical analysis

Statistical analysis was conducted using SPSS 19.0 software (SPSS, Inc., Chicago, IL). Continuous variables were expressed as mean ± standard deviation (SD), or median (interquartile range). Qualitative data were presented as numbers with percentages in parentheses. Statistical differences in quantitative data were determined using the *t* test or Mann-Whitney U test. Fisher's exact probability test or χ^2 analysis was used for qualitative data (Table 2). The sensitivity and specificity for each value of each test were calculated to assess the accuracy of the clinical scoring system in differentiating between advanced and mild fibrosis, and receiver operating characteristic (ROC) curves were constructed by plotting the sensitivity against (1 - specificity) at each value (Figure 1). The diagnostic performances of the scoring systems were assessed by analysis of ROC curves. The most commonly used index of accuracy was the area under the ROC curve (AUROC), with values close to 1.0 indicating high diagnostic accuracy. (Table 3). The sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) were calculated for the two cut-off values (< 1.45 and > 3.25) proposed by Sterling [22] and those (< 1.30 and > 2.67) proposed by Shah [24]. Differences were considered statistically significant at $p < 0.05$.

Results

A total of 576 subjects were included in this analysis. Of these, 280 (49%) were women and 418 (73%) were obese (Table 2); 241 (42%) had type 2 DM and 184 (32%) were hypertensive. A total of 319 subjects had steatohepatitis, of whom 64 subjects had advanced fibrosis. As expected, subjects with more advanced fibrosis were significantly older, predominantly female, and more likely to be hypertensive, to have type 2 DM, to have higher AST, AAR, GGT, FPG, and IRI, and to have lower hemoglobin, platelet count, albumin, ChE, total cholesterol, and triglyceride. Regarding the individual components of the FIB4 score, the mean (± SD) or median [interquartile range] values were as follows: age (52.3 ± 15.4 years); AST (43 [30-67] IU/L); ALT (69 [43-112] IU/L), and platelets (227 ± 67 × 10^9/L) (Table 2). The distribution of fibrosis stages included stage 0 (n = 263), stage 1 (n = 169), stage 2 (n = 80), stage 3 (n = 45), and

Table 2 Characteristics of study population and values of noninvasive fibrosis marker panels[a].

	Total (n = 576)	Fibrosis stage 0-2 (n = 512)	Fibrosis stage 3-4 (n = 64)	*p-value*[b]
Age (yr)	52.3 ± 15.4	51.2 ± 15.5	62.0 ± 10.1	< 0.0001
Gender (female)	280 (49%)	235 (46%)	45 (70%)	0.0003
BMI (kg/m^2)	27.9 ± 4.9	27.8 ± 4.9	28.6 ± 4.8	0.2138
Obesity (BMI > 25)	418 (73%)	369 (72%)	49 (77%)	0.5524
Hypertension (yes)	184 (32%)	150 (29%)	34 (53%)	0.0062
Type 2 diabetes (yes)	241 (42%)	199 (39%)	42 (66%)	0.0001
Hemoglobin (g/dL)	14.6 ± 2.0	14.7 ± 2.0	13.7 ± 2.0	0.0001
Platelet count (×10^9/L)	227 ± 67	235 ± 64	162 ± 52	< 0.0001
AST (IU/L)	43 (30-67)	41 (29-64)	61 (47-77)	< 0.0001
ALT (IU/L)	69 (43-112)	69 (43-69)	62 (46-94)	0.5074
AST/ALT ratio	0.65 (0.52-0.82)	0.63 (0.51-0.78)	0.98 (0.73-1.21)	< 0.0001
GGT (IU/L)	60 (39-99) (n = 572)	57 (36-92) (n = 508)	84 (59-128)	< 0.0001
Albumin (g/dL)	4.4 ± 0.4	4.4 ± 0.4	4.1 ± 0.4	< 0.0001
Cholinesterase (IU/L)	380 (330-433) (n = 527)	385 (337-439) (n = 466)	297 (244-367) (n = 61)	< 0.0001
Total cholesterol (mg/dL)	209 ± 40 (n = 467)	210 ± 39 (n = 409)	198 ± 42 (n = 58)	0.0484
Triglyceride (mg/dL)	147 (107-207) (n = 566)	150 (109-212) (n = 502)	131 (95-184) (n = 64)	0.0364
HDL-C (mg/dL)	50 ± 17 (n = 548)	50 ± 17 (n = 487)	51 ± 13 (n = 61)	0.7516
LDL-C (mg/dL)	128 ± 33 (n = 405)	129 ± 32 (n = 363)	120 ± 42 (n = 42)	0.1666
Ferritin (ng/mL)	173 (92-300)	169 (91-292)	216 (128-349)	0.0627
FPG (mg/dL)	103 (94-122) (n = 524)	103 (94-119) (n = 462)	111 (95-138) (n = 62)	0.0166
IRI (μU/mL)	11.6 (7.8-18.4)	11.3 (7.5-17.4)	17.3 (11.3-26.2)	< 0.0001
FIB4 index	1.23 (0.77-2.02)	1.13 (0.71-1.79)	3.17 (1.88-4.25)	< 0.0001
AST/ALT ratio (AAR)	0.65 (0.52-0.82)	0.63 (0.51-0.78)	0.98 (0.73-1.21)	< 0.0001
AST to platelet ratio index (APRI)	0.61 (0.40-0.98)	0.57 (0.38-0.92)	1.22 (0.86-1.79)	< 0.0001
Age-platelet index (AP index)	4 (2-6)	3 (2-5)	7 (5-8)	< 0.0001
NAFLD fibrosis score	-1.82 (-3.04 to -0.58)	-2.07 (-3.25 to -0.95)	0.25 (-0.60-1.06)	< 0.0001
BARD score				< 0.0001
0	144 (25%)	138 (27%)	6 (9%)	
1	201 (35%)	194 (38%)	7 (11%)	
2	112 (19%)	99 (19%)	13 (20%)	
3	88 (15%)	62 (12%)	26 (41%)	
4	31 (5%)	19 (4%)	12 (19%)	
N score				< 0.0001
0	135 (23%)	135 (26%)	0 (0%)	
1	170 (30%)	157 (31%)	13 (20%)	
2	118 (20%)	96 (19%)	22 (34%)	
3	99 (17%)	82 (16%)	17 (27%)	
4	54 (9%)	42 (8%)	12 (19%)	

BMI, body mass index; AST, aspartate aminotransferase; ALT, alanine aminotransferase; GGT, γ-glutamyl transpeptidase; HDL, high-density lipoprotein; LDL, low-density lipoprotein; FPG, fasting plasma glucose; and IRI, immuno-reactive insulin. *a* Values are mean ± SD, median (interquartile range), counts (%), as appropriate. *b*Values from univariate ordinal logistic regression, Mann-Whitney, or χ^2 analysis, as appropriate.

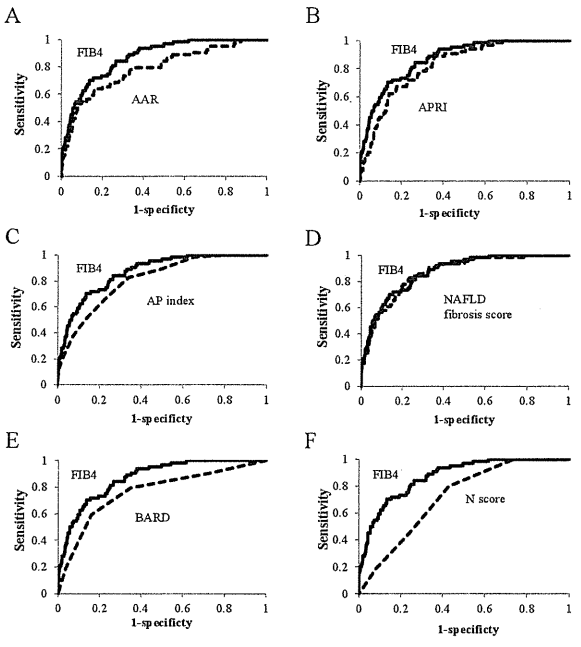

Figure 1 Comparison of ROCs of FIB4 and other panels for the diagnosis of advanced fibrosis.

stage 4 (n = 19). FIB4 values for the whole sample ranged from 0.17-10.74. The median FIB4 score was 1.23 (interquartile range, 0.77-2.02) (Table 3). The mean (interquartile range) FIB4 indices for stages 0, 1, 2, 3, and 4 were 1.09 (0.61-1.34), 1.40 (0.77-1.88), 2.36 (1.44-3.15), 3.23 (1.82-4.04), and 4.48 (3.19-5.17), respectively (p < 0.0001 by analysis of variance). The mean (interquartile range) FIB4 index was 1.13 (0.71-1.79) in patients with stage 0-2 fibrosis and 3.17 (1.88-4.25) in patients with stage 3-4 fibrosis (p <0.0001) (Table 2).

The sensitivity and specificity of FIB4 along the ROC were assessed first. At a sensitivity of 90% (FIB4 = 1.45) the specificity was 35%, while at a specificity of 90% (FIB4 = 2.67), the sensitivity was 52%. ROC curves were then developed for each of the noninvasive marker panels and superimposed, to determine which score would have the most clinical utility (Figure 1). ROC curves were created to determine the utility of the indices for predicting advanced fibrosis (stage 3 and 4 versus lower scores). The AUROC was greatest for FIB4 (0.871), followed by NFS (0.863), APRI (0.823), AP index (0.810), AAR (0.788), BARD score (0.765), and N score (0.715) (Table 3). As the NPVs for FIB4 index, AAR, APRI, AP index, NFS, BARD score, and N score were all greater than 95% using their lower cut-offs, these tests may have sufficient accuracy to be used clinically to exclude advanced fibrosis. Using this approach, a significant proportion of patients could avoid liver biopsy using each of these tests (Table 3). As the PPV were modest for all noninvasive tests, ranging from 19% to 53%, it was felt they were not accurate enough to be used as an alternative to liver biopsy. The PPV for FIB4 is highest among other noninvasive tests.

Using the low cut-off point proposed by Sterling and colleagues (< 1.45)[22], 330 of 336 (98.3%) patients without stage 3 or 4 fibrosis were correctly staged, while only 6 (1.7%) were under-staged (Table 4). All of the 6 patients with advanced fibrosis but FIB4 index below the low cut-off point had stage 3 fibrosis, none had stage 4 fibrosis. The NPV of this cut-off for stage 3 or 4 fibrosis was 98%. Using the high cut-off point proposed by Sterling and colleagues (> 3.25) [24], 31 of 59 (52.5%) patients with stage 3 or 4 fibrosis were correctly staged, while 28 (47.5%) were over-staged. Among the 28 patients without advanced fibrosis but FIB4 index above the high cut-off point, 18 had stage 2 fibrosis, 6 had stage 1, and 4 had no fibrosis. The PPV of this cut-off for stage 3 or 4 fibrosis was 53%. A total of 395 patients (69% of the cohort) had a FIB4 index < 1.45 or > 3.25; FIB4 identified the absence or presence of advanced fibrosis with 91% accuracy in these 361 subjects. A total of 181 subjects (31%) had FIB4 values in the indeterminate range (1.4-3.25).

Table 3 Accuracy of noninvasive fibrosis marker panels.

Fibrosis panel	AUROC	Cut-off values	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
FIB4 index	0.871	1.45	90	64	24	98
		3.25	48	95	53	94
AST/ALT ratio (AAR)	0.788	0.8	66	76	26	95
		1	48	92	44	94
AST to platelet ratio index (APRI)	0.823	1	67	81	31	95
Age-platelet index (AP index)	0.810	6	66	78	27	95
NAFLD fibrosis score	0.863	-1.455	92	63	24	98
		0.676	33	96	50	92
BARD score	0.765	2	80	65	22	97
N score	0.715	2	80	58	19	96

AUROC, area under the receiver operating characteristics curve; PPV, positive predictive value; NPV, negative predictive value

Table 4 Proportion of patients who may potentially avoid liver biopsy using the simple non-invasive tests to exclude advanced fibrosis.

Fibrosis panel	Cut-off values	Patients avoiding liver biopsy[a]	False negative result
FIB4 index	< 1.45	336/576 (58%)	6 (2%)
	< 1.30	308/576 (53%)	4 (1%)
AST/ALT ratio (AAR)	< 0.8	413/576 (72%)	22 (5%)
AST to platelet ratio index (APRI)	< 1	435/576 (76%)	21 (5%)
Age-platelet index (AP index)	< 6	421/576 (73%)	22 (5%)
NAFLD fibrosis score	< -1.455	328/576 (57%)	5 (2%)
BARD score	< 2	355/576 (62%)	13 (4%)
N score	< 2	305/576 (53%)	13 (4%)

[a]Patients with a value below the cut-off.

On the other hand, using the low cut-off point proposed by Shah and colleagues (< 1.30) [24], 304 of 308 (99%) patients without stage 3 or 4 fibrosis were correctly staged, while only 4 (1%) were under-staged (Table 4). All of the 4 patients with advanced fibrosis but FIB4 index below the low cut-off point had stage 3 fibrosis and none had stage 4 fibrosis. The NPV of this cut-off for stage 3 or 4 fibrosis was 99%. Using the high cut-off point proposed by Shah and colleagues (> 2.67), 38 of 89 (43%) patients with stage 3 or 4 fibrosis were correctly staged, while 51 (57%) were over-staged. Among the 51 patients without advanced fibrosis but NAFLD fibrosis scores above the high cut-off point, 28 had stage 2 fibrosis, 14 had stage 1, and 9 had no fibrosis. The PPV of this cut-off for stage 3 or 4 fibrosis was 43%. A total of 397 patients (69% of the cohort) had a FIB4 index < 1.30 or > 2.67; FIB4 identified the absence or presence of advanced fibrosis with 86% accuracy in these 342 subjects. A total of 179 subjects (31%) had FIB4 values in the indeterminate range (1.30-2.67). Thus the prevalence of patients in the indeterminate range was similar using the two different cut-off values, but the number of patients with true positive or true negative predictions (accuracy) was higher using Sterling *et al.*'s cut-off values compared with Shah *et al.*'s (361 patients versus 342 patients). If liver biopsies were only performed in patients with an FIB4 index above the low cut-off point (> 1.45) proposed by Sterling, 336 (58%) of 576 biopsies could be avoided (Table 4).

The diagnostic accuracy of FIB4 index for detecting advanced fibrosis (stage 3-4) was also compared to that of NFS (Table 5). Three hundred and seventy patients (64% of the cohort) had an NFS <-1.455 or > 0.676; NFS identified the absence or presence of advanced fibrosis with 93% accuracy in these 344 subjects. A total of 206 subjects (36%) had NFS values in the indeterminate range (-1.455-0.676). Although the accuracy of NFS was higher (93%) than that of FIB4 (86%), more patients were correctly staged with FIB4 (n = 361) than with NFS (n = 344). Moreover, the percentage of patients in the undetermined range was lower for the FIB4 index (31%) than for NFS (36%). Using the cut-off values reported by Sterling and colleagues, discrepancies between FIB4 index and NFS were observed in 146 (39%) patients (Table 5). Patients were categorized into three groups, "low-risk" (< 10%), "intermediate-risk" (10-30%) and "high-risk" (> 30%), based on the combination of FIB4 index and NFS (Table 5). Only 1 patient (0.4%)

Table 5 Categorized risk groups for advanced fibrosis according to combined FIB4 index and NAFLD fibrosis score (NFS).

		FIB4 index (cut-off values proposed by Sterling et al.)			Total
		Low cut-off point (< 1.45)	Indeterminate (1.45-3.25)	High cut-off point (> 3.25)	
NFS	Low cut-off point (<-1.455)	283 [1 (0.4%)] [a]	42 [4 (9.5%)] [a]	3 [0 (0.0%)] [a]	328 (56.9%) [5 (1.5%)]
	Indeterminate (-1.455-0.676)	53 [5 (9.4%)] [a]	122 [19 (15.6%)] [b]	31 [14 (45.2%)] [c]	206 (35.8%) [38 (18.4%)]
	High cut-off point (> 0.676)	0	17 [4 (23.5%)] [b]	25 [17 (68.0%)] [c]	42 (7.3%) [21 (50.0%)]
Total		336 (58.3%) [6 (1.7%)]	181 (31.4%) [27 (14.9%)]	59 (10.2%) [31 (52.5%)]	576 (100%) [64 (11.1%)]

Total number of patients [stage 3-4 (%)]

Patients were categorized into three groups, "low-risk" (< 10%) [a], "intermediate-risk" (10-30%) [b] and "high-risk" (> 30%) [c], based on the combination of FIB4 index and NFS.

of 243 patients with the low cut-off points for both FIB4 index and NFS had advanced fibrosis.

Discussion

The AUROC of FIB4 was 0.871 for the diagnosis of advanced fibrosis, which was superior to those of the other noninvasive panels tested. For a value < 1.45, fibrosis could be excluded with 98% certainty (NPV 98%) whereas for a value > 3.25, the presence of significant fibrosis could be predicted with 53%. Despite the limited sensitivity of the FIB4 index in a population with a low prevalence of advanced fibrosis, the score was useful for ruling out advanced fibrosis. In our cohort, 58% of the liver biopsies could have been avoided if the procedure was not performed in patients with a FIB4 index below the low cut-off point (< 1.45). The score would therefore be particularly useful for reducing the number of unnecessary liver biopsies performed, and thus the costs of managing NAFLD patients in Asia, where advanced fibrosis is uncommon. A high cut-off FIB4 index of 2.67 which has been proposed by Shah and colleagues [24] had a low PPV (43%) in predicting stage 3 or 4 fibrosis. Our results contrast with those reported by Shah and colleagues [24], where a high cut-off FIB4 index of 2.67 had an 80% PPV in predicting stage 3 or 4 fibrosis; however the prevalence of advanced fibrosis in our study was only 11%, compared to 23% in Shah *et al.*'s study. Our study was therefore unable to reliably validate the high cut-off point, and larger Asian studies are warranted to investigate this. The FIB4 index was higher in our population than in Shah *et al.*'s study; stage 0-2: 1.13 (0.71-1.79) versus 0.97 (0.68-1.37), stage 3-4: 3.17 (1.88-4.25) versus 1.98 (1.28-3.08), probably because of older age, higher levels of ALT, and lower levels of platelets in our population.

The BARD score developed by Harrison *et al.* represents the weighted sum of three easily available variables (BMI ≥ 28 kg/m^2 [1 point], AAR ≥ 0.8 [2 points], and DM [1 point]), and the authors demonstrated that a score of 2-4 was associated with an odds ratio of 17 for predicting advanced fibrosis [19]. Although BARD score is simple to calculate, our validation study failed to detect any advantage of this score over FIB4; a BARD score of ≥ 2 was associated with a sensitivity, specificity, PPV and NPV for detecting advanced fibrosis of 80, 65, 22 and 97%, respectively. Consistent with the present study, Fujii and colleagues reported significantly poorer applicability of BARD in Japanese patients with NAFLD, compared with Caucasian subjects [33]. It has been suggested that BARD score is less predictive of advanced fibrosis in Japanese NAFLD patients because they are less obese than those in western countries. The N score (the total number of the following risk factors: female sex, age > 60 years, type 2 DM, and hypertension), which was established on the basis of data from 182 Japanese NAFLD patients in multiple centers in Nagasaki [20], requires no detailed laboratory measurements, but was not found to be superior to FIB4 index in our validation study. Angulo *et al.* found that the NFS, which consists of six variables (age, BMI, AAR, IFG/DM, platelet count, and albumin), reliably predicted advanced fibrosis in NAFLD patients [21]. In 428 (74%) of the subjects in the present study, FIB4 index was in accordance with NFS. The combination of two scoring systems could help to identify patients likely to have advanced fibrosis. Patients with FIB4 values above the high cut-off point (> 3.25) and NFS values above the low cut-off point (> -1.455) were at high risk (> 30%) for advanced fibrosis. If both FIB4 and NFS were applied to Japanese patients with NAFLD, patients with either FIB4 or NFS values below the low cut-off points (376/576, 65.3%) could avoid liver biopsies. In this way, when FIB4 was combined with NFS, its ability to predict or exclude advanced fibrosis improved further. In summary, the current study demonstrated that the FIB4 index, which can be established using a simple, relatively inexpensive method, correlated with the stage of fibrosis in adult subjects with NAFLD.

Type IV collagen is one of extracellular matrices that are produced by hepatic fibroblasts. The 7S domain in the N-terminus of type IV collagen is inserted in tissues and released into the blood by turnover in connective tissues. Therefore, the serum 7S domain level increases in parallel with the amount of fibrosis and in synthesis from stellate cells and myofibroblasts following increased liver fibrosis. In Japan, type IV collagen 7S is now widely used for assessing the extent of hepatic fibrosis in chronic liver diseases. Our data demonstrated that a cutoff point of 5.4 ng/ml provided a sensitivity and specificity of 86% and 87%, respectively, to detect advanced stage of NASH. The AUROC of type IV collagen 7s was: 0.926 for the diagnosis of advanced fibrosis, which was superior to FIB4 (data not shown). This data suggest that type IV collagen 7S is one of the best parameters among non-invasive parameters, but it costs too much to be determined routinely.

On the other hand, hepatic steatosis is frequently found in patients with HCV infection. Therefore, we also evaluated the value of FIB4 index in 185 HCV-infected patients with hepatic steatosis, including those with 72 advanced and 113 mild fibrosis. The AUROC of FIB4 was 0.808 for the diagnosis of advanced fibrosis. For a value < 1.45, fibrosis could be excluded with 89% certainty (NPV 89%) whereas for a value > 3.25, the presence of advanced fibrosis could be predicted with 82% (data not shown).

This study had several limitations. First, the proportion of subjects with advanced fibrosis was small, as

reported in other Asian studies [34], and further Asian studies with more patients with advanced fibrosis are warranted. Second, patients were recruited from hepatology centers in Japan with a particular interest in studying NAFLD, and the possibility of some referral bias could therefore not be ruled out. Patient selection bias could also have existed, because liver biopsy might have been considered for NAFLD patients who were likely to have NASH. The findings may thus not represent NAFLD patients in the wider community. However, this would introduce a negative bias, as NAFLD patients in the community would be likely to have milder liver disease, thus increasing the NPV of the FIB4 index. We also acknowledge that pathologic diagnosis was mainly determined using liver tissues derived from percutaneous liver biopsies, which are prone to sampling errors or interobserver variability [7,8]. As recent studies suggest that low normal ALT value does not guarantee freedom from underlying NASH with advanced fibrosis [35-37], it remains to be solved whether FIB4 index can be useful for predicting advanced fibrosis in NAFLD subjects with normal ALT. According to our preliminary data by JSG-NAFLD, the AUROC of FIB4 was 0.810 for the diagnosis of advanced fibrosis in 187 biopsy-proven NAFLD patients with normal ALT levels (data not shown). Our data support the hypothesis that FIB4 index could also be used in the Japanese NAFLD population with normal ALT.

Conclusion

The FIB4 index demonstrated a good NPV for excluding advanced fibrosis in Japanese NAFLD patients, and could thus be used to reduce the burden of liver biopsies. Larger Asian studies are required to validate the high cut-off point of the FIB4 index. However, the FIB4 test also has several serious limitations, in common with other noninvasive tests for fibrosis, and further research is needed before simple noninvasive tests, including the FIB4 test, can replace liver biopsies in the vast majority of patients.

Abbreviations

AAR: AST/ALT ratio; AST: aspartate aminotransferase; ALT: alanine aminotransferase; AP index: age to platelet index; APRI- aspartate aminotransferase to platelet ratio index; AUROC: area under the receiver operating characteristic; NAFLD: nonalcoholic fatty liver disease; NASH: nonalcoholic steatohepatitis; NFS: NAFLD fibrosis score; NPV: negative predictive value; PPV: positive predictive value.

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Author details

[1]Center for Digestive and Liver Diseases, Nara City Hospital, Nara, Japan. [2]Division of Gastroenterology, Yokohama City University Graduate School of Medicine, Yokohama, Japan. [3]Department of Medicine and Molecular Science, Graduate School of Biomedical Sciences, Hiroshima University, Hiroshima, Japan. [4]Department of Gastroenterology and Hepatology, Kyoto Prefectural University of Medicine, Kyoto, Japan. [5]Department of Gastroenterology and Hepatology, Kochi Medical School, Kochi, Japan. [6]Department of Hepatology, Graduate School of Medicine, Osaka City University, Osaka, Japan. [7]Department of Internal Medicine, Saga Medical School, Saga University, Saga, Japan. [8]Division of Gastroenterology and Hematology/Oncology, Department of Medicine, Asahikawa Medical College, Asahikawa, Japan. [9]School of Biomedical Informatics, University of Texas Health Science Center at Houston, Houston, TX, USA. [10]Hepatology Center, Saiseikai Suita Hospital, Suita, Japan.

Authors' contributions

YS: study concept and design, drafting of the manuscript, MY: acquisition of data, HH: acquisition of data, YI: critical revision of the manuscript for important intellectual content. MO: study concept and design, HF: acquisition of data, YE: acquisition of data, YS: acquisition of data, NA: statistical analysis, KK: critical revision of the manuscript for important intellectual content, KF: acquisition of data, KC: critical revision of the manuscript for important intellectual content TS: critical revision of the manuscript for important intellectual content NK: critical revision of the manuscript for important intellectual content KF: critical revision of the manuscript for important intellectual content YK: critical revision of the manuscript for important intellectual content, TY: critical revision of the manuscript for important intellectual content, TO: study supervision, JSG-NAFLD: acquisition of data, study supervision. All authors read and approved the final manuscript.

Competing interests

The authors declare that there is no duality of interest associated with this manuscript.

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Original Article

Clinical and pathological progression of non-alcoholic steatohepatitis to hepatocellular carcinoma

Kohichiroh Yasui,[1] Etsuko Hashimoto,[2] Katsutoshi Tokushige,[2] Kazuhiko Koike,[3] Toshihide Shima,[4] Yoshihiro Kanbara,[4] Toshiji Saibara,[5] Hirofumi Uto,[6] Shiro Takami,[7] Miwa Kawanaka,[8] Yasuji Komorizono,[9] Takeshi Okanoue[1,4] and The Japan NASH Study Group*

[1]Department of Molecular Gastroenterology and Hepatology, Kyoto Prefectural University of Medicine, Kyoto, [2]Department of Internal Medicine and Gastroenterology, Tokyo Women's Medical University, [3]Department of Gastroenterology, Graduate School of Medicine, The University of Tokyo, Tokyo, [4]Center of Gastroenterology and Hepatology, Saiseikai Suita Hospital, Suita, [5]Department of Gastroenterology and Hepatology, Kochi Medical School, Kochi, [6]Digestive Disease and Life-style Related Disease Health Research, Human and Environmental Sciences, Kagoshima University Graduate School of Medical and Dental Sciences, Kagoshima, [7]Department of Gastroenterology, Otsu Municipal Hospital, Otsu, [8]Center of Liver Diseases, Kawasaki Hospital, Kawasaki Medical School, Okayama, and [9]Department of Hepatology, Nanpuh Hospital, Kagoshima, Japan

Aim: Non-alcoholic steatohepatitis (NASH) can progress to hepatocellular carcinoma (HCC). We aimed to examine the clinical and pathological course of how NASH progresses to HCC.

Methods: In this retrospective multicenter study conducted in Japan, we examined 19 patients (53% female), who had been previously diagnosed with histologically proven NASH and developed HCC during the follow-up period. The median age of the patients at the time of initial diagnosis of NASH was 65 years.

Results: NASH progressed to HCC after a median follow-up period of 3.8 years (range: 0.5–11.6 years). All patients had been identified as having HCC during screening, which included 12 patients assessed by ultrasound, four patients assessed with computerized tomography, two patients that underwent serum des-γ-carboxy prothrombin testing and one patient that underwent serum α-fetoprotein testing. The median diameter of HCC tumors was 1.8 cm (range: 0.8–3.0 cm). The majority of patients (n = 13; 68%) presented with only one HCC tumor. The stage of liver fibrosis was significantly more advanced at the time of diagnosis of HCC than at the time of initial diagnosis of NASH, whereas there were no significant differences in the degree of steatosis.

Conclusion: Screening for HCC with imaging is necessary not only in NASH patients with advanced fibrosis, but also in those with less advanced forms of fibrosis, particularly if they are old men. Liver fibrosis progresses to a more advanced stage during the development of HCC in NASH patients.

Key words: liver cancer, liver fibrosis, screening

INTRODUCTION

NON-ALCOHOLIC FATTY LIVER disease (NAFLD) is one of the most common causes of chronic liver disease in the world.[1,2] NAFLD is frequently associated with obesity, type 2 diabetes, dyslipidemia and insulin resistance, and is a manifestation of metabolic syndrome. The spectrum of NAFLD ranges from a relatively benign accumulation of lipids (i.e. simple steatosis) to a progressive non-alcoholic steatohepatitis (NASH) that is associated with fibrosis, necrosis and inflammation. Despite its common occurrence and potentially serious nature, little is known about the natural history or prognostic significance of NAFLD.

Hepatocellular carcinoma (HCC) is the fifth most common cancer worldwide and the third leading cause of cancer mortality.[3] Evidence from case reports and case series,[4–7] as well as retrospective[8–11] and prospective studies,[12–16] suggest that NASH can progress to HCC. However, both the incidence of and risk factors for HCC

Correspondence: Dr Takeshi Okanoue, Center of Gastroenterology and Hepatology, Saiseikai Suita Hospital, 1-2 Kawazono-cho, Suita 564-0013, Japan. Email: okanoue@suita.saiseikai.or.jp
*Takeshi Okanoue, Yutaka Kohgo, Sumio Kawata, Kazuhiko Koike, Kohjiro Ueki, Shigeki Arii, Etsuko Hashimoto, Sumio Watanabe, Toshinari Takamura, Kohichiroh Yasui, Fumihiko Matsuda, Tetsuo Takehara, Toshiji Saibara, and Hirofumi Uto.
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in NASH patients remain unclear, and little is known about the clinical and pathological course of how NASH progresses to HCC.

The Japan NASH Study Group,[17] which was established in 2008 by the Ministry of Health, Labor and Welfare of Japan, had previously performed a cross-sectional study that characterized the clinical features of NASH patients that developed HCC.[18] This study revealed that most NASH patients that develop HCC are men, and have high rates of obesity, type 2 diabetes and hypertension. Furthermore, male patients appear to develop HCC at a less advanced stage of liver fibrosis than female patients. In the present study, our study group conducted a multicenter retrospective study to examine the clinical and pathological course of NASH as it progresses to HCC.

METHODS

Patients

THE DIAGNOSIS OF NASH was based on the following criteria: (i) histological features of steatohepatitis (please refer to the histological examinations section below); (ii) negligible alcohol consumption; and (iii) exclusion of liver diseases from other etiologies. Medical records were also reviewed at our institutions to determine alcohol consumption as accurately as possible. Based on the medical records, alcohol consumption was assessed according to the detailed history obtained via physicians, as well as via interviews with family members. The exclusion criteria included the consumption of more than 20 g of alcohol per day, positivity for hepatitis B virus surface antigen, positivity for anti-hepatitis C virus antibody, the presence of other types of liver diseases (e.g. primary biliary cirrhosis, autoimmune hepatitis, Wilson's disease or hemochromatosis), previous treatment with drugs known to produce hepatic steatosis and a history of gastrointestinal bypass surgery. Sections of non-tumor liver tissues were re-analyzed by experienced hepatopathologists, who were blinded to the laboratory parameters and clinical data. We also excluded patients whose histological diagnosis of NASH was not confirmed by central review, as well as those with insufficient or inconclusive information regarding alcohol consumption and clinical data. The diagnosis of HCC was based on liver histology, and, in the absence of histology, on typical features of HCC, as assessed by dynamic computerized tomography (CT) or magnetic resonance imaging (MRI) (i.e. hypervascularity with washout in the portal/venous phase).[19]

Nineteen Japanese patients, who had been previously diagnosed with histologically proven NASH and developed HCC during a follow-up period, were retrospectively identified and reviewed. Patients were identified from nine hepatology centers belonging to the Japan NASH Study Group[17] and their affiliated hospitals. All patients had been identified as having HCC during screening, which included ultrasound and/or CT of the liver, and α-fetoprotein (AFP) and des-γ-carboxy prothrombin (DCP) testing. Of the 19 patients, five patients were diagnosed with HCC following hepatic resection, five other patients were diagnosed following ultrasound-guided tumor biopsy and nine patients were diagnosed with dynamic CT or MRI. In a separate study, we previously reported on parts of the data obtained from 10 of the 19 patients, which were collected at the time of diagnosis of HCC.[18]

This study was approved by the local ethics committee of each participating center and conducted in accordance with the Declaration of Helsinki. Informed consent was obtained from each patient.

Clinical assessment and laboratory tests

Clinical and laboratory data were collected at the time of initial diagnosis of both NASH (i.e. baseline) and HCC. Body mass index (BMI) was calculated using the following formula: weight in kilograms / (height in meters)^2. Obesity was defined as a BMI of 25 kg/m^2 or more, according to the criteria established by the Japan Society for the Study of Obesity.[20] Diabetes, dyslipidemia and hypertension were defined as previously described.[18] Laboratory evaluations included a blood cell count, and measurements of serum aspartate aminotransferase, alanine aminotransferase (ALT), γ-glutamyl transpeptidase and ferritin. Two serum tumor markers of HCC, AFP and DCP, were also measured. These parameters were measured using standard clinical chemistry techniques. There were missing data for ferritin in some patients.

Histopathological examinations

Liver tissues were obtained from all 19 patients at the time of initial diagnosis of NASH, and again non-tumor liver tissues were obtained from 14 of the 19 patients at the time of initial diagnosis of HCC. Additionally, non-tumor liver tissues were surgically resected from five patients that underwent hepatic resection for HCC and were examined. Non-tumor liver tissues away from HCC tumors were separately biopsied in five patients that underwent ultrasound-guided tumor biopsy procedures. Lastly, non-tumor liver tissues away from HCC

tumors were obtained via ultrasound-guided biopsies from four patients that were diagnosed with HCC via dynamic CT or MRI.

The collected specimens were fixed in formalin, embedded in paraffin, and stained with hematoxylin–eosin, Masson's trichrome and silver impregnation. NASH was defined as steatosis with lobular inflammation, hepatocellular ballooning and the presence of Mallory–Denk bodies or fibrosis.[21–23] The necroinflammatory grade and degree of fibrosis were evaluated, and scored according to the criteria proposed by Brunt *et al.*[24] Furthermore, the degree of ballooning degeneration was scored according to the NAFLD activity score (NAS) proposed by the NASH Clinical Research Network.[25]

Statistical analysis

Data were presented as numbers with percentages in parentheses for qualitative data or as medians and ranges for quantitative data. Comparisons were made using a χ^2-test, a Mann–Whitney *U*-test or a Wilcoxon rank sum test. *P*-values below 0.05 from two-sided tests were considered to be significant. All statistical analyses were performed using SPSS ver. 15.0 software (SPSS, Chicago, IL, USA).

RESULTS

THE BASELINE CHARACTERISTICS of the 19 patients at the time of initial diagnosis of NASH are summarized in Table 1. The median age was 65 years (range: 45–76 years). There were nine male (47%) and 10 female (53%) patients. Sixteen patients (84%) were obese (i.e. BMI ≥25 kg/m^2). Type 2 diabetes, dyslipidemia and hypertension were present in 11 (58%), nine (47%) and 12 (63%) patients, respectively. Serum ferritin values were elevated compared to normal values, which were approximately 100 ng/mL for adult men (range: 75–250 ng/mL) and 30 ng/mL for adult women (range: 20–75 ng/mL).[26]

The diagnosis of NASH was histologically confirmed. With respect to the degree of steatosis, 14 patients (74%) had grade 1 (i.e. 5–33%), four patients (21%) had grade 2 (i.e. 34–66%) and one patient (5%) had grade 3 (i.e. >66%). Furthermore, the necroinflammatory grade was mild (i.e. grade 1) in eight patients (42%) and moderate (i.e. grade 2) in 11 patients (58%). Hepatocellular ballooning was scored as 1+ (i.e. few cells) in 16 patients (84%) and 2+ (many cells) in three patients (16%). In regards to the degree of fibrosis, three patients (16%) presented with stage 1, four patients (21%) presented with stage 2, five patients (26%) presented with stage 3 and seven patients (37%) presented with stage 4 (i.e. liver cirrhosis). Data were stratified according to the sex of the patient (Table 1). There were no significant differences between male and female patients with the exception of necroinflammatory grade.

Non-alcoholic steatohepatitis progressed to HCC after a median follow-up period of 3.8 years (range: 0.5–11.6 years). All patients had been identified as having HCC during screening, which included 12 patients assessed with ultrasound, four patients evaluated with CT, two patients that underwent DCP testing and one patient that underwent AFP. The median age at diagnosis of HCC was 71 years (range: 57–78 years). The median diameter of HCC tumors was 1.8 cm (range: 0.8–3.0 cm). Thirteen patients (68%) presented with one HCC tumor, whereas six patients (32%) presented with two or three HCC tumors.

Compared to the baseline, patients had significantly lower BMI, platelet count and ALT levels at the time of diagnosis of HCC (Table 2). Of the two serum tumor markers for HCC, DCP levels, but not AFP, were significantly higher at the time of HCC diagnosis compared to baseline (Table 2). Furthermore, the number of patients with abnormally elevated DCP (>40 mAU/mL), but not with abnormally elevated AFP (>10 ng/mL), was significantly increased at the time of HCC diagnosis compared to baseline (Table 2).

Non-tumor liver tissues were obtained from 14 of the 19 patients at the time of HCC diagnosis. The stage of fibrosis and steatosis grade at baseline and time of HCC diagnosis are presented in Figure 1. The stage of fibrosis was significantly more advanced at the time of HCC diagnosis than at baseline ($P = 0.02$). Specifically, the stage of fibrosis progressed in six patients (43%) and remained unchanged in eight patients (57%). Three patients (21%) presented with stage 2 fibrosis at the time of HCC diagnosis. These patients were all male, aged 75, 77 and 78 years. Hypertension was present in all of these patients, and obesity and type 2 diabetes were present in two of these patients. Furthermore, there were no significant differences in the degree of steatosis at baseline and time of HCC diagnosis ($P = 0.15$). That is, the degree of steatosis decreased in only two patients (14%), and remained unchanged in 12 patients (86%).

DISCUSSION

IN THE PRESENT multicenter retrospective study conducted in Japan, we characterized the clinical and pathological course of NASH patients that developed HCC during the follow-up period. It was found that

Table 1 Baseline characteristics of patients with NASH

Characteristics	Total ($n = 19$)	Male ($n = 9$)	Female ($n = 10$)	*P*-value†
Age (years)	65 (45–76)	67 (53–76)	64 (45–74)	0.25
Obesity	16 (84%)	8 (89%)	8 (80%)	0.59
Diabetes	11 (58%)	5 (56%)	6 (60%)	0.84
Dyslipidemia	9 (47%)	4 (44%)	5 (50%)	0.80
Hypertension	12 (63%)	7 (78%)	5 (50%)	0.21
BMI (kg/m^2)	27.5 (21.5–39.7)	27.2 (21.5–32.0)	28.5 (23.4–39.7)	0.30
AST (IU/L)	57 (22–126)	40 (25–126)	58 (22–101)	0.65
ALT (IU/L)	45 (20–212)	56 (20–212)	42 (22–93)	0.56
γ-GTP (IU/L)	84 (25–230)	71 (25–169)	99 (33–230)	0.28
Platelet count (×10^4/μL)	12.2 (7.0–21.6)	13.0 (8.0–19.0)	9.8 (7.0–21.6)	0.07
Ferritin (ng/dL)	247 (22–1170)‡	335 (96–1170)§	240 (22–430)¶	0.34
Liver tissue				
Steatosis grade				0.32
1: 5–33%	14 (74%)	8 (89%)	6 (60%)	
2: 34–66%	4 (21%)	1 (11%)	3 (30%)	
3: >66%	1 (5%)	0 (0%)	1 (10%)	
Necroinflammatory grade				0.04
1: mild	8 (42%)	6 (67%)	2 (20%)	
2: moderate	11 (58%)	3 (33%)	8 (80%)	
3: severe	0 (0%)	0 (0%)	0 (0%)	
Ballooning degeneration				N/A
0: none	0 (0%)	0 (0%)	0 (0%)	
1: few	16 (84%)	6 (67%)	10 (100%)	
2: many	3 (16%)	3 (33%)	0 (0%)	
Stage of fibrosis				0.42
1	3 (16%)	2 (22%)	1 (10%)	
2	4 (21%)	3 (34%)	1 (10%)	
3	5 (26%)	2 (22%)	3 (30%)	
4	7 (37%)	2 (22%)	5 (50%)	
Follow-up period (years)	3.8 (0.5–11.6)	6.3 (1.3–11.6)	3.3 (0.5–11.6)	0.43

Values are presented as medians (ranges) or numbers (%).
†χ^2-Test or Mann-Whitney *U*-test.
‡$n = 11$.
§$n = 4$.
¶$n = 7$.
ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; γ-GTP, γ-glutamyl transpeptidase; N/A, not applicable; NASH, non-alcoholic steatohepatitis.

HCC could be diagnosed in the early stages of its progression. Specifically, screening with imaging appears to allow for the early detection of HCC in patients with NASH. Furthermore, DCP seemed to be a more useful marker in the detection of HCC than AFP. Lastly, liver fibrosis appeared to progress to a more advanced stage during the development of HCC.

Corroborating previous published work,[8–11] as well as our previous findings,[18] the majority of patients in the present study demonstrated obesity, type 2 diabetes and hypertension. This was not surprising as both obesity and diabetes are significant risk factors for the development of HCC[27–29] as well as NASH.

After a median follow-up period of 3.8 years (range: 0.5–11.6 years), NASH progressed to HCC. The short duration of the follow-up period suggests that these patients were at a higher risk of HCC at the time of diagnosis of NASH. Furthermore, the median diameter of HCC tumors was 1.8 cm (range: 0.8–3.0 cm), which is smaller than the size reported in previous studies on NASH-associated HCC,[8,9,11,30,31] as well as our own cross-sectional study.[18] Additionally, the majority of patients (68%) presented with only one HCC tumor.

Two serum tumor markers for HCC, AFP and DCP, were measured. Of the two markers, serum levels of DCP, but not AFP, were significantly higher at the time

Table 2 Clinical and laboratory data at baseline and time of HCC diagnosis ($n = 19$)

Variable	Baseline	Time of HCC diagnosis	*P*-value†
BMI (kg/m^2)	27.5 (21.5–39.7)	27.2 (21.7–39.0)	0.03
Platelet count (×10^4/μL)	12.2 (7.0–21.6)	10.5 (3.8–17.3)	0.001
AST (IU/L)	57 (22–126)	42 (17–76)	0.10
ALT (IU/L)	45 (20–212)	37 (15–72)	0.02
γ-GTP (IU/L)	84 (25–230)	52 (13–399)	0.35
AFP (ng/mL)	6.0 (3.0–69.4)	10.5 (1.6–2748)	0.09
Abnormal AFP (>10 ng/mL)	5 (26%)	10 (53%)	0.10
DCP (mAU/mL)	18 (10–52)	34 (11–3657)	0.004
Abnormal DCP (>40 mAU/mL)	1 (5%)	9 (47%)	0.003

Values are presented as medians (ranges) or numbers (%).
†Wilcoxon rank sum test or χ^2-test.
AFP, α-fetoprotein; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; DCP, des-γ-carboxy prothrombin; γ-GTP, γ-glutamyl transpeptidase; HCC, hepatocellular carcinoma.

of HCC diagnosis compared to baseline. This finding suggests that DCP may be a more useful marker for the early detection of HCC in patients with NASH than AFP. However, it should be noted that there are many HCC patients that do not have elevated serum DCP levels. Thus, close monitoring of imaging findings is important in the early detection of HCC in aged NASH patients.

Furthermore, it was found that at the time of HCC diagnosis, patients had a significantly lower BMI, platelet count and serum ALT levels than at baseline. The decrease in BMI may be, in part, due to the progression of NASH, the development of HCC and/or due to the implementation of lifestyle changes (i.e. diet and exercise) for the treatment of NASH. The decrease in platelet count and ALT levels may also be accounted for by the progression of NASH (i.e. the progression of fibrosis), as platelet count and ALT levels decrease when NASH progresses to liver cirrhosis.

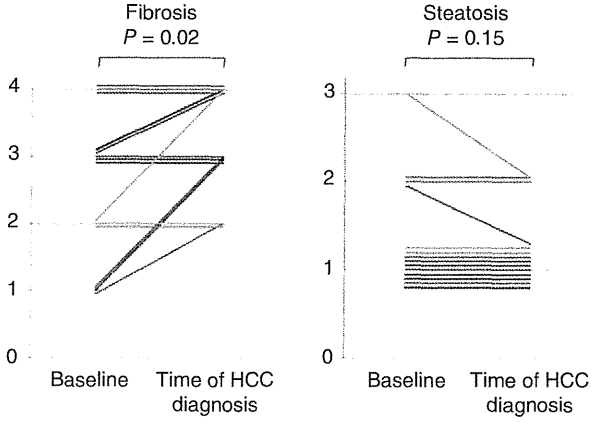

Figure 1 Stage of fibrosis and steatosis grade at baseline and time of hepatocellular carcinoma (HCC) diagnosis.

Most cases of HCC arise in patients with a background of cirrhosis. Our earlier cross-sectional study of 87 patients with NASH-associated HCC demonstrated that cirrhosis (i.e. stage 4 of fibrosis) was present in 51% of cases and advanced stages of fibrosis (i.e. stage 3 or 4) were present in 72% of cases.[18] In the present study, at baseline, cirrhosis was already present in 37% of the patients, and advanced stages of fibrosis (i.e. stage 3 or 4) were present in 63% of the patients. Furthermore, the stage of fibrosis was significantly more advanced at the time of HCC diagnosis than at baseline. Specifically, at the time of HCC diagnosis, cirrhosis was present in 43% of the patients and advanced stages of fibrosis (i.e. stage 3 or 4) were present in 79% of the patients. These findings suggest that fibrosis may be a precursor in the development of HCC in patients with NASH and that the progression of fibrosis is a risk factor for the development of HCC. A report from Japan showed that the annual incidence of HCC was 2.2% and 6.1% in patients with NASH-associated cirrhosis and hepatitis C virus-associated cirrhosis, respectively.[15] Meanwhile, a report from the USA showed that the incidence was 4.0% and 6.3% patients with NASH-associated cirrhosis and hepatitis C virus-associated cirrhosis, respectively.[16]

Interestingly, in our earlier cross-sectional study, we found that 28% of NASH-associated HCC cases had less advanced forms of fibrosis (i.e. stage 1 or 2), though it was more remarkable in males.[18] In the present study, three patients (21%) presented with stage 2 fibrosis at the time of HCC diagnosis. These three patients were all

male and more than 75 years old. These patients also had higher rates of hypertension, obesity and type 2 diabetes. All three patients had been identified as having HCC during screening, with two patients assessed with ultrasound and one patient evaluated with CT. Thus, some patients with NASH may develop HCC without the presence of advanced stages of fibrosis. Paradis *et al.* reported that, in patients whose only risk factors for chronic liver disease were features of metabolic syndrome, HCC usually occurs in the absence of significant liver fibrosis.[31] Therefore, screening for HCC is necessary not only in NASH patients with advanced fibrosis, but also in those with less advanced forms of fibrosis, especially in aged patients.

When NASH progresses to cirrhosis, steatosis tends to disappear and inflammation subsides, and this is referred to as "burn-out" NASH.[4] As expected, because most of the present patients had advanced stages of fibrosis, the grade of steatosis was found to be mild. Furthermore, there were no significant differences in the degree of steatosis at baseline and time of HCC diagnosis, although it decreased in some cases.

Serum ferritin, a marker of iron storage, was elevated in our patients. We had previously reported high frequencies of hyperferritinemia and increased hepatic iron stores in Japanese patients with NASH.[32] Iron overload was positively associated with the prevalence of metabolic syndrome and insulin resistance, and it increased oxidative stress.[33,34] These findings suggest that iron overload is likely to be involved in the pathogenesis and carcinogenesis of NASH. However, the role of iron overload as an underlying etiology or promoter of liver fibrosis and carcinogenesis in NASH continues to be debated.[23]

The carcinogenic mechanisms of NASH are unknown and remain to be elucidated. Some possible mechanisms may include hyperinsulinemia, increased lipid peroxidation, production of reactive oxygen species and subsequent DNA mutations, imbalances in energy and hormonal regulation, and aberrations in regenerative processes.[23]

A few limitations should be considered in the interpretations of our findings: (i) there may have been bias in patient selection, as patients were retrospectively identified as having NASH-associated HCC; (ii) no comparisons between the case and control groups were made, as there was no control group with NASH patients that did not develop HCC during the follow-up period; (iii) the present study examined a small number of patients; and (iv) the incidence of HCC in NASH patients was unknown, as the total number of NASH patients present in our study hospitals during the follow-up period was unknown.

In summary, we demonstrated the clinical and pathological course of NASH, which progressed to HCC. Screening for HCC with imaging is necessary not only in NASH patients with advanced fibrosis, but also in those with less advanced forms of fibrosis, particularly if they are old men. Additionally, liver fibrosis appears to progress to a more advanced stage during the development of HCC. Further prospective studies with a longer follow-up duration and larger cohort of patients are warranted to determine the course of NASH that progresses to HCC.

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Original Article

Usefulness of Technetium-99 m-2-methoxy-isobutyl-isonitrile liver scintigraphy for evaluating disease activity of non-alcoholic fatty liver disease

Kosei Masuda,[1] Masafumi Ono,[1] Mitsutaka Fukumoto,[2] Akira Hirose,[1] Kensuke Munekage,[1] Tsunehiro Ochi,[1] Nobuto Okamoto,[1] Naoki Akagi,[2] Yasuhiro Ogawa[2] and Toshiji Saibara[1]

Departments of [1]Gastroenterology and Hepatology, and [2]Radiology, Kochi Medical School, Nankoku, Kochi, Japan

Aim: Non-alcoholic steatohepatitis (NASH) is a progressive form of non-alcoholic fatty liver disease (NAFLD). Therefore, it is important to evaluate disease activity and distinguish NASH from simple steatosis in NAFLD. Technetium-99 m-2-methoxy-isobutyl-isonitrile (^{99m}Tc-MIBI) is a lipophilic cation designed for myocardial perfusion scintigraphy in the diagnosis of ischemic heart diseases, and its retention reflects mitochondrial function. It was reported that hepatic mitochondrial abnormalities would be an important predictive factor for NASH disease progression. The aim of this study was to examine the clinical usefulness of ^{99m}Tc-MIBI liver scintigraphy for evaluating disease activity of NAFLD and distinguishing NASH from simple steatosis in patients with NAFLD.

Methods: Twenty-six patients with biopsy-proven NAFLD were enrolled. Clinicolaboratory tests and ^{99m}Tc-MIBI liver scintigraphy were performed. To evaluate hepatic uptake, regions of interest were set at the liver and heart, and the uptake ratio of the liver to heart (liver/heart ratio) was calculated.

Results: All patients with NAFLD were classified into three groups according to the NAFLD activity score: non-NASH (simple steatosis) ($n = 4$), borderline NASH ($n = 11$), and NASH ($n = 11$). Liver/heart ratios were significantly lower in NASH than in simple steatosis ($P < 0.05$). Moreover, liver/heart ratios were significantly correlated with NAFLD activity scores among the patients ($r = -0.413$, $P < 0.05$).

Conclusions: The present study indicates that ^{99m}Tc-MIBI liver scintigraphy would be a useful non-invasive functional imaging method with which to evaluate disease activity of NAFLD and distinguish NASH from simple steatosis.

Key words: mitochondrial dysfunction, non-alcoholic fatty liver disease, non-alcoholic steatohepatitis, technetium-99 m-2-methoxy-isobutyl-isonitrile liver scintigraphy

INTRODUCTION

NON-ALCOHOLIC FATTY LIVER disease (NAFLD) is one of the most common causes of chronic liver disease in Asian and Western countries. NAFLD encompasses a wide spectrum ranging from simple steatosis, which is a benign condition, to non-alcoholic steatohepatitis (NASH), which can lead to cirrhosis and/or hepatocellular carcinoma.[1,2] Therefore, it is important to distinguish NASH from simple steatosis to predict future adverse hepatic outcomes. Liver biopsy is currently needed for a definite diagnosis of NASH despite its invasiveness and potential for complications.[3] Thus, the need for non-invasive methods for the diagnosis of NASH is pressing.

Patients with NAFLD frequently have several metabolic disorders, including visceral obesity, diabetes mellitus type 2, hypertension, and dyslipidemia.[3] Therefore, NAFLD is now recognized as the hepatic manifestation of metabolic syndrome (MS).[4] MS is a well-known predictor of cardiovascular disease (CVD).[5] Patients with NAFLD have a high prevalence of cardiovascular risk factors and high risk of cardiovascular events.[6–10]

Correspondence: Dr Masafumi Ono, Department of Gastroenterology and Hepatology, Kochi Medical School, Kohasu, Oko-cho, Nankoku, Kochi 783-8505, Japan. Email: onom@kochi-u.ac.jp

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Moreover, the pathological severity of NAFLD is associated with the degree of atherosclerosis independently of classic cardiometabolic risk factors.[11] Most importantly, several longitudinal studies have shown that CVD occurs more frequently than liver-related diseases as the cause of death in patients with NAFLD.[12–14]

Myocardial perfusion scintigraphy (MPS) is one of the most established non-invasive diagnosis methods for CVD.[15] Technetium-99 m-2-methoxy-isobutyl-isonitrile (^{99m}Tc-MIBI) is a lipophilic cationic agent that was initially designed for myocardial perfusion imaging.[16] This compound predominantly accumulates in mitochondria, where it is retained in response to the electrical potential generated across the membrane bilayer.[17] ^{99m}Tc-MIBI retention in mitochondria is related to mitochondrial function.[18] Furthermore, ^{99m}Tc-MIBI scintigraphy is useful for clinical evaluation of mitochondrial function of myocardium and skeletal muscle.[19–21] Although the precise pathogenic mechanism is obscure, recent data suggest that hepatic mitochondrial abnormalities of NASH are closely related to its pathogenesis.[22–24] On the other hand, these mitochondrial lesions are absent in most patients with simple steatosis and in healthy subjects.[24] Accordingly, hepatic ^{99m}Tc-MIBI uptake in NASH was hypothesized to decrease compared with that in simple steatosis. The aim of this study was to determine the usefulness of ^{99m}Tc-MIBI liver scintigraphy for evaluating disease activity of NAFLD and distinguishing NASH from simple steatosis.

METHODS

Patients

TWENTY-SIX PATIENTS WITH biopsy-proven NAFLD (17 males and nine females; age, 39.7 ± 14.0 years) were enrolled in this study. Informed consent was obtained at the time of liver biopsy or laboratory testing, and the study was conducted in conformance with the Declaration of Helsinki. Patients with an alcohol intake in excess of 20 g per day were excluded from this study. Patients with evidence of hepatocellular carcinoma or with any other known liver disease, including viral hepatitis, hemochromatosis, Wilson disease, and autoimmune liver disease, were excluded. None of the patients were taking any medications that might produce hepatic steatosis (including methotrexate, tamoxifen, corticosteroids, and insulin). Patients with evidence of established cardiopulmonary disease or symptoms of suspected cardiopulmonary disease were excluded. The body mass index (BMI) was calculated using the following formula: weight in kg/(height in meters).[2]

Histological evaluation

Liver biopsies of all patients were performed percutaneously under ultrasonographic guidance, and biopsy specimens were obtained from the liver parenchyma of the upper region of the right lobe using a 15-gauge biopsy needle. Liver biopsy specimens were routinely fixed in 10% phosphate-buffered formalin (pH 7.4), embedded in paraffin, and sectioned for hematoxylin and eosin staining. The histological findings of NAFLD were interpreted and scored according to the NASH Clinical Research Network scoring system.[25] In this scoring system, the degree of disease activity in NAFLD was evaluated using the NAFLD Activity Score (NAS), which was calculated as the unweighted sum of the scores for steatosis (0–3), lobular inflammation (0–3), and hepatocyte ballooning (0–2) and thus ranged from 0 to 8. A NAS of 5 or more was diagnosed as "definitive NASH" (NASH), 2 or less as "non-NASH," and 3 or 4 as "borderline NASH." Hepatic fibrosis was assessed by Brunt's classification,[26] and fibrosis staging was as follows: 0 = no fibrosis; 1 = zone 3 fibrosis only; 2 = zone 3 and portal/periportal fibrosis; 3 = bridging fibrosis; and 4 = cirrhosis. Histological evaluation was performed by two pathologists with no knowledge of the patients' clinical data.

Laboratory test

Peripheral venous blood samples were obtained the morning after an overnight fast. The laboratory evaluation included determination of prothrombin time and measurements of serum aspartate aminotransferase (AST), alanine aminotransferase (ALT), gamma glutamyl transpeptidase (GGT), bilirubin, albumin, and ferritin levels.

^{99m}Tc-MIBI liver scintigraphy

^{99m}Tc-MIBI liver scintigraphy was performed at rest. Following an overnight fast, 600 megabecquerels (MBq) of ^{99m}Tc-MIBI (Cardiolite, FUJIFILM RI Pharma Co., Ltd, Tokyo, Japan) was injected intravenously while the patients lay on a gamma camera bed in a dorsal position. Planar scans including liver and heart areas were performed by a gamma camera (GCA-7100A, Toshiba, Tokyo, Japan) with a low-energy, high-resolution collimator 10 min after bolus injection. Scan conditions were as follows: scan time, 2 min; view, anterior; and

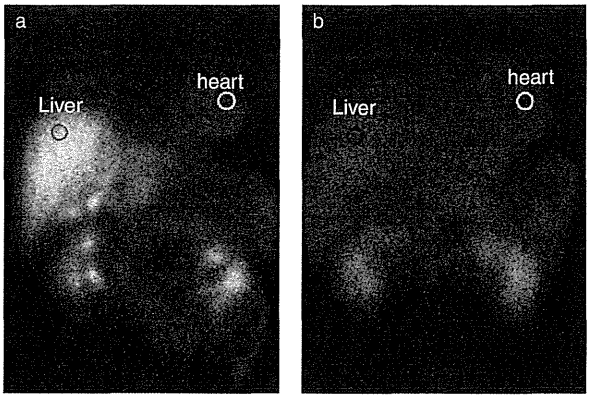

Figure 1 Representative anterior planar images of the liver and heart in "non-NASH" (simple steatosis) (a) and "NASH" (b). Intrahepatic uptake of ^{99m}Tc-MIBI in NASH was lower than that in simple steatosis. The regions of interest were set at the upper right lobe of the liver (black circle) and the anterolateral wall of the left ventricle (white circle). The uptake ratio of the liver to heart (liver/heart ratio) was calculated as an indicator of intrahepatic uptake. NASH, non-alcoholic steatohepatitis.

matrix, 512 × 512. The energy window was set at 140 keV with a 10% symmetrical width. To evaluate hepatic uptake, regions of interest (ROI) were set at the liver and heart by hand to obtain the mean counts per pixel. Liver ROI was set at the right upper lobe of the liver in ^{99m}Tc-MIBI scintigraphy (Fig. 1) to harmonize with the region of liver biopsy, because significant sampling variability occurs between the right and left lobes in NAFLD.[27] Heart ROI with a size identical to that of liver was placed on the anterolateral wall of the left ventricle to avoid the influence of radioactivity from the left lobe of the liver (Fig. 1). Next, the uptake ratio of the liver to heart (liver/heart ratio) was calculated as an indicator of intrahepatic uptake, because there was neither evidence nor suspicion of established heart disease among the patients.

Statistical analysis

Results are presented as mean ± standard deviation (SD) for quantitative data and as numbers or percentages for categorical or qualitative data. Statistical differences in quantitative data were determined using the Mann–Whitney *U*-test or post-hoc test. Qualitative data were compared using the χ^2 test. Spearman's coefficients of correlation were used to evaluate the relationship between NAFLD activity and hepatic fibrosis and the intrahepatic ^{99m}Tc-MIBI uptake. Results were considered significant when the *P*-value was <0.05.

RESULTS

A TOTAL OF 26 patients with NAFLD were classified into three groups based on NAS: non-NASH ($n = 4$), borderline NASH ($n = 11$), and NASH ($n = 11$). Clinical and pathological features were compared among the groups (Table 1). Age, gender, and BMI were not significantly different among the groups. Transaminase levels in the NASH group tended to be higher than those in other groups. Prothrombin time and serum GGT, bilirubin, albumin, and ferritin levels were not significantly different among the groups.

Representative ^{99m}Tc-MIBI planar images of the liver and heart in patients with simple steatosis and those with NASH are shown in Figure 1. Intrahepatic uptake of ^{99m}Tc-MIBI in NASH (Fig. 1b) was remarkably lower than that in simple steatosis (Fig. 1a). Radioactivity of intrahepatic uptake of ^{99m}Tc-MIBI was evaluated as the liver/heart ratio. The liver/heart ratios on ^{99m}Tc-MIBI scintigraphy were significantly lower in patients with NASH (1.42 ± 0.41, $P < 0.01$) and borderline NASH (1.56 ± 0.20, $P < 0.05$) than in patients with non-NASH (2.07 ± 0.29 Fig. 2). Furthermore, liver/heart ratios were significantly correlated with NAS (Spearman's correlation; $r = -0.413$, $P < 0.05$ Fig. 3). Liver/heart ratios in patients with F0 stage hepatic fibrosis (F0) (2.00 ± 0.33, $n = 4$) were significantly higher than those in patients with F2 (1.43 ± 0.26, $n = 6$) stage fibrosis ($P < 0.05$ Fig. 4). However, all patients with F0 were diagnosed as other than NASH, and all patients with F2 were diagnosed as other than simple steatosis (Table 1). NAS in patients with F0 (2.5 ± 0.6, $n = 4$) were significantly lower compared with those in patients with F1 (4.1 ± 1.2, $n = 16$, $P < 0.05$) or F2 (5.0 ± 0.9, $n = 6$, $P < 0.01$ Fig. 5).

DISCUSSION

NON-INVASIVE METHODS THAT can evaluate disease activity of NAFLD and distinguish NASH from other forms of NAFLD are urgently needed, because NASH is a progressive disease that potentially leads to liver cirrhosis and/or hepatocellular carcinoma. Liver biopsy remains the gold standard for diagnosis of NASH despite extensive assessment of non-invasive methods. Serum markers such as type IV collagen and hyaluronic acid can detect the severe liver fibrosis present in patients with late-stage NASH. Radiological