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APPENDIX

The members of the phase III study were as follows: Sapporo Kosei General Hospital, Toranomon Hospital, Juntendo University Hospital, Musashino Red Cross Hospital, Toranomon Branch Hospital, University of Yamanashi Hospital, Shinshu University Hospital, Gifu Municipal Hospital, Ogaki Municipal Hospital, Nagoya University Hospital, Osaka University Hospital, Ikeda Municipal Hospital, Saiseikai Suita Hospital, Hiroshima University Hospital, Shin-Kokura Hospital, Kurume University Hospital and Kagoshima University Medical and Dental Hospital.

Reprinted from Early View
(Online Version of Record published before inclusion in an issue)

ORIGINAL ARTICLES—LIVER, PANCREAS, AND BILIARY TRACT

Characteristics of Patients With Nonalcoholic Steatohepatitis Who Develop Hepatocellular Carcinoma

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This article has an accompanying continuing medical education activity on page e50. Learning Objectives—At the end of this activity, the learner should identify the clinical features of patients with nonalcoholic steatohepatitis who develop hepatocellular carcinoma and the role of hepatic fibrosis in the development of hepatocellular carcinoma.

See related article, Villanueva A et al, on page 1501 in *Gastroenterology*.

BACKGROUND & AIMS: Nonalcoholic steatohepatitis (NASH) can progress to hepatocellular carcinoma (HCC). We aimed to characterize the clinical features of NASH patients with HCC. **METHODS:** In a cross-sectional multicenter study in Japan, we examined 87 patients (median age, 72 years; 62% male) with histologically proven NASH who developed HCC. The clinical data were collected at the time HCC was diagnosed. **RESULTS:** Obesity (body mass index ≥25 kg/m^2), diabetes, dyslipidemia, and hypertension were present in 54 (62%), 51 (59%), 24 (28%), and 47 (55%) patients, respectively. In nontumor liver tissues, the degree of fibrosis was stage 1 in 10 patients (11%), stage 2 in 15 (17%), stage 3 in 18 (21%), and stage 4 (ie, liver cirrhosis) in 44 (51%). The prevalence of cirrhosis was significantly lower among male patients (21 of 54, 39%) compared with female patients (23 of 33, 70%) (P = .008). **CONCLUSIONS: Most patients with NASH who develop HCC are men; the patients have high rates of obesity, diabetes, and hypertension. Male patients appear to develop HCC at a less advanced stage of liver fibrosis than female patients.**

Keywords: Liver Cancer; Incidence; Sex; Retrospective Study.

Hepatocellular carcinoma (HCC) is the fifth most common cancer worldwide and the third leading cause of cancer mortality.[1] HCC mostly occurs within an established background of chronic liver disease and cirrhosis. Although the risk factors for HCC, including infection with hepatitis B and C viruses as well as alcohol consumption, are well-defined, 5%–30% of patients with HCC lack a readily identifiable risk factor for their cancer. It has been suggested that a more severe form of nonalcoholic fatty liver disease (NAFLD), namely nonalcoholic steatohepatitis (NASH), might account for a substantial portion of cryptogenic cirrhosis and HCC cases.[2]

NAFLD is one of the most common causes of chronic liver disease in the world.[3,4] NAFLD is associated with obesity, diabetes, dyslipidemia, and insulin resistance and is recognized as a hepatic manifestation of metabolic syndrome. The spectrum of NAFLD ranges from a relatively benign accumulation of lipid (simple steatosis) to progressive NASH associated with fibrosis, necrosis, and inflammation. Despite its common occurrence and potentially serious nature, relatively little is known about the natural history or prognostic significance of NAFLD. Although prospective studies on the natural history of NAFLD and NASH with a larger cohort are awaited, these

Abbreviations used in this paper: AFP, α-fetoprotein; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; CT, computed tomography; DCP, des-γ-carboxy prothrombin; γ-GTP, γ-glutamyl transpeptidase; HCC, hepatocellular carcinoma; HDL, high-density lipoprotein; MRI, magnetic resonance imaging; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis.

1542-3565/$36.00
doi:10.1016/j.cgh.2011.01.023

studies might be limited by the long and asymptomatic clinical course of these diseases, by their high prevalence in the general population, and by the lack of serologic markers for NASH. The evidence suggesting that NASH can progress to HCC comes from (1) case reports and case series,[5-8] (2) retrospective studies,[9-12] and (3) prospective studies.[13-17] These studies generally examined limited numbers of cases and follow-ups; therefore, the incidence of HCC and risk factors for HCC in NASH patients remain unclear.

The Japan NASH Study Group (representative, Takeshi Okanoue)[18] was established in 2008 by the Ministry of Health, Labour and Welfare of Japan to address unmet research needs in the area of liver diseases. As a part of this mandate, the study group conducted a cross-sectional multicenter study to characterize the clinical features of histologically proven NASH patients who developed HCC.

Methods

Patients

We retrospectively identified and reviewed 87 Japanese patients with NASH, who developed HCC between 1993 and 2010, at 15 hepatology centers that belong to the Japan NASH Study Group[18] and their affiliated hospitals in Japan. The diagnosis of NASH was based on (1) the histologic features of steatohepatitis, (2) negligible alcohol consumption, and (3) exclusion of liver diseases of other etiology. To determine alcohol consumption as accurately as possible, we reviewed medical records in our institutions, and when patients had been transferred from other institutions, we also reviewed a summary of medical records from those institutions. According to the medical records, alcohol consumption was assessed on the basis of a detailed history that was obtained by physicians and by interviewing family members. Exclusion criteria included 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 (eg, 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. The sections of nontumor liver tissues were reanalyzed by experienced hepatopathologists (T.O., E.H.) who were blinded to the laboratory parameters and clinical data. We excluded patients whose histologic diagnosis of NASH was not confirmed by central review and patients with insufficient or inconclusive information concerning alcohol consumption, body mass index (BMI), and laboratory data including fasting glucose and lipid.

Of the 87 patients, 14 patients had been previously diagnosed as NAFLD or NASH and had been followed at our institutions; 73 patients had been transferred from other institutions to our institutions for investigation and treatment of HCC. Most patients had been identified as having HCC during screening, which included ultrasound and/or computed tomography (CT) of the liver and alpha-fetoprotein (AFP) testing.

The diagnosis of HCC was based on liver histology and, in the absence of histology, on typical features of HCC as assessed by dynamic CT or magnetic resonance imaging (MRI) (ie, hypervascular with washout in the portal/venous phase).[19] Of the 87 patients, 49 patients were diagnosed as HCC after hepatic resection, 21 patients were diagnosed after ultrasound-guided tumor biopsy, and 17 patients were diagnosed by dynamic CT or MRI.

The Ethics Committees of each participating center approved this study. Informed consent was obtained from each patient in accordance with the Declaration of Helsinki.

Clinical Assessment and Laboratory Tests

The clinical and laboratory data were collected at the time HCC was diagnosed. BMI was calculated by using the following formula: weight in kilograms/(height in meters)^2. Obesity was defined as BMI $\geq$25 kg/m^2 according to the criteria of the Japan Society for the Study of Obesity.[20] Diabetes was defined as fasting plasma glucose concentration of $\geq$126 mg/dL or 2-hour plasma glucose concentration of $\geq$200 mg/dL during an oral glucose (75 g) tolerance test or by the use of insulin or oral hypoglycemic agents to control blood glucose.[21] Hypertension was defined as systolic blood pressure $\geq$130 mm Hg or diastolic blood pressure $\geq$85 mm Hg or by the use of antihypertensive agents.[22] Dyslipidemia was defined as serum concentrations of triglycerides $\geq$150 mg/dL or high-density lipoprotein (HDL) cholesterol $<$40 mg/dL and $<$50 mg/dL for men and women, respectively, or by the use of specific medication.[22]

Venous blood samples were taken in the morning after 12-hour overnight fast. The laboratory evaluations included blood cell count and measurement of serum aspartate aminotransferase (AST), alanine aminotransferase (ALT), γ-glutamyl transpeptidase (γ-GTP), fasting plasma glucose, HbA1c, total cholesterol, HDL cholesterol, triglyceride, ferritin, hyaluronic acid, AFP, and des-γ-carboxy prothrombin (DCP). These parameters were measured by using standard clinical chemistry techniques.

Histopathologic Examination

Nontumor liver tissues were obtained from all 87 patients to diagnose the background liver tissue at the time HCC was diagnosed. In 49 patients who underwent hepatic resection for HCC, we examined nontumor liver tissues that were surgically resected. In 21 patients who underwent ultrasound-guided tumor biopsy, nontumor liver tissues far from HCC tumors were biopsied separately. In 17 patients who were diagnosed as HCC by dynamic CT or MRI and did not undergo either hepatic resection or tumor biopsy, only nontumor liver tissues far from HCC tumors were obtained by ultrasound-guided biopsy.

The specimens were fixed in formalin, embedded in paraffin, and stained with hematoxylin-eosin, with Masson trichrome, and by silver impregnation. NASH was defined as steatosis with lobular inflammation, hepatocellular ballooning, and Mallory's hyaline (Mallory's body) or fibrosis.[23-25] The necroinflammatory grade and the degree of fibrosis were evaluated and scored according to the criteria proposed by Brunt et al.[26]

Statistical Analysis

Results are presented as numbers with percentages in parentheses for qualitative data or as the medians and ranges (25th–75th percentiles) for quantitative data. Comparisons were made by using a χ^2 test for qualitative factors or a Mann-

Table 1. Patient Characteristics

Characteristic	Total (n = 87)	Male (n = 54)	Female (n = 33)	*P* value[a]
Age (*y*)	72 (69–75)	72 (69–75)	72 (68–75)	.52
BMI (kg/m^2)	26.0 (23.8–28.3)	26.0 (23.8–28.8)	26.2 (23.9–27.7)	.54
Obesity	54 (62%)	35 (65%)	19 (58%)	.50
Diabetes	51 (59%)	31 (57%)	20 (61%)	.77
Dyslipidemia	24 (28%)	13 (24%)	11 (33%)	.35
Hypertension	47 (54%)	22 (41%)	25 (76%)	.001
Platelet count ($\times 10^4/\mu L$)	13.9 (10.1–18.0)	14.5 (11.7–18.0)	10.9 (7.8–18.0)	.05
AST (*IU/L*)	47 (30–59)	46 (27–60)	47 (35–58)	.45
ALT (*IU/L*)	36 (26–55)	43 (26–69)	34 (26–42)	.11
γ-GTP (*IU/L*)	75 (40–115)	68 (36–177)	75 (40–115)	.90
Fasting glucose (*mg/dL*)	114 (99–145)	112 (99–144)	120 (97–152)	.59
HbA1c (%)	6.1 (5.4–7.1)	5.9 (5.4–7.0)	6.3 (5.2–7.1)	.78
Total cholesterol (*mg/dL*)	169 (147–202)	169 (147–202)	169 (147–202)	.62
HDL cholesterol (*mg/dL*)	50 (41–60)	45 (41–58)	55 (50–73)	.03
Triglyceride (*mg/dL*)	100 (76–138)	118 (80–147)	96 (74–116)	.06
Ferritin (*ng/dL*)[b]	197 (74–401)	273 (154–703)	98 (23–172)	.005
Hyaluronic acid (*ng/mL*)[c]	166 (67–241)	151 (69–244)	174 (61–332)	.85
AFP (*ng/mL*)	7.1 (5.0–18.0)	6.0 (4.0–14.7)	10.8 (5.9–18.0)	.02
DCP (*mAU/mL*)	66 (22–298)	48 (22–243)	81 (21–942)	.42
HCC tumor size (*cm*)	3.0 (2.0–4.0)	3.1 (2.2–4.5)	2.6 (1.9–4.0)	.18
Number of HCC tumors				.78
1	65 (75%)	39 (72%)	26 (79%)	
2 or 3	16 (18%)	11 (20%)	5 (15%)	
≥4	6 (7%)	4 (8%)	2 (6%)	
Background liver tissue				
Steatosis grade				.64
0: <5%	1 (1%)	1 (2%)	0 (0%)	
1: 5%–33%	60 (69%)	36 (67%)	24 (73%)	
2: 34%–66%	19 (22%)	11 (20%)	8 (24%)	
3: >66%	7 (8%)	6 (11%)	1 (3%)	
Necroinflammatory grade[d]				.22
1: mild	31 (35%)	22 (41%)	9 (27%)	
2: moderate	45 (52%)	26 (48%)	19 (58%)	
3: severe	11 (13%)	6 (11%)	5 (15%)	
Fibrosis stage[d]				.003
1	10 (11%)	10 (18%)	0 (0%)	
2	15 (17%)	10 (18%)	5 (15%)	
3	18 (21%)	13 (25%)	5 (15%)	
4	44 (51%)	21 (39%)	23 (70%)	

NOTE. Values are medians (25th–75th percentiles) or numbers (%). Where no other unit is specified, values refer to number of patients.
[a]χ^2 test or Mann–Whitney *U* test.
[b]Missing data for 27 patients.
[c]Missing data for 29 patients.
[d]According to reference 26.

Whitney *U* test on ranks for quantitative factors with non-equal variance. *P* values less than .05 from two-sided tests were considered to be significant. All statistical analyses were performed by using SPSS 15.0 software (SPSS Inc, Chicago, IL).

Results

The characteristics of the 87 NASH patients who developed HCC are summarized in Table 1. The median age was 72 years (25th percentile, 69; 75th percentile, 75); the mean age (standard deviation) was 71.2 (6.7) years. There were 54 male patients (62%) and 33 female patients (38%); the male:female ratio was 1.6:1. The median BMI was 26.0 kg/m^2, and 54 patients (62%) were obese (BMI ≥25 kg/m^2). Diabetes, dyslipidemia, and hypertension were present in 51 (59%), 24 (28%), and 47 (55%) patients, respectively.

The diagnosis of NASH was proved by histologic examination of nontumor liver tissues at the time HCC was diagnosed. The degree of steatosis was grade 1 (5%–33%) in 60 patients (69%), grade 2 (34%–66%) in 19 (22%), and grade 3 (>66%) in 7 (8%). One patient who showed less than 5% steatosis was diagnosed as "burn-out" NASH, because a previous liver biopsy that was performed before development of HCC had demonstrated typical histologic features of NASH. The necroinflammatory grade was mild (grade 1) in 31 patients (35%), moderate (grade 2) in 45 (52%), and severe (grade 3) in 11 (13%). The degree of fibrosis was stage 1 in 10 patients (11%), stage 2 in 15 (17%), stage 3 in 18 (21%), and stage 4 (ie, liver cirrhosis) in 44 (51%).

The median diameter of HCC tumors was 3.0 cm (25th percentile, 2.0; 75th percentile, 4.0). A single HCC lesion was present in 65 of 87 patients (75%).

Data were stratified according to sex (Table 1). Compared with female patients, male patients had significantly less hypertension, lower HDL cholesterol and AFP, higher ferritin, and a less advanced stage of fibrosis. The prevalence of cirrhosis was significantly lower in male patients (21 of 54, 39%) than in female patients (23 of 33, 70%) (P = .008).

Discussion

In this cross-sectional multicenter study in Japan, we showed the clinical features of a relatively large number (n = 87) of NASH patients with HCC. The male:female ratio was 1.6:1. Men have higher HCC rates than women in almost all populations, with male:female ratios usually averaging between 2:1 and 4:1.[2] In the latest nationwide survey of HCC in Japan,[27] this ratio was 2.5:1. The reasons underlying higher rates of HCC in men might relate to sex-specific differences in exposure to risk factors. Men are more likely to be infected with hepatitis B and C viruses, consume alcohol, smoke cigarettes, and have increased iron stores.[2] Moreover, androgens are considered to influence the development of HCC. With regard to the male: female ratio of HCC associated with NASH, a male:female ratio of 1.3:1 was reported in a summary of 16 published cases of HCC associated with NASH.[28] Ratios of 2.8:1 and 0.67:1 were reported in 2 retrospective studies of HCC arising from cryptogenic cirrhosis in Italy (n = 44)[10] and the United States (n = 30),[9] respectively, and a ratio of 1.6:1 was reported for 36 cases of NASH-associated HCC from a single center in Japan.[15] Overall, NASH patients with HCC are more often men. However, these male:female ratios might be lower than the ratios for HCC of other etiologies, including viral hepatitis and alcohol consumption.

Although it is well-known that male gender is a risk factor for HCC in patients infected with hepatitis B and C viruses,[2] it remains unclear whether male gender is a factor associated with the development of HCC in NASH patients. It is now suspected that there is an even distribution of NASH among men and women.[29] In another study by our group,[30] the male:female ratio was 0.85:1 in 342 NASH patients without cirrhosis and HCC. The male:female ratio (1.6:1) of NASH patients with HCC in the present study is higher than this ratio. In agreement with our observations, a case-control study showed that the male: female ratio was 1.6:1 in 34 NASH patients with HCC, whereas the ratio was 0.69:1 in 348 NASH patients without HCC.[15] A recent prospective study indicated that older age and alcohol consumption were independent risk factors for the development of HCC in patients with NASH-cirrhosis and that male gender tended to be associated with the development of HCC, although this trend did not reach statistical significance.[17]

The median age of our patients was 72 years. There was no significant difference in age between men and women. Although the global age distribution of HCC varies by geographic region, sex, and etiology, in almost all areas the peak female age group in HCC patients is 5 years older than in male HCC patients.[2] In a nationwide survey of HCC in Japan,[27] the mean ages were 65.5 years for men and 69.4 years for women. The male patients in the present study are slightly older than the mean ages reported in these previous studies.

Consistent with the literature,[9-12] more than half of our patients displayed obesity, diabetes, and hypertension. Obesity constitutes a significant risk factor for cancer mortality in general and is an increasingly recognized risk factor for HCC in particular.[31,32] In the present study, body weight was measured at the time HCC was diagnosed. Because advanced HCC might cause weight loss, it is likely that our patients were obese before the development of HCC. Diabetes has also been proposed as a risk factor for HCC.[2] Thus, HCC shares 2 major risk factors, obesity and diabetes, with NASH.

Once cirrhosis and HCC are established, it is difficult to identify pathologic features of NASH. As NASH progresses to cirrhosis, steatosis tends to disappear, so-called burn-out NASH.[5] As expected, the grade of steatosis was mild in most of our cases. It was possible to diagnose 1 case without steatosis as burn-out NASH, because a previous liver biopsy specimen (liver biopsy was performed 25 years prior) was preserved and available. It is likely that many cases of NASH-associated HCC might have been missed because of loss of the telltale sign of steatosis.

Most HCC arises on a background of cirrhosis. It is less clear whether cirrhosis is a necessary predisposition for the development of HCC in patients with NASH. Case reports of HCC arising from NAFLD and NASH patients without fibrosis or cirrhosis have been accumulating.[33-36] Cirrhosis (fibrosis stage 4) was present in 51% of cases, and advanced stages of fibrosis (stage 3 or 4) were found in 72% of cases in the present study. Indeed, cirrhosis or advanced fibrosis appeared to be the predominant risk factors for HCC development. However, in the remaining 28% of cases, HCC developed in patients with less fibrosis (stage 1 or 2). Interestingly, male patients developed HCC at a less advanced stage of fibrosis than female patients, and the prevalence of cirrhosis was significantly lower in men (39%) than in women (70%). Although the reason for the sex differences is unclear, these findings indicate that screening for HCC is needed not only in NASH patients with advanced fibrosis but also in those with less fibrosis, particularly if they are men. Further studies are needed to confirm this potentially important observation. Paradis et al[37] reported that in patients whose only risk factors for chronic liver disease are features of metabolic syndrome, HCC usually occurs in the absence of significant liver fibrosis. In addition, they found that some of these HCCs developed on preexisting liver cell adenomas. However, no preexisting adenomas were observed in the present cases.

Compared with female patients, male patients had significantly higher serum ferritin value. The normal value for ferritin varies according to the age and gender of the individual. Adult men have serum ferritin values averaging approximately 100 ng/mL (range, 75–250), whereas adult women have levels averaging approximately 30 ng/mL (range, 20–75).[38] Thus, normal men have higher ferritin levels than women. Elevation of ferritin levels is associated with NASH.[39] Because we excluded patients with alcohol consumption as rigorously as possible, we believe that alcohol consumption did not contribute to the elevation of ferritin levels in our patients.

The median diameter of the HCCs in the present study was 3.0 cm, which is equal to or smaller than the size of previously reported HCCs.[9,10,12,28,37] This is probably because most of our patients had been identified as having HCC during screening. A single HCC lesion was present in 75% of patients. For early detection of NASH-associated HCC, vigilant screening is important,[9] and the development of serologic markers for NASH is necessary.

The mechanisms of carcinogenesis in NASH remain to be elucidated. Possible mechanisms include hyperinsulinemia

caused by insulin resistance in NASH, increased levels of insulin-like growth factor that promotes tumor growth, increased susceptibility of the steatotic liver to lipid peroxidation, production of reactive oxygen species and subsequent DNA mutations, disordered energy and hormonal regulation in obesity, and aberrations in regenerative processes occurring in cirrhosis.[25]

Certain limitations should be considered in the interpretation of our findings. First, the cross-sectional study design hinders the ability to draw inferences regarding the causality of NASH in HCC. Second, the study did not include a control group of HCC patients with other liver diseases. Third, there might be a bias in patient selection, because patients were retrospectively identified as having NASH-associated HCC. Finally, although our patients were negative for hepatitis B virus surface antigen, it is still possible that occult hepatitis B virus infection might be associated with the development of HCC in some of our cases.

In summary, we showed the clinical features of NASH patients with HCC. NASH patients with HCC were more often men and frequently displayed obesity, diabetes, and hypertension. Our results suggest that male patients might develop HCC at a less advanced stage of fibrosis than female patients. Further prospective studies with a longer follow-up time and larger cohorts are needed to determine the causal association of NASH with HCC and to identify risk factors for the development of HCC in NASH patients.

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Conflicts of interest

The authors disclose no conflicts.

Funding

This work was supported by a grant from the Ministry of Health, Labour and Welfare of Japan (H20-hepatitis-008 to Takeshi Okanoue).

doi:10.1111/j.1440-1746.2010.06547.x

REVIEW

Nonalcoholic fatty liver disease and nonalcoholic steatohepatitis in Japan

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Key words
diabetes mellitus, obesity, metabolic syndrome.

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Conflict of interest
The authors do not have any conflicts of interest to disclose.

Abstract

During the past 20 to 30 years, the frequency of patients presenting with nonalcoholic fatty liver diseases (NAFLD) has increased gradually in Japan in proportion to the increase in the population with life-style related diseases. We describe here the current status of the clinical and basic aspects of research into NAFLD in Japan.

The increase in the incidence of life-style-related diseases has resulted in an increase in NAFLD throughout the past 20 to 30 years. The rate of obesity in the population is not high compared to western countries but the incidence of NAFLD is similar to those countries. In 2008 we started a nationwide study of NAFLD which has been supported by the Ministry of Labor and Welfare Japan. In this project, we planned to investigate the epidemiology, genetic backgrounds and biochemical markers, and liver injury in patients with diabetes mellitus (DM) and hepatocellular carcinoma in NASH, and treatment of NASH. Approximately 20 to 25% of DM patients showed NAFLD in which the prevalence of NASH might be more than 30 to 40%. Fortunately, we have been able to obtain very interesting results from our group studies, including single necleotide polymorphisms (SNPs) which will be published in the near future.

Introduction

In 1980, Ludwig *et al.* proposed a new disease concept called nonalcoholic steatohepatitis (NASH), a condition which may progress to cirrhosis and hepatocellular carcinoma (HCC). In Japan, much attention has been paid over the past few decades to patients infected with hepatitis B virus (HBV) and hepatitis C virus (HCV), because the rates of carriage of these viruses are high and most cases of cirrhosis and hepatocellular carcinoma (HCC) in Japan are associated with persistent HBV and HCV infection. In recent years, however, with the westernization of the Japanese lifestyle, public interest in lifestyle-related diseases has increased rapidly metabolic syndrome has attracted attention for its association with underlying insulin resistance, and the risk of dyslipidemia, and hypertension even in non-obese Japanese. In 2007, the Japan Society of Diabetes Mellitus reported that, among the causes of death for 18 385 individuals with diabetes, liver cancer was the leading cause (8.6%), while death from liver cirrhosis also was very common (4.7%). Altogether, 13.3% of death among diabetes patients were attributable to liver disease (Fig. 1);[1] however, the prevalence of hepatitis virus infections and heavy alcohol drinking were not analyzed in that paper.

Seventy to seventy-five percent of HCC in Japan is associated with HCV infection, approximately 15% of patients are positive for hepatitis B surface B antigen (HBsAg), and the remaining 10–15% are so-called non-B non-C HCC. The proportion of non-B non-C HCC increased from 6.8% in 1992 to almost twice that during the subsequent ten years. Total alcohol consumption in Japan has not increased in the past 15 years, the possibility arises that NASH is responsible for this apparent increase in non-B non-C HCC (Fig. 2).

Most Japanese are not obese but nonalcoholic fatty liver disease (NAFLD) is becoming more common. At present, NASH is one of the most important liver diseases in Japan. In 2008, the Japan NASH Study Group (of which Takeshi Okanoue is a member) was founded, supported by the Ministry of Labor and Welfare, Japan. The purpose was to elucidate the epidemiology, pathophysiology, genetic backgrounds and long-term prognosis of NAFLD. Other objectives are to establish biochemical markers for differential diagnosis between simple steatosis (SS) and NASH, and devise treatment guidelines based on the individual pathophysiology of NASH.

Epidemiology

In the last two decades, patients diagnosed with fatty liver by image analysis and with elevated serum alanine aminotransferase (ALT) increased in number in proportion to the increase in

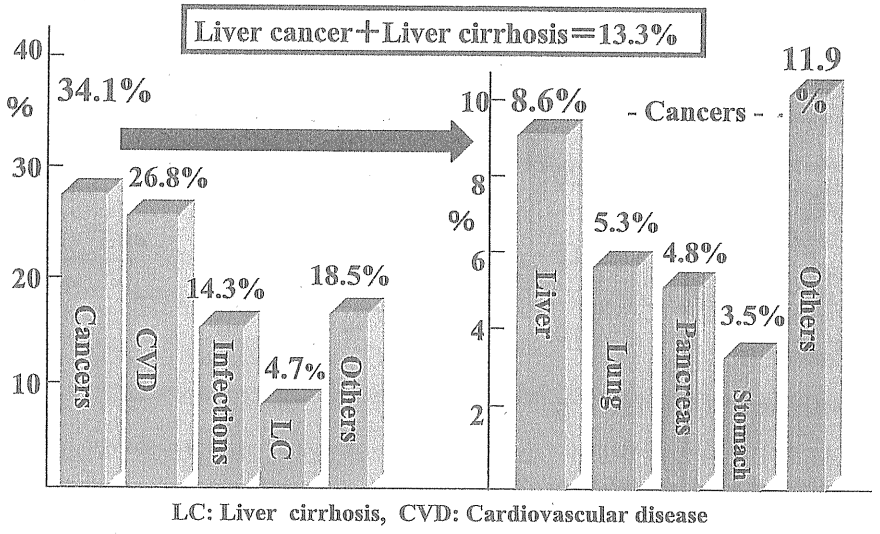

Figure 1 Cause of death in diabetes mellitus patients in Japan. The leading cause of death in 18 385 diabetes mellitus patients, who died from 1991 to 2000, was cancer, the second was cardiovascular diseases, the third was infection and the fourth was liver cirrhosis. Among the cancer deaths, the highest rate was of hepatocellular carcinoma (8.6%; males: 10.5% and females: 5.2%). In the general Japanese population, the primary cause of cancer death is lung cancer, the next is gastric cancer, the third is colon cancer and the fourth is HCC in males.

lifestyle-related diseases, such as obesity, diabetes and dyslipidemia. The Japan Society of Ningen Dock (health check-up organization) reported in 2008[2] that the prevalence of liver dysfunction, including fatty liver, was 31.9% in men and 17.1% in women, based on a study carried out on 1 814 864 adult men and 1 136 903 adult women. The prevalence of obesity, liver dysfunction, and high levels of cholesterol and triglyceride showed no significant differences in distribution with age in men, but the prevalence increased with age in women; for those in their 60s, it reached a high level comparable to that in men. Glucose intolerance and high blood pressure increased with age in both men and women (Fig. 3a/3b). A comparison of annual variations showed increase of all these factors, but the increase was especially marked in the incidence of liver dysfunction, obesity, and hypercholesteremia, and these became prominent in the late 1990s (Fig. 4).

Kojima *et al.* reported that the prevalence of fatty liver detected by medical health checks increased year after year, from 12.6% in 1989 to 30.3% in 1998.[3] According to the report by the Japan Society of Ningen Dock in 2008, 26.2% of subjects who underwent health check-ups showed fatty liver by abdominal ultrasonography.[2]

The majority of fatty liver disease comprises alcoholic fatty liver and NAFLD, including NASH. Tanaka *et al.* reported that approximately 25% of the health check-up examinees had fatty liver.[4] Hamaguchi *et al.* reported that the prevalence of NAFLD was 23.3% in Japanese adults.[5] There is a gender difference in the incidence of NAFLD; men are more likely to develop fatty liver. There is also a gender difference in the age distribution; in men, the incidence of fatty liver is about 25% and remains unchanged from the 30s to the 60s, whereas in women, the prevalence of fatty liver increases gradually with age and, in the 60s and beyond, reaches nearly the same level as in men. According to previous reports, the number of NAFLD patients is estimated to be 10 million (the population in Japan is around 130 million), and, from recent studies around 2% of them are considered to have NASH.

In 2008, a fact-finding survey was conducted on the causes of cirrhosis at the 44th Annual Meeting of the Japan Society of Hepatology; 33 379 cirrhotic patients were enrolled in 58 hospitals, and 2.1% were diagnosed with NASH-induced cirrhosis (Fig. 5). According to that survey, the proportion of NASH cirrhosis is 1.4% in males and 3.4% in females, and there is a significant gender difference ($P < 0.005$). In that study, obese subjects were few and, at that time, the concept of NASH was not yet commonly accepted by many Japanese doctors. Furthermore, many cases of advanced stage NASH show no fatty deposit, so-called "burn-out NASH", resulting in the diagnosis of cryptogenic liver cirrhosis. Therefore, the actual incidence of NASH-related cirrhosis might be higher than was reported.

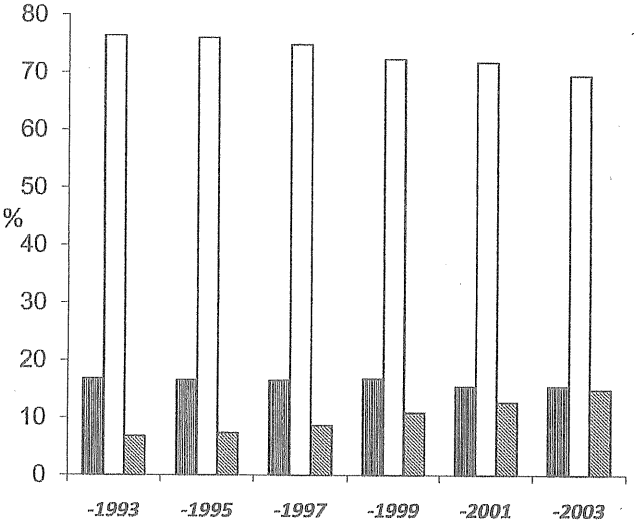

Figure 2 Change of the etiology of hepatocellular carcinoma (HCC) in Japan from 1993 to 2003. Among the causes of HCC, the HCV infection rate was decreasing gradually and HBV infection showed no significant change; however, the prevalence of non B, non C HCC has doubled over the past 10 years. HBV, hepatitis B virus; HCV, hepatitis C virus; NBNC, non B, non C. ▥, HBV; □, HCV; ▨, NBNC.

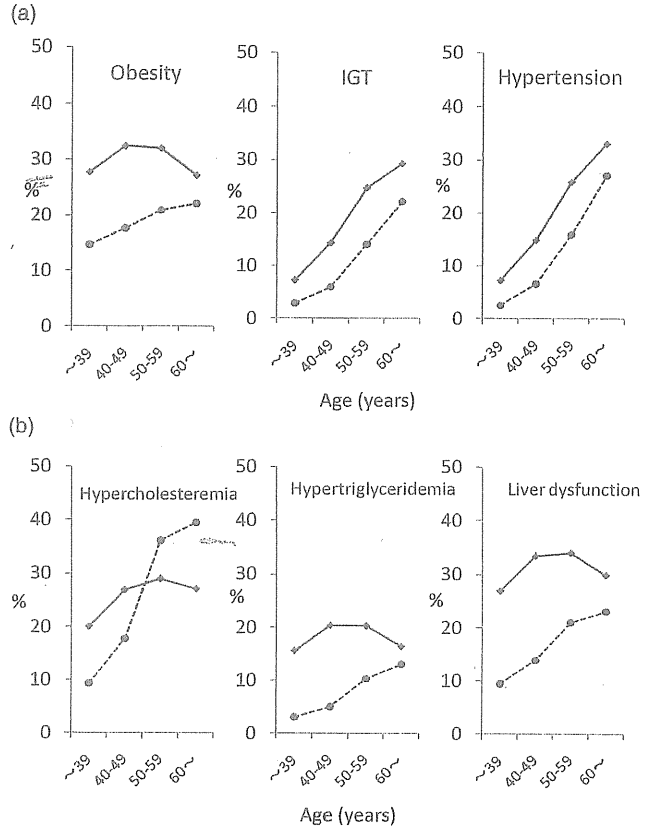

Figure 3a/3b The complication rates of life-style related diseases in the general population receiving health check-ups in 2008. In women, the prevalence of obesity, hypercholesteremia, hypertriglyceridemia, and liver dysfunction increased with age and, for women in their 60s, these reached a high level comparable to those in men. Glucose intolerance and high blood pressure increased with age in both sexes. IGT, impaired glucose tolerance. —◆—, Male; --●--, Female.

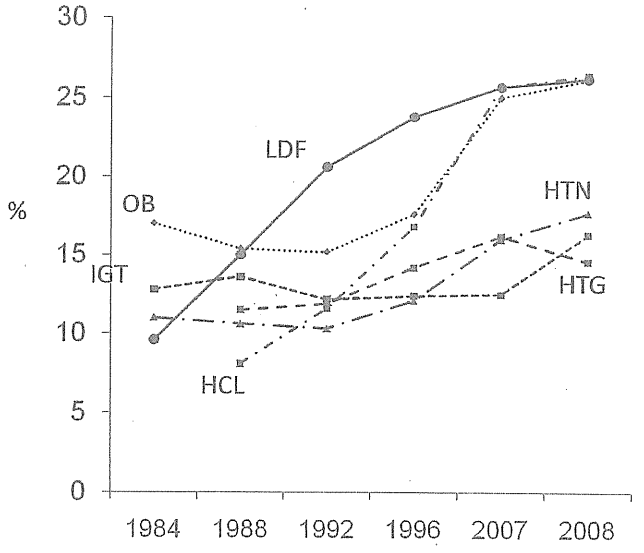

Figure 4 Annual variation of life-style related diseases in the general population. During the past 20 years all diseases significantly increased year by year. ---◆---, Obesity(OB); --■--, Impaired Glucose Tolerance (IGT); —▲-, Hypertension(HTN); —■—, Hypercholesteremia(HCL); —■-, Hypertriglyceridemia(HTG); —●—, Liver dysfunction(LDF).

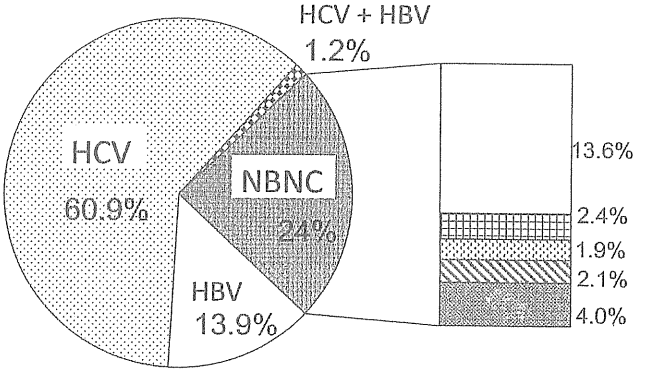

Figure 5 Etiology of liver cirrhosis in Japan. A nationwide survey was carried out in 2008 at the 44th Annual Meeting of the Japan Society of Hepatology. The non B, non C group occupied 24%, of which 2.1% were diagnosed as definite NASH. HBV, hepatitis B virus; HCV, hepatitis C virus; NBNC, non B, non C; PBC, primary biliary cirrhosis; AIH, autoimmune hepatitis; NASH, nonalcoholic steatohepatitis. □, Alcohol; ▦, PBC; ▩, AIH; ▧, NASH; ■, Others.

Metabolic syndrome

Patients with metabolic syndrome are increasing in number in Japan (Figs. 4,6). Visceral fat accumulation and insulin resistance are usual in these patients. The enhanced insulin resistance caused by the excessive accumulation of body fat (especially visceral fat) is considered to be important in the pathogenesis of fatty liver.

The diagnostic criteria for metabolic syndrome established by the Japanese Society of Internal Medicine are as follows:[6] an umbilical abdominal circumference (men: 85 cm or more; women: 90 cm or more) which reflects visceral fat accumulation (a visceral fat area of 100 cm^2 or more), and any two of the following four criteria: (i) elevated serum triglyceride level; (ii) reduced HDL cholesterol; (iii) elevated blood pressure; and (iv) elevated fasting plasma glucose. According to the National Health and Nutrition Examination Survey conducted in Japan in 2008, the prevalence of patients afflicted by metabolic syndrome was 25.3% among men and 10.6% among women, whereas patients with pre-metabolic syndrome (patients with an abdominal circumference of ≥85 cm in men and 90 cm in women, and who fulfill one other criterion) accounted for 21.9% of the men and 8.3% of the women. Therefore, approximately half of Japanese men and about 20% of Japanese women might have metabolic syndrome or be predisposed to metabolic syndrome.[7]

The criteria for metabolic syndrome are useful for the screening of NAFLD. The previous report by Ishibashi *et al.* stated that abdominal circumference was well correlated with NAFLD in men, but not in women.[8] Waist circumference has been reported to be smaller in men than women and there has been considerable debate regarding whether this criterion is appropriate or not.[9] There is the possibility that the amount of visceral fat might be underestimated and that the estimate may detect fewer than the actual number of

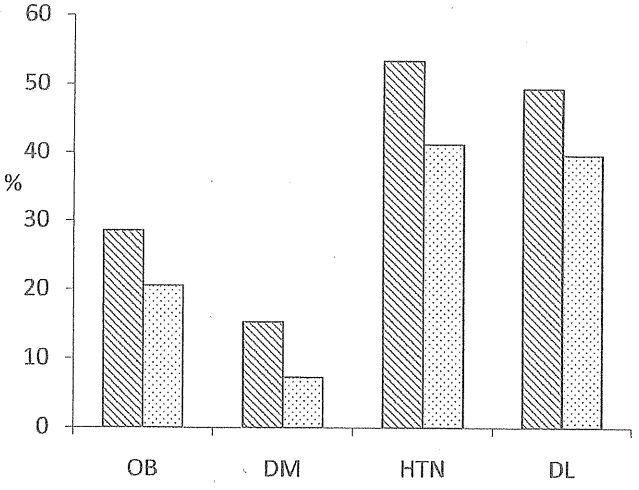

Figure 6 The complication rate of lifestyle-related diseases in Japan in 2007 to 2008. OB, obesity; DM, diabetes mellitus; HTN, hypertension; DL, dyslipidemia. ▨, Male; ▩, Female.

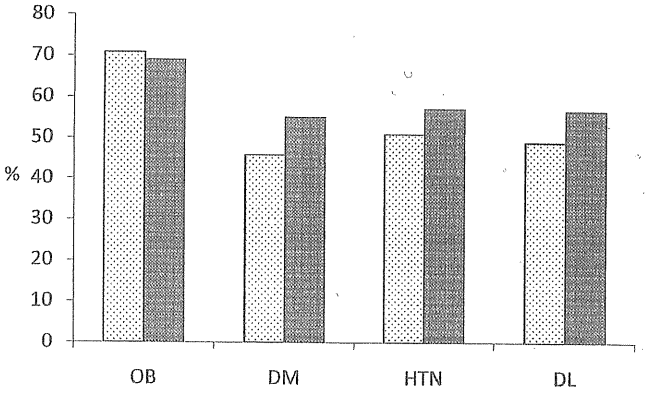

Figure 7 The complication rates of life-style related diseases among 283 NAFLD patients subjected to liver biopsy at Saiseikai Suita Hosptital from 2008 to 2010. SS, simple steatosis; NASH, nonalcoholic steatohepatitis; OB, obesity; DM, diabetes mellitus; HTN, hypertension; DL, dyslipidemia. ▨, SS n=96; ▩, NASH n=187.

women with NAFLD. In women, caution is required when the abdominal circumference is used instead of the visceral fat area.

Epidemiologically, it is clear that the risk of cardiovascular diseases is increased markedly in people with multiple risk factors for life-style related diseases. In addition, Hamaguchi *et al.* showed that NAFLD patients were at high risk of cardiovascular diseases and NAFLD, but not metabolic syndrome (MS), and showed a statistically significant correlation with cardiovascular disease in a multivariate model (OR = 4.12; 95% CI, 1.58–10.75).[10] Having multiple life-style related diseases is considered to be a risk factor for developing NASH.

The incidence of complications of life-style related diseases among 283 biopsy-proven NAFLD patients in Saiseikai Suita Hospital from April 2007 to March 2010 was high. However, no significant difference was seen in the incidence of individual factors between 187 NASH patients and 96 SS patients; obesity: NASH 69.0% *versus* SS 70.8%, diabetes: NASH 55.1% *versus* SS 45.8%, hypertension: NASH 57.2% *versus* SS 51.0%, and dyslipidemia: NASH 56.7% *versus* SS 49.0% (Fig. 7). Most NASH-cirrhotic patients have been reported to be obese with an average BMI of 27.6 ± 4.5 kg/m^2; the prevalence of the complications of diabetes and hypertension were 66.6% and 50.2%, respectively.[11]

Hamaguchi *et al.* also showed that the presence of metabolic syndrome was related to the new onset of NAFLD, with a 4-fold increase in men and an 11-fold increase in women compared to non-metabolic syndrome subjects.[5] Mitsumune *et al.* reported that obesity (6.3 fold), dyslipidemia (2.4 fold), hyperglycemia (1.8 fold), and hypertension (1.4 fold) all increased the odds of having NAFLD.

Obesity

The National Health and Nutrition Examination Survey[7] conducted in 2008 showed that the proportion of obese subjects with a BMI of 25 kg/m^2 or more was 28.6% of men and 20.6% of women. Classified by age, this category accounted for over 29% of men aged 30–69 years, whereas, for women, the obesity rate increased with age: 11.8% in their 30s, 18.0% in their 40s, 21.1% in their 50s, 24.4% in their 60s, and 26.8% in their 70s. The prevalence of fatty liver according to age shows the same tendency as obesity. Fatty liver was noted in only 2.7% of non-obese subjects with a BMI less than 23 kg/m^2 and was 10.5% in those with a BMI ≥ 23 but < 25 kg/m^2, 34.6% in those with a BMI ≥ 25 but < 30 kg/m^2, and 77.6% in highly obese subjects (BMI > 30 kg/m^2).[12] Kojima *et al.* carried out an analysis of 39 151 people who underwent a health check-up over a period of 12 years from 1989 to 2000. They reported that the grade of obesity correlated with the development of fatty liver. The prevalence was 12.8% in non-obese subjects (BMI < 25 kg/m^2), 51.4% in subjects with BMI ≥ 25 but < 30 kg/m^2, and 80.4% in highly obese subjects (BMI > 30 kg/m^2). In subjects with BMI values of 25 kg/m^2, the odds ratio for fatty liver was 6.3 compared with of non-obese subjects.[3]

Hamaguchi *et al.* reported that, in a group receiving a health check-up, 18% showed NAFLD at the time of the initial health check-up, and 10% (14% of men and 5% of women) developed NAFLD during the follow-up period of an average 414-days.[5] Patients with newly developed NAFLD showed weight gain of 1.7 ± 1.7 kg for men and 1.3 ± 1.4 kg for women. Logistic regression analysis showed that weight gain was an independent risk factor for the newly developed NAFLD, with an OR = 1.51 (95% CI, 1.40–1.63) for men and OR = 1.62 (95% CI, 1.39–1.89) for women.

Diabetes/impaired glucose tolerance

In the National Health and Nutrition Examination Survey conducted in Japan in 2007, 8 900 000 people were strongly suspected of diabetes (HbA1c ≥ 6.1%, or currently under treatment); the number of people with an undeniable possibility of diabetes (HbA1c ≥ 5.6% but < 6.1%) was 13 200 000, in total, the number of people possibly with diabetes was 22 100 000, which was 1.6-fold higher than 10 years earlier.[13] Kojima *et al.* reported that the prevalence of fatty liver was 18.6% in subjects with normal glucose metabolism (FBS < 110 mg/dL), 43.7% in borderline subjects (FBS ≥ 110 but < 126 mg/dL), and 53.3% in diabetic

patients (FBS ≥ 126 mg/dL). FBS ≥ 110 mg/dL was an independent risk factor for fatty liver (OR = 3.1).[3] Likewise, Jimba *et al.* reported that the overall prevalence of NAFLD was 29% among 1950 Japanese people receiving a health check-up; the prevalence was 27% in the normal glucose metabolism group (FBG < 6.1 mmol/L) and rose to 43% for the borderline type (FPG ≥ 6.1 but < 7.0 mmol/L) and 62% for the diabetic type (FBG ≥ 7.0 mmol/L or a medical history of diabetes). In addition, the incidence of complications with abnormal glucose metabolism (borderline type and diabetic type) was 19.1% in NAFLD patients, which was higher than the 5.6% of patients without NAFLD ($P < 0.001$).[14]

Miyaaki *et al.* examined the relationship between the stage of hepatic fibrosis and the prevalence of diabetes in Japanese patients. In the mild fibrosis group, 42% were complicated with diabetes, whereas in the severe fibrosis (bridging fibrosis or cirrhosis) group, the prevalence was as high as 71% ($P = 0.020$). Diabetes might be a factor responsible for the development of hepatic fibrosis in NAFLD.[15] Shiga *et al.* performed a 75-g oral glucose tolerance test on the participants of a health check-up. They found that blood glucose levels at one and two hours after glucose load showed a closer relationship with NAFLD than the fasting blood glucose level. Therefore, they stated the importance of the evaluation of impaired glucose tolerance (IGT) in detecting NAFLD.[16]

Hypertension

According to the criteria of the Japanese Society of Hypertension, systolic blood pressure under 130 mm Hg/diastolic blood pressure under 85 mm Hg is normal, pressure higher than 140/90 mm Hg is diagnosed as hypertension, and pressure 130–139/85–89 mm Hg is high-normal blood pressure.

In the National Health and Nutrition Examination Survey conducted in 2007, the prevalence of subjects with hypertension (including 24.0% currently under treatment) was 46.2%, the prevalence of high-normal blood pressure was 13.8%, and the normal pressure group was 40.0%.

Hypertension is frequently seen in NASH/NAFLD patients, but there are no reports describing the prevalence of NAFLD among hypertensive patients in Japan. Reports from overseas stated that systolic and diastolic blood pressures were correlated with liver fat content, and that the risk of developing NAFLD was 2-fold higher in patients with systolic hypertension, and 1.7-fold higher in patients with diastolic hypertension.[17,18] In addition, Donati *et al.* reported that even in non-obese, non-diabetic high blood pressure patients, the prevalence of fatty liver was three times higher than in healthy individuals. Further such patients showed high levels of HOMA-IR, indicating insulin resistance.[19] The pathogenesis of hypertension is influenced by various factors, such as salt intake, and also is associated with insulin resistance. It is important to know that even non-obese high blood pressure patients with no other lifestyle-related diseases are likely to develop NAFLD if they have insulin resistance. In Japan, large-scale studies on hypertension and NAFLD are currently underway, including among subjects with chronic kidney disease (CKD).

Recently, we reported the prevalence of CKD in 174 NAFLD patients. The prevalence of CKD was significantly higher in NASH patients (19 of 92; 21%) than SS patients (5 of 82; 6%), and associated with a higher body mass index and the presence of hypertension.[20]

Dyslipidemia

Dyslipidemia is a generic term describing a clinical condition in which the levels of cholesterol esters or triglycerides increase in the blood: high levels of triglycerides (150 mg/dL or higher) and LDL cholesterol (140 mg/dL or higher), with decreased levels of HDL cholesterol (less than 40 mg/dL) are each risk factors for other diseases. In the National Health and Nutrition Examination Survey conducted in 2007, the percentage of subjects suspected of dyslipidemia (including 9.7% currently under treatment) was 44.1%, and that of normal subjects was 55.9%.

Dyslipidemia in NAFLD often involves hypertriglyceridemia and decreased blood levels of HDL cholesterol. This is due to the insufficient effects of lipoprotein lipase (LPL), which leads to a decreased metabolism of triglyceride-rich lipoproteins into HDL cholesterol. In addition, there is also an increased synthesis of very-low density lipoprotein (VLDL).

Pediatric NASH/NAFLD

The incidence of pediatric NAFLD in Japan is increasing in proportion to the increase in the prevalence of childhood obesity. In a previous study conducted on children aged 6–15 years, Tominaga *et al.* reported that the prevalence of NAFLD was 3.4% in children aged 6–10 years and 5.2% in those aged 11–15 years.[21] In addition, the prevalence of NAFLD in children who met the diagnostic criteria for pediatric metabolic syndrome was 40.0% in those diagnosed with pre-metabolic syndrome, and 76.8% in those who fulfilled the criteria for metabolic syndrome. Tsuruta *et al.* also reported that, in a similar study conducted in 2007 on 288 junior high school students (13–15 years old), 5.9% were obese, the prevalence of NAFLD was 4.5%, and obesity and ALT levels of 30 IU/L or higher were independent risk factors for NAFLD in children.[22] In addition, the prevalence of complications with obesity (degree of obesity ((body weight—standard body weight)/standard body weight × 100) ≥ 20%) showed a higher frequency in NAFLD patients (58.3%) than those without NAFLD (5.7%, $P < 0.001$).

Pathophysiology and genetic background

Racial differences might affect the onset and pathophysiology of NAFLD. Weston *et al.* reported that the prevalence of obesity, dyslipidemia, and diabetes in NAFLD was similar among racial and ethnic groups, except that body mass index was lower in Asians compared to Whites, Hispanics, and African Americans ($P < 0.001$). Compared with the base population, Hispanics with NAFLD were overrepresented and Whites were underrepresented.[23] In addition, Mohanty *et al.* reported that African Americans showed a lower degree of steatosis than Whites. In contrast, it has been considered that Asians showed higher grades of ballooning and Hispanics showed higher grades of Mallory-Denk bodies, than Whites and other ethnicities combined.[24] These findings indicate the importance of racial differences for the development and progression of NAFLD.

There are many reports concerning the genetic predisposition to the development of NASH and NAFLD, and most of them refer to functional genetic polymorphisms. Tumor necrosis factor-alpha (TNF-α) is known to be produced by adipocytes in visceral fat and Kupffer cells in the liver. It inhibits insulin receptor substrate-1 (IRS-1) of target cells, and insulin receptor kinase in skeletal muscles and adipocytes, thereby cause or exacerbating insulin resistance. Increased blood levels of TNF-α have been reported in NAFLD and NASH patients whose BMI and insulin resistance were matched, thereby suggesting a relationship between increased levels of TNF-α and the development of NAFLD or the progression of NASH.[25] It has been reported in Japanese subjects that functional genetic polymorphisms of TNF-α are present at positions T-1031C and C-856A in the promoter region, and these were more frequent in patients with NASH, potentially mediating progression of the disease.[26]

Adiponectin has an insulin sensitivity effect by opposing fatty acid accumulation which causes insulin-resistance, an anti-atheriosclerotic effect, and an anti-inflammatory effect. Therefore, hypoadiponectinemia associated with obesity has been considered to play a crucial role in the development of metabolic syndromes. In addition, the serum adiponectin level has been shown to be lower in NASH patients than in healthy groups and simple fatty liver groups.[27] The presence of functional polymorphisms G45T and G276T is the adiponectin gene have been reported to be associated with diabetes.[28,29] Regarding Japanese subjects with NASH, it has been reported that the G/G homo-allele at the 45th base of the exon of adiponectin was more frequent in NASH with advanced fibrosis than that in mild fibrosis, and that insulin resistance was distinctly more prominent.[30] Yoneda *et al.* reported that genetic variations in angiotensin II type1 receptor (ATGR1) may influence the risk of NAFLD and liver fibrosis in NAFLD.[31]

Functional polymorphisms in the β3-adrenergic receptor gene, microsomal triglyceride transfer protein (MTP), phosphatidylethanolamine *N*-methyltransferase (PEMT), interleukin-1β (IL-1β), and manganese superoxide dismutase (MnSOD) have also been reported in Japan.[32–34] MTP and PEMT are important factors for the metabolism in triglyceride.

In addition, sex hormones are involved in gender differences in the incidence of NAFLD, and in postmenopausal women the decreased level of estrogen results in the accumulation of visceral fat and insulin resistance.[35] This may explain why postmenopausal women appear to be at a higher risk for the development of NAFLD.

Pathological diagnosis

NAFLD can be diagnosed in patients from whom hepatitis virus infection, alcoholic liver disease and autoimmune hepatitis have been excluded when over 5% of hepatocytes contain fatty droplets.

NAFLD encompasses a histological spectrum ranging from simple steatosis (SS) to NASH, the latter showing hepatocyte degeneration (ballooning hepatocyte), necrosis, inflammation and fibrosis.[36]

Recently, Matteoni *et al.* categorized NAFLD into four types; type 1 (simple fatty liver), type 2 (steatohepatitis), type 3 (steatonecrosis) and type 4 (steatonecrosis + Mallory-Denk body (MDB) or fibrosis). They proposed that types 1 and 2 should be categorized as SS, and types 3 and 4 as NASH, according to the prognosis based on their follow-up study.[37] Actually we sometimes encounter difficulty in the differential diagnosis between type 2 and type 3 NAFLD, and between type 3 and type 4 NAFLD. This is because the criteria of ballooning hepatocytes and presence of pericentral and pericelluar fibrosis are unclear when these morphological changes are very mild.

In 2005, Kleiner *et al.* proposed a new scoring system, the so-called NAFLD activity score (NAS), according to the extent of the three features: steatosis, hepatocellular ballooning and lobular inflammation. By the NAS, NASH is defined as having a score of five or more.[38] This score is based on disease activity and the evaluation of fibrosis is excluded; this might be not suitable for the diagnosis of advanced staged NASH. Brunt and others proposed a grading and staging system according to the grade of inflammation and fibrosis,[39] and this method is widely accepted in Japan.

Ten to 30% of NASH cases have the potential to develop to cirrhosis within 10 years. However, much attention should be paid to so-called "burn-out NASH", in which fatty droplets have disappeared during the progression of hepatic fibrosis, resulting in difficulty making a precise diagnosis of NASH. In such a case, we must make an effort to collect the detailed background and previous patient history. This difficulty could lead to an underestimation of the prevalence of NASH-cirrhosis the Mallory-Denk bodies (MDB) are one of the morphological hallmarks for the diagnosis of type 4 NAFLD: they are an abnormal flocculent producter in degenerated hepatocytes and are comprised of intermediate filaments (IF).[40] We consider that the frequency of MDB in Japanese NASH is lower than in western countries.

Biochemical markers: non-invasive score assessment

Liver histology is the gold standard for the diagnosis of NASH; however, it is invasive and there is a risk of sampling errors in some cases. It has been anticipated that it should be possible to use serum biochemical markers to diagnose NASH, and various parameters reflecting oxidative stress, insulin resistance, inflammation, apoptosis, and fibrosis have been proposed to discriminate between SS and NASH. A NASH test that allows prediction on the basis of 13 parameters has been reported in Europe but, in recent years, Gholam *et al.* designed a more convenient differential formula based on only two criteria: the AST level and the presence or absence of diabetes mellitus (DM).[41] Campos *et al.* proposed a clinical scoring system for NASH[42] in which the scored criteria consist of hypertension (HTN), type 2 DM, AST, ALT, sleep apnea syndrome, and race (exception for blacks). However, these reports are from Europe and the USA. Recently, it was reported that the serum level of soluble fraction in cytokeratin 18 (soluble CK-18) was able to discriminate between SS and NASH,[43] and this has been adopted for our Japanese patients (unpublished data).

We reported previously the importance of serum ferritin and thioredoxin levels, reflecting status of oxidative stress, in the differential diagnosis between SS and NASH.[44,45] Recently, Sumida *et al.* proposed the NAFIC (NASH, Ferritin, Insulin, Collagen) scoring using Japanese patients. This comprises three measurements: serum ferritin, insulin, and type-4 collagen 7s.[46] To determine the utility of this score, we conducted a validation study in collaboration with ten centers all over Japan (Japan Study Group of NAFLD; JSG-NAFLD).[46]

Various indicators have been proposed for the evaluation of the degree of fibrosis in NASH. From a study based on the analysis of 50 NASH patients including nine with cirrhosis, Fujii *et al.* reported that the cirrhosis determinant score (CDS) and the hepatitis C antiviral long-term treatment against cirrhosis (HALT-C) model were valuable for the differentiation of cirrhosis induced by NASH and HCV infection.[47] A French group proposed the BAAT score[48] and Fibrotest,[49] which assign one point to each of the following items: BMI, ALT, age, and triglycerides. Angulo *et al.* proposed the NAFLD fibrosis score which can be calculated from parameters such as age, platelet count, albumin, AST/ALT ratio, fasting hyperglycemia/DM, and BMI.[50] The NAFLD fibrosis score is simple and has advantages. However, the major problem is that liver biopsy cannot be avoided in around 25% cases, which are classified as intermediate because of scores halfway between the high cut-off level and the low cut-off level. Harrison *et al.* proposed the simple and easy BARD score based on BMI $\geq$ 28 kg/m^2, AST/ALT ratio, and DM; and reported that the odds ratio increased 17-fold for cases with scores of two points or higher, associated with F3 or higher stages of fibrosis.[51] However, Fujii *et al.* suggested that the BARD score was not useful in Japanese patients because the average BMI is significantly lower than western people.[52] The N (Nippon) score[15] is very simple; it can be calculated on the basis of only gender, age, and the presence or absence of type 2 DM and HTN, and has been evaluated by a multicenter study in Japan.[16] Recently we showed that senescence marker protein 30 (SMP-30), which has an antiapoptotic activity and an effect on Ca^{++} efflux, was significantly decreased in NASH compared to SS. Thus, SMP-30 is a useful marker for the differential diagnosis between SS and NASH. However, at present we cannot detect it in serum.[53]

Prognosis

It has been reported that cardiovascular-related death and liver-related death are significantly higher in NAFLD patients than with the general population.[54] A cohort study conducted in 2006, reported a development of cancers among 97 771 individuals in the general Japanese population; 6.7% of men and 3.1% of women had DM, in diabetes patients, the hazard ratio of developing liver cancer was 2.24 (95% CI, 1.64–3.04) in men, and 1.94 (95% CI, 1.00–3.73) in women during an average follow-up period of 10.7-years.[55]

In a comparative study between HCV and NASH cirrhosis matched by gender and age, obesity, diabetes, and dyslipidemia were significantly more frequent in NASH cirrhosis. The 5-year cancer rate was 11.3% in NASH cirrhosis and 30.5% in HCV cirrhosis.[55] The leading cause of death in these two types of cirrhosis was HCC, 47% in NASH and 68% in HCV, and the second cause was hepatic failure, 32% in NASH and 25% in HCV.[56,57] The annual incidence of HCC in Japan is 2.2% in NASH cirrhosis and 6.1% in HCV cirrhosis. Meanwhile, Ascha *et al.* reported that the annual incidence of HCC was 2.6% in patients with NASH cirrhosis, compared to 4.0% in HCV cirrhosis in the USA.[58]

Treatment

Diet and exercise therapy

Weight loss achieved by diet and exercise is the most important aspect of treatment in obese patients with NAFLD, including NASH. In those treated weight, blood biochemical data such as ALT, albumin, cholinesterase, total cholesterol and fasting blood glucose values, and steatosis decreased significantly after significant weight loss.[59] The recommended daily energy intake is 25–35 kcal/kg, daily protein intake is 1.0–1.5 g/kg and fat should be less than 20% of total calories.

Antihyperlipidemic drugs

Saibara *et al.* showed that bezafibrate for tamoxifen-induced NASH resulted in biochemical and histological improvement.[60] Dohmen *et al.* reported that administration of fenofibrate for fatty liver complicated with dyslipidemia improved dyslipidemia and led to a decrease in the levels of ALP, whereas the levels of ALT showed no significant change.[61] Hyogo *et al.* reported that atorvastatin led to an improvement in liver function, fibrosis marker, adipocytokine, and improvement of fatty liver and hepatic inflammation.[62] Nozaki *et al.* reported the utility of ezetimibe and acarbose in mouse models of NAFLD.[63] Recently, we also demonstrated the histological improvement using 96 weeks of ezetimibe monotherapy in the 45 biopsy-proven NAFLD patients.[64]

Angiotensin II type-1 receptor blockers (ARB)

There also have been a number of reports from Japan regarding the utility of angiotensin II type-1 blockers (ARB) in NASH. This application is derived from basic studies which showed the inhibitory effect of ARB on the progression of fibrosis via inhibition of the activation of hepatic stellate cells.[65–67]

Antidiabetic drugs

Morita *et al.* demonstrated the effect of nateglinide on glucose metabolism, liver function, and liver histology in NASH patients with type 2 diabetes.[68] The effects of metformin and thiazolidine derivatives such as pioglitazone and rosiglitazone on NASH were reported in Japan, however, the numbers were small and the trials were uncontrolled.

Other drugs

There is the possibility that combination therapies using pantethine and probucol,[69] colestimide[70] and α-tocopherol[71] are useful for NASH; however, the subjects were in small numbers and there was no histological analysis after treatment. Recently, Sanyal *et al.* reported that administration of vitamin E for 96 weeks administration for non-DM NASH patients significantly improved liver histology compared to placebo, this result being more promising than pioglitazone administration.[72] Phlebotomy might be effective in NASH with excessive iron deposition in the liver.[73]

Group survey in NASH in Japan

As mentioned above, the Japan NASH Study Group founded in April 2008 (the representative: Takeshi Okanoue, Table 1), has started the following research projects: (i) nationwide study of 5000 cases of diabetes mellitus; (ii) SNP study of 1000 cases of SS and NASH; (iii) long-term follow-up study of 1000 cases of SS

Table 1 The members of Japan NASH study group

The representative of this study group:
Takeshi Okanoue MD, PhD., Hepatology Unit, Saiseikai Suita Hospital
The members of this study group:
Yutaka Kohgo M.D, PhD., Division of Gastroenterology and Hematology/Oncology, Department of Medicine, Asahikawa Medical College,
Sumio Kawata M.D, PhD., Department of Gastroenterology, Yamagata University Faculty of Medicine,
Kazuhiko Koike, M.D., PhD, Department of Gastroenterology, Graduate School of Medicine, The University of Tokyo,
Kohjiro Ueki, M.D., PhD., Department of Diabetes and Metabolic Diseases, Graduate School of Medicine, the University of Tokyo,
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Sumio Watanabe, M.D., PhD., Department of Gastroenterology, Juntendo University School of Medicine,
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Hirofumi Uto, M.D., PhD., Department of Digestive and Life-Style Related Disease, Kagoshima University Graduate School of Medical and Dental Sciences

and NASH; (iv) collection of 100 cases of NASH-HCC; (v) biochemical markers of differential diagnosis between SS and NASH; and (vi) therapeutic guidelines based on the individual pathophysiology. Projects i, ii, iii, and iv are going well and we are expecting to present these results, including SNPs, in the near future.

Conclusion

Recently, much attention has been paid to NAFLD in Japan because the number of NAFLD patients has been increasing, while non-B, non-C HCC also is increasing gradually. We suspect that NASH might be responsible for this increase in HCC in Japan; however, the precise cause of the increased non B, non C HCC has not yet been established. In this review, we have described the epidemiology and the present status of clinical and basic aspects of NASH/NAFLD in Japan.

Acknowledgments

This study was funded by the grant from by the Ministry of Labor and Welfare Japan. The authors thank all members of the Japan NASH Study Group.

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J Gastroenterol (2011) 46:257–268
DOI 10.1007/s00535-010-0305-6

ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

A simple clinical scoring system using ferritin, fasting insulin, and type IV collagen 7S for predicting steatohepatitis in nonalcoholic fatty liver disease

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Received: 12 April 2010 / Accepted: 2 August 2010 / Published online: 15 September 2010

Abstract
Background Liver histology is the gold standard for the diagnosis of nonalcoholic steatohepatitis (NASH). Noninvasive, simple, reproducible, and reliable biomarkers are greatly needed to differentiate NASH from nonalcoholic fatty liver disease (NAFLD).
Methods To construct a scoring system for predicting NASH, 177 Japanese patients with biopsy-proven NAFLD were enrolled. To validate the scoring system, 442 biopsy-proven NAFLD patients from eight hepatology centers in Japan were also enrolled.
Results In the estimation group, 98 (55%) patients had NASH. Serum ferritin [≥200 ng/ml (female) or ≥300 ng/ml (male)], fasting insulin (≥10 μU/ml), and type IV collagen 7S (≥5.0 ng/ml) were selected as independent variables associated with NASH, by multilogistic regression analysis. These three variables were combined in a weighted sum [serum ferritin ≥200 ng/ml (female) or ≥300 ng/ml (male) = 1 point, fasting insulin ≥10 μU/ml = 1 point, and type IV collagen 7S ≥5.0 ng/ml = 2 points] to form an easily calculated composite score for predicting NASH, called the NAFIC score. The area under the receiver operating characteristic (AUROC) curve for predicting NASH was 0.851 in the estimation group and 0.782 in the validation group. The NAFIC AUROC was the greatest among several previously established scoring systems for detecting NASH, but also for predicting severe fibrosis.
Conclusions NAFIC score can predict NASH in Japanese NAFLD patients with sufficient accuracy and simplicity to be considered for clinical use.

All authors are members of the Japan Study Group of NAFLD (JSG-NAFLD).

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Keywords Iron overload · Hepatic fibrosis · Nonalcoholic steatohepatitis

Introduction

Nonalcoholic fatty liver disease (NAFLD) is the most common chronic liver disease (CLD) in many developed countries and is a serious public health problem worldwide. NAFLD includes a wide spectrum of liver diseases that range from simple steatosis, which is usually a benign and nonprogressive condition, to nonalcoholic steatohepatitis (NASH), which can progress to liver cirrhosis (LC) and hepatocellular carcinoma (HCC) despite the absence of significant alcohol consumption [1–5]. Liver biopsy remains a reliable tool for the diagnosis of NASH [1, 6], and the most sensitive and specific method of providing prognostic information. Practically speaking, however, it is difficult to perform liver biopsy for every patient with NAFLD to ascertain the presence of NASH [7]. Moreover, biopsy itself has significant limitations such as pain, risk of severe complications, sampling error [8, 9], cost, and patient unwillingness to undergo invasive testing. Therefore, there is an urgent need to develop and validate simple, reproducible, noninvasive tests that accurately distinguish NASH from NAFLD and determine the stage of the disease [7]. Noninvasive approaches for this purpose have included a combination of clinical features and routine laboratory investigations, as well as some readily available serum markers of fibrosis [6, 7, 10, 11]. Most of these noninvasive approaches have consisted of small sample sizes and have lacked rigorous external validation.

The purposes of this study were (1) to develop a simple noninvasive scoring system aimed at differentiating NASH from NAFLD patients by using easily available clinical and biochemical variables and (2) to validate the results in a separate cohort of patients.

Methods

Patients

A total of 177 patients with well-characterized and liver-biopsy-confirmed NAFLD were included in this study to establish a simple method to detect NASH. They were consecutively biopsied patients who were seen at the Center for Digestive and Liver Diseases, Nara City Hospital from 2002 to 2008. To validate the model, 442 patients with biopsy-proven NAFLD from 2002 to 2008 were enrolled from eight Hepatology Centers in Japan: 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.

The diagnosis of NAFLD was based on the following criteria: (1) liver biopsy showing steatosis in at least 5% of hepatocytes [12] and (2) appropriate exclusion of liver diseases of other etiology including viral hepatitis, autoimmune hepatitis, drug-induced liver disease, primary biliary cirrhosis, biliary obstruction, hemochromatosis, Wilson's disease, and 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. Diabetic patients treated with exogenous insulin or insulin sensitizers (metformin or pioglitazone) were also excluded. Written informed consent was obtained from all patients at the time of their liver biopsy, and the study was conducted in accordance with the Helsinki Declaration.

Anthropometric and laboratory evaluation

Venous blood samples were taken in the morning after a 12-h overnight fast. The laboratory evaluation in all patients included a blood cell count and the measurement of aspartate aminotransferase (AST), alanine aminotransferase (ALT), γ-glutamyl transpeptidase, cholinesterase (ChE), total cholesterol, triglyceride, albumin, fasting plasma glucose (FPG), immunoreactive insulin (IRI), ferritin, hyaluronic acid (HA), and type IV collagen 7S. These parameters were measured using the standard techniques of clinical chemistry laboratories. Body mass index (BMI) was also calculated. Obesity was defined as BMI >25, according to the criteria of the Japan Society for the Study of Obesity [13]. Patients were assigned a diagnosis of diabetes mellitus (DM) if they had documented use of oral hypoglycemic medication, a random glucose level >200 mg/dl, or FPG >126 mg/dl [14]. Dyslipidemia was diagnosed if the cholesterol level was >220 mg/dl and/or triglyceride level was >160 mg/dl. Hypertension was diagnosed if the patient was taking antihypertensive medication and/or had a resting recumbent blood pressure ≥140/90 mmHg on at least two occasions.

The HAIR score [15] was calculated by summation of the scores of hypertension (1 point), ALT >40 IU/l (1 point), and insulin resistance (IR) index >5 (1 point). IR

index was calculated using the formula: 1/quantitative insulin sensitivity check index (QUICKI) [16] = log fasting IRI (μU/ml) + log FPG (mg/dl). Palekar's score [17] was calculated by summing the risk factor of age ≥50 years, female sex, AST ≥45 IU/l, BMI ≥30 kg/m^2, AST/ALT ratio (AAR) ≥0.80, and HA ≥55 ng/ml. Gholam's score [18] was calculated by the formula: 2.627 × ln AST + 2.13 for DM. The BAAT score [19] was calculated by summing the risk factor of BMI ≥28, age ≥50 years, ALT levels measuring twice normal or higher, and triglyceride level ≥1.7 mmol/l (150 mg/dl). The BARD score [20] is a weighted sum of three easily available variables [BMI ≥28 kg/m^2 (1 point), AAR ≥0.8 (2 points), and DM (1 point)]. Modified scores (with cutoff values of BMI changed to 25 kg/m^2) of Palekar's, BAAT, and BARD were also calculated. The NAFLD fibrosis score (NFS) [21] was calculated according to the following formula: −1.675 + 0.037 × age (years) + 0.094 × BMI + 1.13 × impaired fasting glycemia (IFG)/DM (yes = 1, no = 0) + 0.99 × AAR − 0.013 × platelet (×10^9/l) − 0.66 × albumin (g/dl). The N (Nippon) score [22] was calculated as the total number of the following risk factors: female sex, older age (>60 years), type 2 DM (T2DM), and hypertension.

Histological evaluation

All patients enrolled in this study underwent a percutaneous liver biopsy under ultrasonic guidance. The liver specimens were embedded in paraffin and stained with hematoxylin and eosin, Masson-trichrome, and reticulin silver stain. Two hepatopathologists (T.O. and Y.S.) who were blinded to the clinical data reviewed the liver biopsy specimens. Adequate liver biopsy samples were defined as >1.5 cm long and/or having more than six portal tracts. NASH was defined as steatosis with lobular inflammation and ballooning degeneration, with or without Mallory-Denk body or fibrosis [2, 3]. Patients whose liver biopsy specimens showed simple steatosis or steatosis with nonspecific inflammation were identified as the nonNASH cohort [2, 3]. The presence or absence of hepatocyte ballooning degeneration is influenced by the variability in pathologists' interpretation. The NAFLD Activity Score (NAS) proposed by Kleiner et al. [12] was the unweighted sum of the scores for steatosis (0–3), lobular inflammation (0–3), and ballooning degeneration (0–2). If liver histology was too atypical to make a judgment, cases with an NAS of ≥5 were considered to be NASH. The severity of hepatic fibrosis (stage) was defined as follows: stage 1, zone 3 perisinusoidal fibrosis; stage 2, zone 3 perisinusoidal fibrosis with portal fibrosis; stage 3, zone 3 perisinusoidal fibrosis and portal fibrosis with bridging fibrosis; and stage 4, cirrhosis [23].

Statistical analysis

Results are presented as the means and standard deviation (SD) for quantitative data, or as numbers with percentages in parentheses for qualitative data. Statistical differences in quantitative data were determined using the t test. Fisher's exact probability test or χ^2 analysis was used for qualitative data. Multivariate analysis was performed by logistic regression analysis to identify variables independently associated with the presence of NASH. Those variables with $P < 0.05$ by multivariate analysis were used to construct a scoring system to predict NASH. The scoring system was a weighted sum of significant variables on the basis of odds ratio (OR) obtained from logistic regression analysis. To assess the accuracy of the clinical scoring system in differentiating NASH from NAFLD, we calculated the sensitivity (Se) and specificity (Sp) for each value of each test, and then constructed receiver operating characteristic (ROC) curves by plotting the Se against (1 − Sp) at each value. The diagnostic performance of the scoring systems was 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. To evaluate the overall accuracy of our score and NFS in detecting significant or advanced fibrosis, the sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) were calculated. Differences were considered statistically significant at $P < 0.05$.

Results

Characteristics of the patient population in the estimation group

Table 1 summarizes the clinical, laboratory, and liver biopsy data of the patient population in the estimation group. Eighty-six (49%) patients were female, and 120 (68%) were obese. Of 177 NAFLD patients involved in this estimation group, 98 (55%) were histologically diagnosed with NASH, and 79 (45%) had nonNASH NAFLD. NASH patients were significantly older, predominantly female, heavier, hypertensive, and more likely to have T2DM; had lower hemoglobin (Hb), platelet count and ChE; and had higher levels of AST, ALT, AAR, ferritin, FPG, IRI, HA, and type IV collagen 7S.

Predictors of NASH

Table 2 shows the univariate comparison and the results of the multivariate analysis performed in the 177 patients in the estimation group. Univariate analysis showed that age